



Increased size and energy reserves in diapausing eggs of temperate *Aedes aegypti* populations

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ABSTRACT

Many insects overwinter in diapause, a pre-programmed anticipated response to unfavorable environmental conditions, often induced by a short-day photoperiod. Diapause involves morphological changes and increased energy stores required for metabolic demands during winter. In diapausing mosquito eggs, the accumulation of lipids plays an important role, because these molecules are the primary fuel consumed during embryogenesis and pharate larvae metabolism, and have a key role in egg desiccation resistance. The supposed inability of the mosquito *Aedes aegypti* to lay diapausing eggs has been recently challenged by a study on a temperate population, which showed that the inhibition of egg hatching in response to short days is possible in this species. Thus, the aim of the present study was to assess the effects of parental photoperiod on embryonic diapause-related traits, such as the triglyceride content and size of eggs laid, of two populations whose localities of origin differ in their winter length. Two colonies were maintained for each population: one under a Short-Day Photoperiod (SD: 10 h:14 h – Light:Dark) and the other under a Long-Day Photoperiod (LD: 14 h:10 h – Light:Dark). The eggs obtained from each combination of population and light treatment were used for size measurement (length, width and volume) and for the quantification of triglyceride content. Egg size showed differences between photoperiod treatments, with larger width and volume in eggs from the SD treatment. Remarkably, eggs from the SD treatment accumulated twice as many triglycerides as those from the LD treatment. Also, the eggs derived from the population having the longer winter accumulated larger amounts of triglycerides. The higher lipid content is probably contributing to a better survival during the cold season in both populations. The photoperiod-induced response in egg size and amount of triglycerides observed in this study support the hypothesis that the *Ae. aegypti* populations studied are able to lay diapausing eggs, a fact that provides physiological bases for the further expansion of this species to colder regions.

1. Introduction

At high latitudes, ectotherms exploit the warm season for growth, development and reproduction, and avoid the negative effects of winter cold through dormancy or migration (Bradshaw and Holzapfel, 2010). In insects, dormancy can be classified into quiescence and diapause, depending on whether the hypometabolic state is driven either by an anticipatory programmed response or by a direct response to environmental adversities (Koštal, 2006). Many temperate insect species overwinter in diapause, a hormonally mediated arrest of development in response to a token stimulus, often induced by the length of the day as an anticipatory cue to prepare for the changing seasons (Koštal, 2006). In

mosquitoes, diapause has independently evolved several times and likely contributed to the establishment, maintenance and spread of natural populations (Denlinger and Armbruster, 2014; Diniz et al., 2017). Although diapause can occur at various developmental stages in different mosquito species, the most common type is embryonic diapause, with cases in different genera like *Aedes*, *Psorophora* and *Ochlerotatus* (Diniz et al., 2017). One of the mosquito species most studied in relation to embryonic diapause is *Aedes albopictus*, and the results of these studies have largely contributed to our understanding of the processes underlying this seasonal adaptation (reviewed in Armbruster, 2016). In this species, the inhibition of hatching, the increases in egg volume and lipid content, and the changes in lipid

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metabolism are hallmarks of embryonic diapause (Reynolds et al., 2012; Lacour et al., 2014).

In diapausing insects, the capacity for lipid accumulation plays an important role in winter survival because it allows fulfilling the energy demands during harsh conditions (Hahn and Denlinger, 2011). For example, in adult Dipterans, temperate species of the *Drosophila melanogaster* group, which undergo reproductive diapause for overwintering, can accumulate large amounts of triglycerides during the prediapause period (Ohtsu et al., 1992). Also, diapausing adult females of the mosquito *Culex pipiens* store twice the lipid content of non-diapausing individuals during the first 7 days after eclosion (Mitchell and Briegel, 1989). A similar pattern has been reported in diapausing eggs of the mosquito *Ae. albopictus*, which have been found to have 30% more total lipids than quiescent eggs (Reynolds et al., 2012). In mosquitoes, lipids, mostly triglycerides, are the primary fuel consumed during embryogenesis, making up 30–40% of the dry weight of insect oocytes (Van Handel, 1993). In addition, lipids have a key role in egg desiccation resistance (Sota and Mogi, 1992; Urbanski et al., 2010).

In embryos and pharate larvae in diapause, fuel conservation is especially critical because insect eggs are a closed system, i.e. only water and gases are exchanged between enclosed embryos and their environment, and there is no opportunity to obtain additional fuels (Reynolds et al., 2012). During oogenesis, energy reserves for embryos and pharate larvae are uptaken by receptor-mediated endocytosis, mainly in the form of yolk proteins and lipids (Ziegler and Van Antwerpen, 2006). These egg energy reserves are modulated by photoperiod-induced maternal effects, which enhance the accumulation of large amounts of metabolic reserves required for long non-feeding periods (e.g., embryogenesis and the dormant pharate larval stage). Some of the differences in lipid content are due to the maternal provisioning, whereas others appear to be due to the control of embryos and pharate larvae themselves. In effect, major transcriptional changes in genes related to lipid metabolism promote lipid conservation during embryogenesis and the pharate larval stage by decreasing lipid catabolism and increasing the biosynthesis of unsaturated fatty acids (Reynolds et al., 2012).

This increase in lipid content (mainly triglycerides) in diapausing eggs is accompanied by morphological differences. In *Aedes atropalpus*, for example, egg width and length are consistently larger in diapausing eggs than in non-diapausing eggs (Linley and Craig, 1994). In *Ae. albopictus*, egg volume is significantly larger in diapausing eggs than in non-diapausing eggs (Reynolds et al., 2012), mostly related to differences in width, but not in length (Lacour et al., 2014).

The yellow fever mosquito *Aedes aegypti* is the main vector of arboviruses such as the dengue, Zika and chikungunya viruses (Souza-Neto et al., 2019). This species, originally an African mosquito species, is abundant in tropical and subtropical regions (Kraemer et al., 2015). However, in the last decades it has increased its geographic range, colonizing also temperate climate regions (Eisen et al., 2014) in North America (Lima et al., 2016), Europe (Medlock et al., 2015), Asia (Kot-sakiozi et al., 2018) and South America (Zanotti et al., 2015; Fischer et al., 2017; Carbajo et al., 2019; Rubio et al., 2020). Unlike that observed in *Ae. albopictus*, dormancy in *Ae. aegypti* is exclusively considered as egg quiescence (Perez and Noriega, 2012; Denlinger and Armbruster, 2014), a non-seasonal dormant stage in which the inhibition of hatching of pharate larvae is interrupted immediately upon exposure to the appropriate stimulus (e.g., the presence of water in breeding containers). The lack of true diapause in this species has always been claimed as the reason of its inability to colonize temperate regions (Denlinger and Armbruster, 2014; Kramer et al., 2020; Mogi, 2011). However, we have recently shown that *Ae. aegypti* females from a temperate population (Buenos Aires City, Argentina) reared under a short-day (SD) photoperiod laid eggs with low hatching response. In fact, after one month of storage, 64% of the eggs from long-day (LD) photoperiod hatched, as compared to only 19% of hatching of the eggs from SD photoperiod, which maintained a hatching response below 40% up to three months, suggesting that, in this species, embryonic diapause

is possible (Fischer et al., 2019). Interestingly, the results of that study also showed that the hatching response of the eggs laid by different females reared under the SD photoperiod was not uniform, a fact that could indicate that such variability, if heritable, may be subjected to natural selection. In order to evaluate other features of this putative seasonal adaptation, further research should address the genetics and eco-physiological basis of embryonic dormancy of *Ae. aegypti*.

Thus, the aim of the present study was to assess the trans-generational effects of parental photoperiod on embryonic dormancy-related traits by comparing the triglyceride content and size of eggs laid by females reared under contrasting photoperiods (i.e., short-day [SD] and long-day [LD] photoperiods). To this end, two populations of the southernmost distribution in South America were analyzed. In this region, the populations of *Ae. aegypti* are characterized by a seasonal activity (Fischer et al., 2017), most likely as a consequence of the high mortality of larvae and adults due to cold conditions (Campos et al., 2020; Montini et al., 2021), and eggs are the main overwintering stage. The localities from where the populations were collected, i.e. Buenos Aires and San Bernardo (Argentina), differ in the duration of the winter season. Although in both localities the environmental temperature drops below the lower developmental threshold (i.e. the unfavorable period for juvenile development) during the winter season, the low-temperature period has a longer duration in San Bernardo than in Buenos Aires. For these reasons, our expectations were that the eggs laid by females reared under SD: 1) accumulate higher amounts of triglycerides and 2) exhibit larger size than those laid by females reared under LD. We also expected that: 3) SD eggs differ between populations in both traits under study, as a consequence of the different winter duration (i.e., we expected larger size and higher triglyceride content in eggs from San Bernardo given that the dormant stage in this population should be longer than that in the population from Buenos Aires).

2. Methods

2.1. Localities of the populations studied

Two populations of *Ae. aegypti* were studied: one from Buenos Aires City (BA) and the other from San Bernardo (SB), cities located about 280 km apart from each other, both in the temperate region of Argentina, but with some differences in their winter temperatures and durations. Climate descriptions are based on average data provided by the National Meteorological Service (2020). The information available comprises 30 years (1981–2010) for BA and 10 years (2001–2010) for SB.

Buenos Aires City (34° 36' 13.26" S – 58° 22' 53.61" W) is located in the northeast of Buenos Aires province, on the coast of the Río de la Plata River. The climate is temperate humid, with an annual mean temperature of 18.2 °C. Mean monthly temperatures from June to July are near or below 12 °C, which is the lowest known temperature for larvae to complete development (De Majo et al., 2019). Thus, the period unfavorable for *Ae. aegypti* development lasts approximately two months, and, during this period, the overall average temperature is 11.7 °C. The annual cumulative rainfall is 1237 mm, with higher mean monthly values in the warmer period (October to April: 127 mm) than during the cold period (May to September: 70 mm). Regarding the photoperiod, the maximum and minimum daylight duration are 14 h:29 min on December 21st and 9 h:31 min on June 21st, respectively (Servicio de Hidrografía Naval, 2020).

San Bernardo (36° 41' 10.92" S – 56° 40' 45.11" W) is located in the east of Buenos Aires province, on the coast of the Atlantic Ocean. The climate is temperate oceanic, with an annual mean temperature of 15.2 °C, and mean monthly temperatures are below 12 °C from June to September. Thus, in this case, the period considered unfavorable for *Ae. aegypti* development lasts approximately four months, and, during this period, the overall average temperature is 10.6 °C. Annual cumulative rainfall is 1061 mm, with slightly higher mean monthly values in the warmer period (October to April: 96 mm) than during the cold period

(May to September: 77 mm). Regarding the photoperiod, the maximum and minimum daylight duration are 14 h:40 min on December 21st and 9 h:20 min on June 21st, respectively (Servicio de Hidrografía Naval, 2020).

2.2. Colonies and collection of eggs

The colonies of BA were started from eggs collected during early fall (from March 15th to May 15th 2019), from different ovitraps placed throughout the city. The colonies of SB were started from eggs laid by field individuals. These individuals were collected from local breeding sites as larvae and pupae during mid-summer (early February 2019), and maintained in cages in the laboratory up to the emergence of the adults. The females were blood-fed on a human host (one of the authors: SF), and eggs were obtained from about 50 females from late summer through early fall (from February 15th to May 15th 2019). Both groups of eggs (BA and SB) were stored at 18 °C to ensure their conservation until their immersion.

2.3. Experimental design

The eggs analyzed in this study were the F1 and F2 generations from individuals collected in the field in BA and SB respectively. The conditions for induction of deep dormancy of the eggs were those adjusted previously (Fischer et al., 2019). These consisted of the rearing of two colonies for each population (BA and SB), one maintained under a Short-Day Photoperiod (SD: 10 h:14 h – Light:Dark) and the other maintained under a Long-Day Photoperiod (LD: 14 h:10 h – Light:Dark), under a temperature of 21 °C. These photoperiods represent the natural conditions of the fall-winter transition and the spring-summer transition in both localities.

The four colonies were simultaneously initiated by immersing approximately 3000 eggs from each locality in water with powdered baker's yeast. After 24 h, all larvae from each origin were separated in two groups, and each group was assigned to a rearing photoperiod that was maintained during the whole development. The larvae were maintained in pans and fed *ad libitum* with powdered baker's yeast. The pupation time in each colony was monitored to confirm the simultaneity of development among the four groups, and the pupae were transferred to rearing boxes where they emerged and the adults were maintained. Adult mosquitoes were provided with raisins as a source of sugar. After the emergence of the adults, females were provided access to a guinea pig for 2 h to favor blood feeding in two opportunities, separated by a 72-hour interval.

After the second blood meal, a group of females were separated in individual cages (6 cm in height × 3 cm in diameter), lined with a humid paper conditioned for egg laying and a raisin as a source of sugar. The individual cages were maintained under the same photoperiod at which the females were reared. The individual cages were inspected daily up to 6 days, and papers with eggs maintained under the same photoperiod and temperature for 9 additional days to ensure the complete development of the embryos, after which they were used for size measurements.

The remaining adults were maintained in the rearing cages, which were transferred to an intermediate photoperiod (12 h:12 h – Light:Dark) after the second blood meal, because there was not enough space available to maintain all the adults under their original photoperiod. Each cage was provided with containers lined with humid papers where females could lay their eggs. A few days later (4–7 days), a third blood meal was provided. Eggs were collected from the cages twice (once after one week, and then after 9 additional days), and each group of eggs was stored and analyzed separately. The two groups of eggs represent different durations of the exposure of females to the intermediate photoperiod, i.e. the first group corresponded to early laid eggs, by females exposed to the 12 h:12 h – Light:Dark photoperiod for one week or less, whereas the second group corresponded to late laid eggs, by females exposed for at least one week to that photoperiod before

oviposition. Although it was not originally the aim of the study, the change of all colonies to an intermediate photoperiod (12 h:12 h – Light:Dark) provided the opportunity to assess whether that intermediate photoperiod might reverse the effect of the previous parental photoperiod on the amount of stored lipids. These two groups of papers with eggs from each locality and rearing photoperiod combination were stored humid in plastic bags at 21 °C up to their use for the quantification of reserve substances (triglycerides).

2.4. Egg size estimation

Eggs from 20 females were used for each combination of population (BA, SB) and rearing photoperiod (SD, LD). Five or six eggs from each female were selected for measurement, attaining a total of 110–118 eggs for each combination. Eggs were placed in a horizontal position by wetting the paper and moving the eggs with an entomological needle. Eggs were photographed using a dissecting microscope (Leica APO S8), equipped with a digital camera. Measurements were performed on the digital photographs with the Leica Application Suite software V 4.0.0, and for each egg the maximum length and the maximum width were measured to the nearest 0.001 mm. These data were used to calculate the volume of each egg as $V = \text{it} * \text{Length} * \text{Width}^2 / 6$ (Armbruster et al., 2001).

2.5. Triglyceride content quantification

Five replicate pools of 400 eggs were used for each combination of laying time (early laid, late laid eggs), population (BA, SB), and rearing photoperiod (SD, LD). Eggs were homogenized in 700 µl of phosphate buffer (25 mmol l⁻¹ KH₂PO₄, pH 7.4) and centrifuged for 2 min at 14,300g. Then, 600 µl of the supernatant was removed and frozen. Homogenates for triglyceride quantification were performed 24 and 43 days after the collection of eggs for early and late laid eggs, respectively. Such difference in the times of homogenate preparation was due to logistical constraints. Triglyceride content was measured using the Colorimetric Assay Kit (GPO-PAP Method) by Wiener Lab (Rosario, Argentina). Samples (400 µl of homogenate + 500 µl of reaction buffer) were incubated at 37 °C for 10 min prior to absorbance quantification at 505 nm in a microplate reader (Biotek ELx808, Bad Friedrichshall, Germany). As this study focused on energy storage compounds, total lipid content (which includes structural lipids) was not determined, and only triglycerides were measured. Samples were measured in triplicate and mean values per egg were calculated and used for further analysis. Although during the first step of the quantification method the lipoprotein lipase enzyme hydrolyzes both triglycerides and diacylglycerides to glycerol and free fatty acids (Tennesen et al., 2014), we considered the total estimation as triglycerides because they are the primary form of lipid accumulation in diapausing eggs of *Aedes* mosquitoes such as *Ae. albopictus* (Batz and Armbruster, 2018).

2.6. Statistical analyses

The effects of the treatments on the size (length, width, and volume) and triglyceride content of eggs were analyzed with Generalized Linear Models (GLM), using the R package Version 3.6.2 (R Core Team, 2019), accessed through a user friendly interface in Infostat Software (Di Rienzo et al., 2019). In all cases, the normal distribution and the identity link function were used. Model assumptions were tested by visual analysis of the residuals as a function of predicted values, and the Q-Q plot. Post-hoc comparisons were performed with Fisher's LSD test on ranks (Conover, 1999), adjusting the significance of the test with the Holm-Bonferroni correction for multiple comparisons (Holm, 1979). For the egg size variables (length, width, and volume), the population, photoperiod and their two-way interaction were included as fixed effects. Furthermore, the identity of the female that laid the eggs was included as a random variable, to account for the lack of independence

among eggs from the same female.

For the triglyceride content of the eggs, the population, photoperiod, laying time, and their two- and three-way interactions were included in the model as fixed effects. Variances among photoperiod and laying time treatments were stabilized with the varident function (Pinheiro and Bates, 2000).

3. Results

3.1. Egg size

For the three size variables analyzed (length, width, and volume), variability among eggs within and between treatments was observed (Fig. 1). The length of the eggs showed no significant effect of the photoperiod ($F_{1,76} = 0.0001$, $p = 0.99$), the population ($F_{1,76} = 0.83$, $p = 0.36$) or their two-way interaction ($F_{1,76} = 0.0006$, $p = 0.98$), thus no differences among treatments were detected (Fig. 1a). The width of the eggs was affected by the photoperiod ($F_{1,76} = 40.72$, $p < 0.001$), but not by the population ($F_{1,76} = 1.05$, $p = 0.31$) or the two-way interaction ($F_{1,76} = 0.33$, $p = 0.57$). SD eggs showed a significantly larger width than LD eggs (Fisher LSD test: $p < 0.05$) (Fig. 1b). Finally, the volume of the eggs was affected by the photoperiod ($F_{1,76} = 26.92$, $p < 0.001$), but not by the population ($F_{1,76} = 0.26$, $p = 0.61$) or the two-way interaction ($F_{1,76} = 0.17$, $p = 0.68$). SD eggs showed a significantly larger volume than LD eggs (Fisher LSD test: $p < 0.05$) (Fig. 1c).

3.2. Triglyceride content

The triglyceride content was affected by the photoperiod ($F_{1,32} = 271.66$, $p < 0.001$), the population ($F_{1,32} = 34.75$, $p < 0.001$), the laying time ($F_{1,32} = 7.80$, $p < 0.01$), and the interaction of photoperiod and laying time ($F_{1,32} = 4.22$, $p < 0.05$), but not by the interaction of photoperiod and population ($F_{1,32} = 1.83$, $p = 0.19$), the interaction of population and laying time ($F_{1,32} = 1.43$, $p = 0.24$), or the three-way interaction ($F_{1,32} = 0.63$, $p = 0.43$). SD eggs showed a higher triglyceride content than LD eggs (mean \pm S.E. = 26.08 ± 0.75 for SD, 13.17 ± 0.24 for LD), and, on average, the eggs from SB contained more triglycerides than those from BA (mean \pm S.E. = 21.93 ± 0.55 for SB and 17.32 ± 0.55 for BA). Although late laid eggs had a higher triglyceride content than early laid eggs, this was only observed for LD eggs, but not for SD eggs, which did not differ in their triglyceride content across times of oviposition (Fig. 2).

4. Discussion

Many mosquito species of the genus *Aedes*, such as *Ae. atropalpus* (Anderson, 1968), *Ae. sollicitans* (Anderson, 1970), *Ae. vexans* (Wilson and Horsfall, 1970), *Ae. dorsalis* (McHaffey and Harwood, 1970), *Ae. caspius* (Vinogradova, 1975) and *Ae. albopictus* (Mori et al., 1981), display maternal effects on the control of offspring diapause induced by the photoperiod. The aim of the present study was to investigate how the

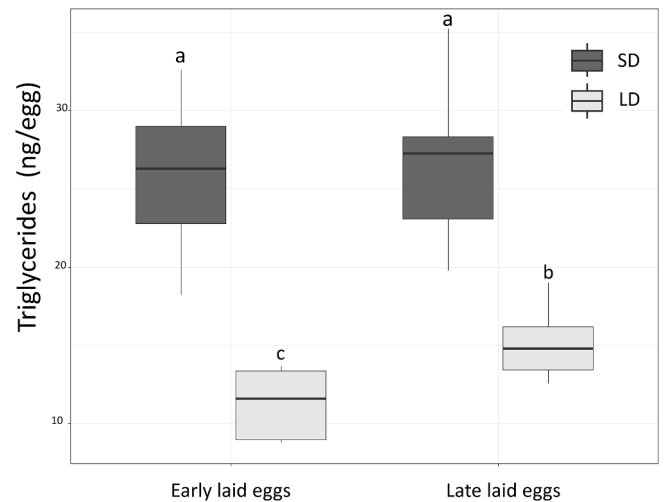


Fig. 2. Box plot of triglyceride content in different replicates ($n = 5$) of 400 eggs from the two populations studied (pooled) laid by females reared under two photoperiods (short-day [SD] and long-day [LD]), in two laying times (early and late). Different letters indicate significant differences according to Fisher's LSD ($P < 0.05$). Boxplot lines represent the median, and box boundaries are the upper and lower quartiles.

parental photoperiod affects the size and triglyceride content of *Ae. aegypti* eggs of temperate populations, looking for signatures of embryonic diapause.

4.1. Roles of energy reserves in overwintering and their relationship with egg size

Females reared under the SD photoperiod laid eggs with larger volume, as a consequence of the width enlargement, but not the length. Similarly, in a previous study by Lacour et al. (2014), SD eggs of temperate and tropical strains of *Ae. albopictus* showed a positive association between egg width and volume. In that study, since both temperate (diapausing) and tropical (non-diapausing) strains showed similar patterns, the authors argued that maternal photoperiod, and not diapause, influences egg size in *Ae. albopictus* (Lacour et al., 2014). Our findings, however, showed that the larger size of eggs laid by females reared under SD is accompanied by an increased accumulation of triglyceride content, comparing with eggs laid by females reared under LD. In most insects, these accumulated lipid stores are the primary fuel for overwintering and post-overwintering activities (Sinclair and Marshall, 2018). Given that eggs and pharate larvae consist of a closed system, energy conservation is especially important because overwintering eggs rely on a tight energy budget, as there is no opportunity to obtain additional fuels (Reynolds et al., 2012). In our study, since both diacylglycerides and triglycerides were jointly quantified, we might have over-estimated the amount of triglycerides in the samples. Thus,

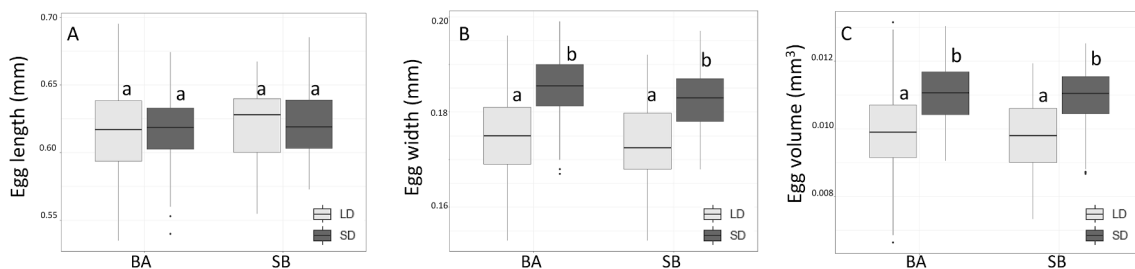


Fig. 1. Box plot of the a) length, b) width and c) volume of the eggs from two populations (Buenos Aires City [BA] and San Bernardo [SB]) laid by females reared under two photoperiods (short-day [SD] and long-day [LD]). Different letters indicate significant differences within each size variable according to Fisher's LSD tests ($P < 0.05$). Boxplot lines represent the median, and box boundaries are the upper and lower quartiles.

although the latter are the most important components of lipid reserves in diapausing eggs of *Ae. albopictus* (Batz and Armbruster, 2018), further lipidomic experiments will allow us to better distinguish the amount of each glyceride and the specific fatty acid composition in diapausing and non-diapausing *Ae. aegypti* eggs. High levels of triglycerides can be obtained through a decreased lipid catabolism and increased lipid storage, as previously observed in diapausing eggs of *Ae. albopictus* (Batz and Armbruster, 2018). Triglycerides provide energy reserves that can potentially serve as fuel sources to mitigate the metabolic demands linked to stress tolerance (e.g. cold, dehydration) during overwintering through oxidation of free fatty acids (Sinclair and Marshall, 2018).

In aedine mosquitoes, as well as in other insects, diapausing eggs are more resistant to desiccation (Sota and Mogi, 1992; Urbanski et al., 2010) and to low temperatures (Hanson and Craig, 1995; Thomas et al., 2012), which increases their probability of survival during the cold season. Although this has not yet been studied in *Ae. aegypti*, a similar increase in egg survival under stressful conditions should be expected, since the response observed should be adaptive in regions with cold and relatively long-lasting winters.

The ability to enter diapause varies among populations of the same species and across different insect orders, including mosquitoes (Shroyer and Craig, 1983; Urbanski et al., 2012; Bradshaw and Holzapfel, 2001). In general, populations that inhabit regions with more drastic winter conditions exhibit a higher ability to enter diapause and longer critical photoperiods (Tauber and Tauber, 1972; Bennett et al., 2005; Schmidt et al., 2005; Urbanski et al., 2012; Hou et al., 2016). The observed effect of the photoperiod, with a larger size and higher triglyceride content in eggs from SD females, similar in the two populations studied, might also occur in other temperate populations in Argentina. Since there are no similar previous studies on either tropical or temperate populations of *Ae. aegypti*, it is not possible to know whether this response to the SD photoperiod, i.e. the anticipation to unfavorable conditions, is exclusive to temperate populations. A slight (~15%) hatching inhibition in SD eggs has been recently shown in a subtropical population of *Ae. aegypti* from northeastern Argentina (Garzón et al., 2021), but these results should be taken with caution, since, in that study, the immersion was performed at 27 °C, a temperature significantly higher than that used in the present study. In this study, the high temperature might have interfered with the induction of diapause under a SD photoperiod, similar to that previously documented for *Ae. albopictus* (Pumpuni et al., 1992). Although our results are in line with embryonic diapause, further experiments analyzing strains of tropical origin, but reared under the same conditions as in the present study, will allow us to better understand whether the inhibition of hatching (Fischer et al., 2019), accumulation of triglycerides, and increase in egg size are only associated with overwintering individuals of temperate environments.

4.2. Contrasting variation between egg size and triglyceride content across diapausing and non-diapausing eggs

One intriguing result of the present study was that the populations studied showed differences in the average content of triglycerides, but not in the size of the eggs. Although the relationship of egg size with latitude has not been extensively studied in mosquitoes, studies in other insects have shown contradicting trends. Some have shown larger eggs at higher latitudes for multiple species, whereas others have shown either opposite clines or no clines in egg size (reviewed in Bauerfeind et al., 2018). In the present study, egg size exhibited a relatively small percentage of difference even between eggs from contrasting photoperiod treatments, where SD eggs had only an 11% larger volume than LD eggs. In contrast, the change in the triglyceride content between photoperiods was much larger, with increases of 93% (SB) and 102% (BA) in the content of triglycerides in SD eggs as compared with LD eggs. These differences in the triglyceride content between SD and LD eggs might have been even larger if the rearing cages had not been changed to the 12 h:12 h Light:Dark photoperiod after the second blood meal. This

suggests that, in *Ae. aegypti*, egg size (width or volume) has less variability among individuals or treatments than lipid content, similarly to that previously observed for *Ae. albopictus* (Reynolds et al., 2012). Presumably, these differences in responses also apply to variability among populations, and may thus explain why differences in the triglyceride content, but not in egg size, were detected between the populations studied.

4.3. Variation in triglyceride content between populations

The higher content of triglycerides in the SB population observed in eggs from both photoperiods could be related to the higher demands of energy reserves in that population. In the case of SD eggs, this might be explained by the longer duration of the winter season experienced in SB, which imposes a longer period of permanence in the egg stage on late-fall eggs. Since there is no clear explanation for the higher amount of triglycerides in the LD eggs of SB, this pattern should be confirmed and further explored in future studies.

Although lipids constitute an important part of the reserve substances in insects, other macronutrients such as carbohydrates and proteins also form part of the metabolic reserves both in diapausing and non-diapausing individuals (Hahn and Denlinger, 2007). Taking in consideration the lack of differences here observed in egg size between populations, one possibility is that the differences in the content of triglycerides between populations could be due to a difference in the composition of energy reserves in the eggs. Variation among populations in the composition of proteins, carbohydrates and lipids has been suggested in stoneflies, mayflies and dragonflies (Rotvit and Jacobsen, 2014), and demonstrated in snails (Baur and Baur, 1998) and dung flies (Bauerfeind et al., 2018). Further studies should address whether the lower lipid content in the eggs of *Ae. aegypti* of BA is compensated by higher amounts of other reserve substances. In insects, both proteins and carbohydrates are related to cold and desiccation resistance (Hahn and Denlinger, 2007). To our knowledge, no studies on the variation in the content of egg components among populations of mosquitoes have been performed, and thus there are no published results that could be compared with the results of our study.

4.4. Possible role of the critical photoperiod in the differences of triglyceride reserves between early and late laid eggs

Another interesting result was the increase in the amount of triglycerides in late laid eggs from the LD photoperiod, which could be a response to different situations. The most plausible explanation for the pattern observed is that the exposure to the 12 h:12 h Light:Dark photoperiod affected the amount of lipids stored in the eggs. The rationale of this explanation is that females reared under a LD photoperiod provide a low amount of triglycerides to their eggs, and that, as the photoperiod decreases, the amount of triglycerides is expected to increase (as confirmed in the present study). Females laying early eggs were exposed to the shorter intermediate photoperiod for less than a week, while females laying late eggs were exposed to that photoperiod between one and two weeks. If females were receptive to this cue during the maturation of their eggs, an increase in the amount of triglycerides laid by females exposed to a shortened photoperiod for a longer time would be expected. In contrast to eggs from the LD photoperiod, late laid SD eggs did not show a significant change in the amount of triglycerides as compared with early laid ones, i.e. the increase in the number of hours with light did not decrease their transfer of triglycerides to their eggs. Such results would be consistent with a critical photoperiod that is somewhere between the LD conditions (14 h:10 h Light:Dark) and the intermediate photoperiod (12 h:12 h Light:Dark). In this case, LD females should have experienced a change in their induction conditions from their rearing photoperiod to their oviposition photoperiod, but SD females remained under diapause-inducing conditions during the whole experiment.

An alternative explanation to the change and the higher variability in the triglyceride content in late laid eggs could be the fact that females laying late eggs probably had the chance to take more (up to three) blood meals, and thus might have transferred a higher amount of reserves to their eggs. In the case of the LD eggs, this could have increased the triglyceride content because females were better fed, and this effect could be (or not) additive to the effect of the change in the female photoperiod. In the case of SD eggs, two opposing processes might be acting: on the one hand, the effect of lengthening the photoperiod from SD to intermediate (i.e., 12 h:12 h Light:Dark) during the maturation of the eggs, possibly causing a decrease in the amount of reserve substances transferred to the eggs, and, on the other hand, the effect of repeated blood feeding, which might have increased the amount of reserve substances transferred to the eggs. In either case, our design might have underestimated some differences and should be therefore considered conservative, since the early laid eggs were analyzed at a shorter age (24 days) than late laid eggs (43 days), and it is likely that the amount of triglycerides in the late laid eggs had decreased more than that in early laid eggs, simply as a consequence of the different time elapsed until the analyses. We are not aware of studies on the nutrient depletion with age of aedine eggs, but a study analyzing the lipid content of recently hatched *Ae. aegypti* larvae demonstrated that larvae from older eggs had an impressive decrease in their lipid content as compared with those from younger eggs, suggesting a depletion in the egg reserves with time (Perez and Noriega, 2012).

Determining which of these explanations is more likely needs further investigation, firstly on the critical photoperiod for different populations, and secondly on whether this critical photoperiod changes or not along a latitudinal gradient in Argentina, as previously reported for *Ae. albopictus* (Urbanski et al., 2012) and *Wyeomyia smithii* (Bradshaw and Holzapfel, 2001). In *Ae. Aegypti*, no studies have yet been performed on this subject, but results from a previous study of our research group suggest that eggs from females reared under a 12 h:12 h Light:Dark photoperiod from SB have a stronger inhibition to hatch than those from BA, suggesting different critical photoperiods for both populations (Campos et al., 2020).

Another feature that deserves future research is the developmental stage/s sensitive to the photoperiod conditions that induce diapause. In *Ae. aegypti*, we have recently described the effect of the photoperiod on diapause induction and have shown that this physiological change is induced by the photoperiod experienced either by the parental generation and/or by the eggs during the development of the embryos (Fischer et al., 2019). Until now, no studies have been performed to elucidate the exact stage sensitive to the photoperiodic conditions, and it is not possible to infer this from studies on other mosquito species, since the stage sensitive to the photoperiod varies among species. For example, the most sensitive stages in *Ae. canadensis* are recently laid eggs (Pinger and Eldridge, 1977), whereas those in *Ae. albopictus* (Mori et al., 1981) and *Ae. atropalpus* (Anderson, 1968) are 4th instar larvae and pupae, and those in *Ae. vexans* are adults (Wilson and Horsfall, 1970).

5. Conclusions

In conclusion, our study provides new evidence of the unique ability of *Ae. aegypti* populations from temperate Argentina to induce changes in their eggs, which are compatible with diapause in response to the photoperiodic conditions experienced by their parents. Since these changes are likely to affect the performance and survival of the eggs during the cold season in temperate regions, it is expected that they will affect the ability of this species to continue colonizing new regions with harsher and longer cold seasons.

CRedit authorship contribution statement

Julián Mensch: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing - original

draft, Writing - review & editing. **Cristian Di Battista:** Investigation, Writing - review & editing. **María Sol De Majo:** Investigation, Writing - review & editing. **Raúl E. Campos:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Funding acquisition. **Sylvia Fischer:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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