When mothers anticipate: Effects of the prediapause stage on embryo development time and of maternal photoperiod on eggs of a temperate and a tropical strains of Aedes albopictus (Diptera: Culicidae)

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When mothers anticipate: Effects of the prediapause stage on embryo development time and of maternal photoperiod on eggs of a temperate and a tropical strains of *Aedes albopictus* (Diptera: Culicidae)

Guillaume Lacour [a,b,*], Florian Vernichon [a], Nicolas Cadilhac [a], Sébastien Boyer [c], Christophe Lagneau [a], Thierry Hance [b]

[a] EID Méditerranée, 165 Avenue Paul-Rimbaud, 34184 Montpellier, France
[b] Earth and Life Institute, Biodiversity Research Centre, Université catholique de Louvain, 1348 Louvain-la-Neuve, Belgium
[c] UMR-MIVEGEC, Institut de Recherche pour le Développement, 34394 Montpellier, France

ABSTRACT

Background: The diapause of *Aedes albopictus* is maternally induced by photoperiod and initiates at the pharate larvae stage in eggs. This pre-diapause results in enhanced survival eggs during the winter. This study aims to disentangle the effects of photoperiod and diapause on embryonic developmental time and egg size in *A. albopictus*. A temperate strain capable to perform diapause and a tropical strain unable of diapause were reared at 21 °C with long-(LD) and short-day (SD) lengths. Four distinct traits were studied on embryos and eggs were measured at the end of embryogenesis.
Results: The chronologies of embryo development for both strains were influenced by maternal photoperiod, especially in the temperate strain in which the development of SD eggs took longer than LD eggs. The delay increased gradually in the temperate strain, and reached up to 38 h at the end of embryogenesis. The kinetics of embryogenesis differed among the temperate and the tropical strains, each one of the 4 studied traits showing differences. For example the serosal cuticle was secreted precociously in the tropical strain. Egg width and volume are influenced by the maternal photoperiod and the strain × photoperiod interaction. For both strains, larger eggs were laid by female reared under SD when compared to LD.
Conclusions: The influence of several maternal effects was demonstrated in this study. The diapause process modifies greatly the length of embryogenesis in the temperate strain, whereas the maternal photoperiod has a direct influence on egg size and embryogenesis regardless of the strain considered. These findings provide useful data on chronology of embryonic development for integrative biology studies of egg pre-diapause stages.

1. Background

The Asian tiger mosquito *Aedes albopictus* is an emerging model for the study of the eco-physiology of egg diapause. It is a successful invasive species now present in all continents excepted Antarctica (Bonizzoni et al., 2013). It was first introduced in tropical countries around the 18th century, in water-stock of migrants' ships, and a second wave of invasion is still ongoing in tropical areas (Delatte et al., 2011). The colonization of temperate areas began in the middle of the 20th century, through used tire traffic by ships from Asian subtropical areas (Kuno, 2012; Urbanelli et al., 2000). As it is an effective vector for many arboviruses (Gratz, 2004), this species represents a new public health threat for the US and Europe. Its role as the exclusive vector in epidemics in temperate areas has been proven for chikungunya and dengue fevers (Rezza et al., 2007; Vega-Rua et al., 2013). There is a remote risk that diapausing eggs will be infected by arboviruses (Guo et al., 2007); this could lead to the persistence of arboviruses in mosquito populations in temperate countries.

Diapause is an adaptation and a complex physiological process defined as "a form of dormancy that is hormonally programmed in advance of its onset and is not immediately terminated in response of favorable conditions" (Denlinger and Armbruster, 2014). Diapausing individuals are indeed usually more resistant to harsh environmental conditions than non-diapausing ones. Desiccation (Sota and Mogi, 1992a; Urbanski et al., 2010a) and cold resistance are generally enhanced during diapause (Hanson and Craig, 1995) and post-diapause (Thomas et al., 2012) as for *A. albopictus* eggs. Diapause also enhances nutritive resources (Hahn and Denlinger, 2011), and even irradiation resistance (Brower, 1980) in insects. Numerous examples show that the diapause status of the progeny is determined by the exposure of ovipositing females to short photoperiod (Mousseau and Fox, 1998). The annual variation in day length is the most reliable indicator of seasonality in temperate areas. The photoperiodism, defined as the ability to determine day length with precision and to react to changes, is in most organisms a major adaptation to avoid harsh periodical environmental conditions (Danilevskii, 1965; Tauber et al., 1986). As a result it is used to initiate biological events essential to survival, such as reproduction, migration and dormancy (Danilevskii, 1965).

Egg diapause is a useful phenotype to study photo-induced maternal effects. Maternal effect is environmentally modulated transgenerational phenotypic plasticity (Mousseau and Fox, 1998). Investigating the pre-diapause process in the egg is of particular interest to elucidate the molecular process of the photo-induced maternal effects, from maternal induction to phenotypic initiation. Egg diapause is currently associated with any condition of suspended hatchability in temperate species that overwinter as cold hardy eggs. As described in Lepidoptera, egg diapause can be initiated early in the embryogenesis phase in the late gastrula stage as in the silkworm *Bombyx mori*, or at the end of the embryogenesis in the pharate larva stage, with a fully-developed larva still contained and compacted in the egg, as in *Lymantria dispar* and *Antheraea yamamai* (Denlinger and Armbruster, 2014). The Asian tiger mosquito has only one clearly defined stage of diapause, the pharate larva (Vinogradova, 2007). The changes in the eggs occurs during diapause preparation, before the initiation *sensu stricto* (Koštál, 2006), resulting in phenotypes with differences in morphology, development time and physiology. The developmental period preceding the stage of diapause initiation is frequently prolonged in insects (Denlinger, 2002; Harrat and Petit, 2009). This increased duration is linked to changes in metabolism, including protein synthesis and additional lipid storage (Denlinger, 2002).

In addition to diapause effects, photoperiod generates direct impacts on mosquito development and life history traits. For example, some larvae of *Aedes* and *Culiseta* species cannot reach maturity in the absence of light exposure (Clements, 1963), and the development time of *A. albopictus* larvae from the US is affected by the rearing photoperiod (Yee et al., 2012). Nevertheless it can be difficult to discriminate between effects of the mechanisms of a photoperiod-induced diapause and direct effects of photoperiod on organisms. It is the case for *Aedes mariae*, where diapause-programmed females preferentially seek sheltered holes in rock pools, providing them an appropriate hibernaculum against winter events like storms (Coluzzi et al., 1975). We can use tropical and temperate populations of *A. albopictus* to study this type of phenomenon in mosquitoes. Tropical strains are unable to perform diapause, contrary to temperate and subtropical strains which perform photo-induced diapause (Pumpuni, 1989). This fundamental difference between strains occurs naturally, contrary to other biological models of insects where strains must be artificially selected (Lee et al., 1997). It allows for the set-up of a discriminative comparison test of the consequences of change in daylength on the embryonic development of eggs with or without diapause.

The objective of this paper is to disentangle the effects of photoperiod and diapause on egg size and embryonic developmental time in *A. albopictus*. We predict that diapause induction in *A. albopictus* eggs will generate a prolonged embryo development sometime before the diapausing initiation. To test this prediction, we will investigate the effects of photoperiod and of the diapause syndrome by recording the size of eggs as related to an indicator of mother size (maternal wingspan), and by hourly monitoring the appearance of four features representing successive steps in the embryo development. The simultaneous study of a diapausing temperate strain and a non-diapausing tropical strain under long and short daylengths will allow us to disentangle the effects on development of the daylength experienced by the mother.

2. Methods

2.1. Ethic statement

The animal facility of the "Entente Interdépartementale pour la Démoustication du littoral méditerranéen" has received accreditation from the French Ministry of Agriculture to perform experiments on live guinea pig (permit number B34-172-29) in appliance of the French and European regulations on care and protection of Laboratory Animals.

2.2. Mosquitoes

Two strains of *A. albopictus* were used in this study. The European temperate strain named SPAM was collected in 2007 in the coastal area of Nice, France (43° 41′ 45″ N, 7° 16′ 17″ E). The tropical strain is native of La Reunion Island, located south-east of Africa near the Madagascar island, and was collected in 2011 in the coastal area of Saint-Denis Providence city (20° 52′ 44″ S, 55° 26′ 53″ E). The F16-F17 and F2-F3 maternal generations were used respectively for the temperate and tropical strains.

2.3. General rearing protocol

Mosquitoes of both strains were maintained in a laboratory room under a constant environment of 21.5 ± 0.3 °C, 80.1 ± 2.4% relative humidity, a photoperiod of 16 h of light and 8 h of darkness. Larvae were reared in batches of 500 larvae per pan (30.5 × 20 × 6 cm) in 2 l tap water and fed with 3.5 g of milled dog food during larvae development. This standardized protocol was chosen to produce an optimal expression of photoperiodic response, as it has been shown that this response is sensitive to temperature and larval diet (Pumpuni et al., 1992). After pupation, 500 pupae were placed per pan and transferred in cages in photoperiodic chambers. They were either submitted to non-diapausing long-days conditions (LD) with a light:dark cycle of 16 h:8 h, or short-days conditions (SD) inducing diapause in temperate strain with a light:dark cycle of 9 h:15 h. Photoperiodic chambers consisted of windowless plastic boxes (65 × 65 × 40 cm) with a zipper opening in black-cloth placed in the rearing room. Individual chambers were maintained at a constant temperature of 21.5 ± 0.4 °C and 79.1 ± 2.3% relative humidity, using a fan-produced air flow and a periodic air dampening system made of a water pot stirred using an aquarium air-pump. Light cycle was computer controlled and generated with a 3 watt bar of 42 white LED per photoperiodic chamber. There was no light transition between photophase and scotophase. Adult mosquitoes were left in the cages in photoperiodic chambers and were supplied with a 10% sucrose solution. The first blood meal was provided on anesthetized guinea pig 10 days after emergence, to make sure enough

time was given to strongly induce diapause in SD temperate females (Pumpuni, 1989).

2.4. Synchronous egg laying

Constraining females to lay eggs synchronously is necessary to know precisely the age of embryos. The protocol employed is adapted from Rezende et al. (2008). One day sugar-deprived females were blood-fed on anesthetized guinea pig. In order to hasten the laying of eggs when transferred into a suitable oviposition surface (nest-box), females were forced into egg retention during the 6 following days. Nest-boxes consisted of cotton filled cups humidified with larval rearing water and covered with a Whatman N°1 paper disk. Cups are closed by a piece of cloth, creating a space of 20/30 mm of height and 75 mm of diameter for about 7 female mosquitoes per cup. Nest-boxes containing mosquitoes were placed in an incubator to begin oviposition at 21 °C in darkness. Egg laying was allowed during 30 min for eggs destined to the study of serosal cuticle appearance, and during 60 min for eggs used to determine timing of segmentation, eyes and egg burster apparition and egg volume, afterwards females were removed from nest-boxes. The middle of the synchronous egg laying period determines the 0 h after egg laying (HAE).

Humid paper disks with eggs were stored in Petri dishes in incubators at 21 °C, and in darkness to avoid any possible reaction due to embryonic light sensitivity. Each replicate in the following experiments used different paper disks, with eggs laid by different females. Eggs were transferred out at different hours after egg laying for egg hatching calculation, egg volume measurements and embryonic observations.

2.5. Diapause incidence

This experiment was performed to verify that diapause was initiated only in temperate strain under maternal short days. Three replicates of at least 400 10-days old eggs produced in the first gonotrophic cycle were submitted to the following hatching protocol: eggs were immersed in oxygenated tap water during 30 min, for each test group of strain type and maternal photoperiod. A dose of 100 mg of ascorbic acid per liter of water was added to consume dissolved oxygen, in order to suppress the quiescence, a form of dormancy directly triggered and terminated by environmental conditions (Sinègre, 1974; Denlinger and Armbruster, 2014). The next day, eggs were brought out and let out to dry during 2 h, and were submitted to the hatching protocol a second time. Hatched eggs were counted. Unhatched eggs were bleached in a bath of Trpiš solution (Trpiš, 1970) during 30 min. The presence of ocelli and egg burster (a spine on the clypeus of embryo used to break the egg chorion in hatching) was investigated, as indicator of embryogenesis completion (Farnesi et al., 2009). Unhatched eggs were considered to be in diapause only when embryos were fully developed and without deformity. Egg hatching rate was calculated with embryonated and hatched eggs:

$$\text{Hatch } \% = (\text{hatched eggs} \times 100)/(\text{embryonated unhatched eggs} + \text{hatched eggs})$$

2.6. Wing size measurement

In insects, female size can strongly affect reproductive output, including egg size (Berrigan, 1991). Wing length was investigated to confirm that our standardized rearing protocol produced adults of similar size. At least 30 A. albopictus females were killed by freezing in each test group of strain type and maternal photoperiod. Their right wings were removed and flattened between microscope slide and cover glass. A stereomicroscope Zeiss Stemi SV6 fitted with a CCD camera Sanyo VCC-2972 was used to capture pictures of each wing using the software Ellix™ (Version 6.1.5, Microvision Instruments, Evry, France). Wing length was measured from the notch between the alula and the posterior margin of the wing to the distal tip of the wing excluding the apical fringe.

2.7. Egg volume determination

Sixty eggs in each test group were isolated from 6 nest-boxes at the rate of 10 eggs per petri dish nine days after egg laying. Each egg was placed on a damp Whatman paper onto dorsoventral position with a micro teasing needle, in order to show its side of prolate spheroid shape. Maximal width measurement can be subjective in the absence of landmark on the egg chorion. In order to limit the observational error, egg length and width measurements were repeated 3 times per egg and means were used to calculate each egg volume (Urbanski et al., 2012):

$$\text{Volume} = 1/6 \cdot \pi \cdot \text{Length} \cdot \text{Width}^2$$

Measurements were performed with the system described in the Section 2.6.

2.8. Embryonic development speed determination serosal cuticle formation

The first trait studied in order to determine the developmental time of embryos was the serosal cuticle. The desiccation resistance of Aedes species eggs is acquired with the complete formation of the serosal cuticle, an extracellular matrix that is resistant to chlorine digestion contrary to the dark colored chorion (Rezende et al., 2008). Three replicates of 150 eggs per HAE from temperate and tropical strains reared under SD and LD conditions were exposed to chorionic digestion with a 3.6% chlorine solution during 30 min. Eggs without their serosal cuticle lose their cellular content; on the contrary eggs with their serosal cuticle remain intact. Eggs were killed by the treatment.

The three other morphological traits used were studied by direct observation of embryo morphology. Three batches of 50 eggs/HAE bleached by Trpiš solution (Trpiš, 1970) during 30 min were used to observe embryo morphology under Zeiss stereo microscope STEMI SV6. Presence or absence of 3 morphological criteria was noted: embryo segmentation, pigmented ocelli and egg burster (Fig. 1). The presence of embryo segmentation was validated when the germ band presented 8 regular abdominal segments on its ventral side and an anterior lobe with cephalic segments. The egg burster, also called hatching spine, is the last morphological trait to appear (Farnesi et al., 2009). Morphological observations were compared to referenced stages of embryogenesis in Aedes sticticus (Trpiš et al., 1973), Aedes aegypti (Vital et al., 2010), Culex molestus (reviewed in Christophers, 1960), Culex pipiens (reviewed in Clements, 1963) and Aedes caspius (Sinègre, 1974).

2.9. Statistical analysis

Statistical analyses were performed using R 3.0.2 software (R Development Core Team, 2013) and a predetermined significance level of 0.01. Female wing size was compared between strains and rearing photoperiod using a two-way analysis of variance (ANOVA) including an interaction term. The effects of maternal photoperiod and strains origin on egg size descriptors (length, width and volume) were evaluated by a multivariate analysis of variance (MANOVA). Wing size was not included in the MANOVA due to a smaller number of replicates which would have unbalanced the data. Whenever a MANOVA was significant on 4
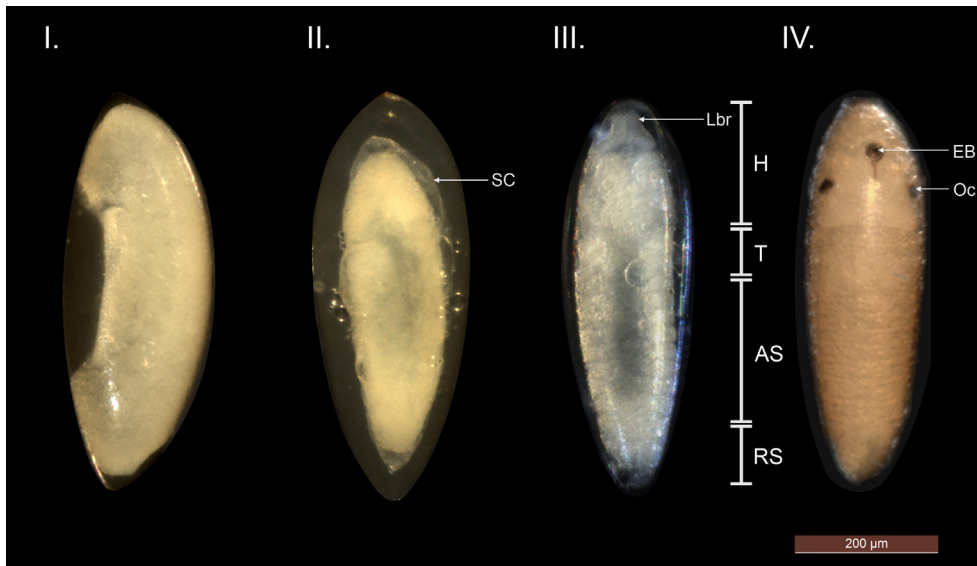
Fig. 1. Embryo morphology of temperate eggs of *Aedes albopictus* developed at 21 °C and bleached with Trpiš solution. (I) Lateral view of early embryogenesis without serosal cuticle 30 h after egg laying (HAE). (II) Lateral view of egg with serosal cuticle (SC) 47 HAE. (III) Ventral view of embryo with appearance of the head (H) with labrum (Lbr), thorax (T), eight abdominal segments (AS) and respiratory siphon (RS) and associated structure at 87 HAE. (IV) Dorsal view of first-instar-larvae with ocelli (Oc) and egg burster (EB) 160 HAE.

multivariate statistics (Pillai–Bartlett criterion, Wilk's lambda, Roy's largest root and Hotelling-Lawley trace), an ANOVA was realized for each dependent variable. The Kruskal–Wallis tests were used to confirm the results of the ANOVAs on significant effect of variables.

Embryonic development time was analyzed through reaction norms modeling of the frequency of occurrence for desiccation resistance, segmentation, ocelli or egg burster (Y), fitted by HAE with an iterative logistic model with a four parameters (a, b, c, d) logistic regression:

$$Y = a + \frac{b - a}{1 + \exp((c - \mathrm{HAE})/d)}$$

The parameters c and d were rendered dependent of the maternal photoperiod to test their influence on the embryogenesis time:

$$Y = a + \frac{b - a}{1 + \exp\left(\frac{c0 + c1 * \mathrm{photoperiod} - \mathrm{HAE}}{d0 + d1 * \mathrm{photoperiod}}\right)}$$

The parameters a, b, $c0$ and $d0$ were estimated per trait and per strain separately.

3. Results

3.1. Diapause incidence

As expected, only the eggs of the temperate strain maternally reared under short days were found to be in diapause, with a hatching ranging from 0.1% to 13.6% (Fig. 2). The egg hatching rate of each biological replicate ranged from 92.1% to 99.5%, both in the tropical strain eggs under both photoperiodic treatments and the temperate strain eggs under long day treatment.

3.2. Wing size

The MANOVA results showed a significant effect of the strain variable on female wing size, the latter is therefore not affected by the photoperiodic rearing conditions nor the strain × photoperiod interaction (Table 1, Fig. 3D). The Kruskal–Wallis test confirms
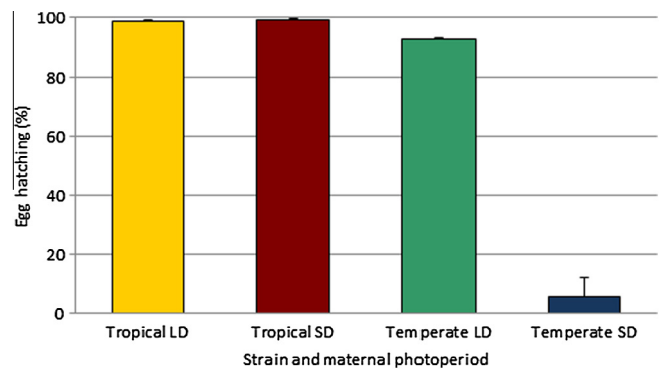

Fig. 2. Percentage (mean ± sd) of egg hatching in *Aedes albopictus* of tropical and temperate strains reared under long-days (LD) and short-days (SD) conditions at 21 °C. Only the temperate strain exposed to SD laid eggs in diapause process which do not hatch. Diapause was not induced by LD exposure of the temperate strain, and the tropical strain is not able to perform diapause whatever the rearing photoperiod.

the significant difference between wing size (means ± sd) of temperate (2503 ± 82 μm) and tropical (2433 ± 76 μm) strains (KW = 27.6, df = 1, $p < 0.01$) and the lack of difference between wing size of SD and LD groups ($p > 0.01$).

3.3. Eggs size

The MANOVAs revealed a significant main effect on egg measurements of the rearing maternal photoperiod (Pillai's criterion = 0.25, $F_{3,238} = 26.87$, $p < 0.01$), the strain origin (Pillai = 0.05, $F_{3,238} = 4.01$, $p < 0.01$) and the strain × photoperiod interaction (Pillai = 0.35, $F_{9,714} = 10.37$, $p < 0.01$). The other multivariate statistics (Wilk's lambda, Roy's largest root and Hotelling-Lawley trace) were all significant. The ANOVAs showed an effect of the maternal photoperiod and the strain × photoperiod interaction on egg width and egg volume, as opposed to egg length that is not influenced by the explanatory variables (Table 1). A strain effect on egg size was only revealed by the MANOVA and not by the ANOVA, underlining that this effect is probably small. The maternal photoperiod is also

Table 1
ANOVA extracts the effects of maternal photoperiod, strain and interaction of strain and maternal photoperiod on the size of eggs of *Aedes albopictus*. Univariate ANOVA on egg size was extracted from MANOVA test; ANOVA on wing size were done separately.

Response variable	Explanatory variable	df	SS	F	p[a]
Wing length	Strain	1	247963	35.71	**<0.01**
	Photoperiod	1	10341	1.49	0.22
	Strain × Photoperiod	1	0	0.00	0.10
Egg length	Strain	1	1747	2.48	0.12
	Photoperiod	1	90	0.13	0.72
	Strain × Photoperiod	3	7857	3.82	0.01
Egg width	Strain	1	145.9	1.69	0.19
	Photoperiod	1	5092.7	77.55	**<0.01**
	Strain × Photoperiod	3	5254.2	26.72	**<0.01**
Egg volume	Strain	1	521.62×10^{10}	3.41	0.07
	Photoperiod	1	6783.8×10^{10}	53.48	**<0.01**
	Strain × Photoperiod	3	7505.8×10^{10}	20.03	**<0.01**

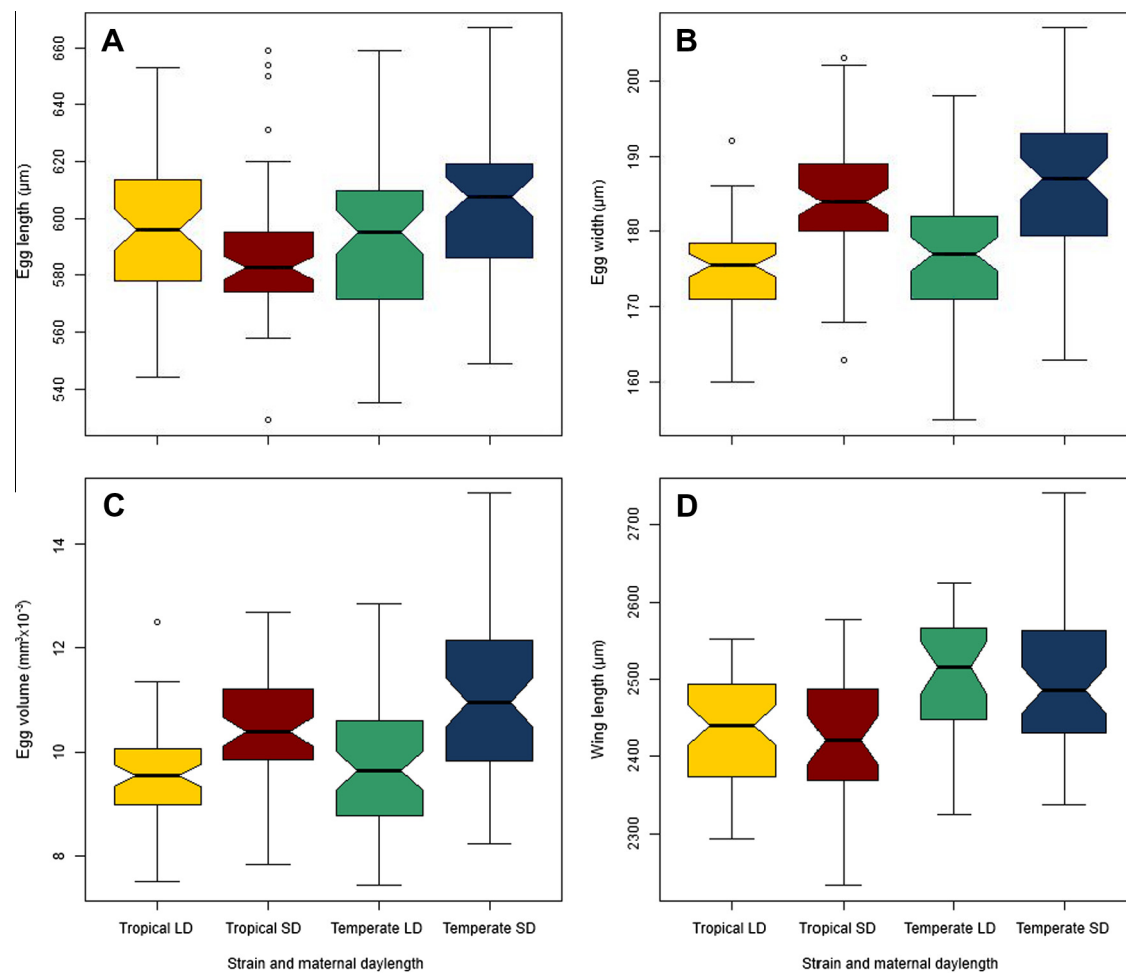[a] Significant *p*-values are in italic and boldface.

Fig. 3. Boxplots of *Aedes albopictus* egg and wing size measurement. Length (A), width (B) and volume (C) of 9-days old eggs. Female wing size (D) of tropical and temperate strains of *A. albopictus* reared under long days LD and short days SD at 21 °C. The solid horizontal line represents the median, the box encompasses the lower and upper quartiles, and the boxplot whiskers encompasses the data with values inferior or equal to 1.5 times the interquartile range from the box. Atypical data which values superior to 1.5 times are represented with empty circles. Eggs laid under short days conditions are wider and more voluminous, independently of population origin. Maternal wing size depends of strain origin.

the factor having the main effect on egg size, even if other factors may still interact. The mean volume of SD eggs is larger than LD eggs, regardless of the temperate or tropical origin of strains (Kruskal–Wallis = 43.5, df = 1, *p* < 0.01) (Fig. 3C). The egg length is not linearly related to egg volume ($R^2 = 0.31$) and represents a relatively small contribution in the calculation formula of the egg volume, as opposed to egg width which is positively correlated with the egg volume ($R^2 = 0.87$).

3.4. Embryonic development speed

The parameters values used to model reaction norms are presented in appendix (Table A.1). The reaction norms for the tropical strain reared under different day lengths remained closed for each studied trait while reaction norms for the temperate strain reared under SD and LD conditions moved away from each other during embryogenesis (Fig. 4).

The difference in the timing of the serosal cuticle, segmentation, ocelli and egg burster appearance is described below as measured when 50% of the analyzed embryos had acquired these traits (Table 2). The LD temperate embryos' serosal cuticle appeared 5 h later than in the tropical strain ones, and in temperate embryos reared under diapause-inducing conditions it appeared almost 10 h later. This difference between SD and LD temperate eggs was supported by direct observations: embryos aged from 18 to 47 HAE showed different stages of development between diapause-induced and non-diapause-induced embryos (Fig. A.2). The abdominal segmentation appears on the third day of embryonic development, first on LD embryos of the temperate strain. In this strain, diapause-programmed embryos are segmented 19 h after those reared under non-diapause-inducing photoperiod. The segmentation of SD and LD tropical embryos presents a temporal development intermediate to temperate embryos' development. Pigmented ocelli first appeared at 93 HAE in temperate strains and at 102 HAE in the tropical strain. The ocelli development began at the same time for LD and SD temperate strains, but finished 2 days earlier in the LD temperate strain. Egg burster formation in SD temperate strains took 38 h longer than in LD strains. The maternal photoperiod has a significant effect on the embryogenesis time for all the studied traits in the temperate strain and estimates of c_1, representing the distance between turning points, all being positive for SD model (Table 3). There is also an effect of the photoperiod in the tropical strain but not generalized to all the studied traits. In addition, c_1 estimates for the tropical SD models are not structured and have lower values than the temperate SD models, which demonstrate a lack of noticeable tendency in the differentiation of embryogenesis timing for the tropical strain. This corroborates the hypothesis that the diapause syndrome is responsible for the large embryonic developmental delay.

The delay between traits appearance during embryonic development of LD and SD temperate strains increases of approximately 10 h for each of the analyzed trait (Table A.3). This increase also seems not to be periodic but continuous during embryogenesis: whatever the strain and the maternal photoperiod considered, abdominal segmentation appeared among 61–65% of total embryogenesis and ocelli were formed among 82–89% of total embryogenesis (Table 2).

Regardless of the morphological feature investigated in the embryo, there are 4 constants: Firstly, temperate and tropical strains have different embryonic kinetics. Secondly, maternal photoperiod modifies the developmental time in both strains, but to a larger extent in the temperate strain. Thirdly, for the temperate strain, females with LD conditions produce eggs with a faster embryonic development that female exposed to diapause-inducing photoperiod. Fourthly, in all test groups the studied traits (except the serosal cuticle) appeared at the same percentage of total development, although the entire embryo development period differs among strains and temperate photoperiods. These results argue in favor of the effect of a progressive diapause preparation process rather than just punctual changes in the embryonic program of the temperate strain.
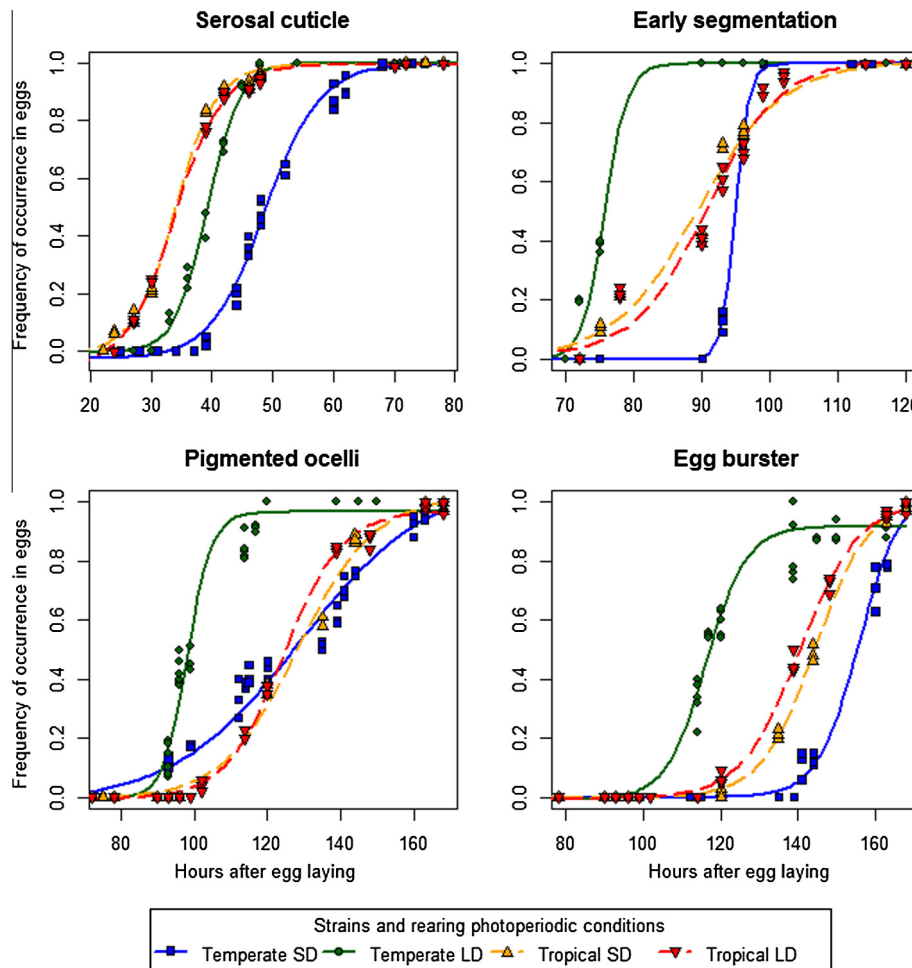
Fig. 4. Photoperiodically-induced egg diapause increases embryogenesis duration in temperate strain of *Aedes albopictus*. Experimental results (plots) and sigmoid nonlinear models (line) of reaction norms for development time of serosal cuticle, early segmentation, pigmented ocelli and egg burster during embryogenesis in tropical and temperate strains of *A. albopictus* reared under long days LD (16 h daylength) and short days SD (9 h daylength) at 21 °C.

Table 2

Developmental duration of *Aedes albopictus* embryos of temperate and tropical strains under different photoperiodic conditions. Development embryonic time is determined by following the formation of 4 morphological indicators: the serosal cuticle providing desiccation resistance, the abdominal segmentation, the ocelli and the egg burster. HAE values were assessed from sigmoid nonlinear models of traits appearance.

Strain	Maternal photoperiod	Moment of trait acquisition in 50% of embryos							
		Serosal cuticle		Abdominal segmentation		Ocelli		Egg burster	
		HAE[a]	%[b]	HAE[a]	%[b]	HAE[a]	%[b]	HAE[a]	%[b]
Temperate	LD	39.3	*34*	75.8	*65*	98.4	*84*	117.2	*100*
Temperate	SD	48.9	*31*	94.9	*61*	127.2	*82*	155.3	*100*
Tropical	LD	34.3	*24*	89.3	*64*	125.1	*89*	140.5	*100*
Tropical	SD	34.1	*24*	90.6	*64*	128.2	*89*	144.4	*100*

[a] HAE: hours after egg laying.

[b] Percentage of total embryonic development time, relative to egg burster formation, are in italic.

4. Discussion

Based on a detailed morphological analysis, we demonstrated for the first time the modulation of embryonic developmental rate due to diapause preparation in *A. albopictus* eggs. The preparation stage of diapause syndrome implies numerous physiological adaptations which necessarily involve an energetic investment. Recent transcriptional works already suggested the existence of a developmental delay of embryos during diapause preparation: a delayed expression of cell-cycle regulators and genes in diapausing SD eggs compared to LD eggs was put in evidence in a US temperate strain of *A. albopictus* (Poelchau et al., 2013a). However, these delays in physiological processes were not correlated to visible morphological differences in the development (Reynolds et al., 2012; Poelchau et al., 2013a). Hence, regardless of the origin of the strain, embryogenesis is also slightly sensitive to the maternal photoperiod.

The embryonic time varies between tropical and temperate strains. Both strains have been crossed and gave a viable and fertile offspring, confirming that tropical and temperate strains are of the same species, as it was already attested on other strains (Hanson et al., 1993). Globally, at lower temperatures tropical strains of *A. albopictus* have an expanded embryonic developmental time compared to temperate strains (Waldock et al., 2013). Variability in the studied developmental patterns could be the result of an absence of directional selection or can reflect a selection process to the local environment (Bradshaw et al., 2004). As this study's experimental rearing temperature corresponds to the natural coldest developmental conditions on La Reunion Island shores, the tropical strain was reared under a more stressful environment than the temperate strain whose fitness is optimized for this average temperature. However, even among tropical strains of *A. aegypti*, developmental rates have been demonstrated to be heterogeneous under the same temperature (Couret and Benedict, 2014). Different larval developmental rates between populations have previously been observed in other mosquito species like *A. (Oc.) triseriatus* (Holzapfel and Bradshaw, 1981), but not in *A. albopictus* (Waldock et al., 2013). This underlines the need to compare several temperate and tropical strains in order to confirm our observations.

Aedes species are particularly exposed to desiccation, and its serosal cuticle development is more effective than in other mosquito genera (Vargas et al., 2014). Interestingly, only the serosal cuticle's complete secretion took place faster in our tropical strain as compared to our temperate strain. The first hypothesis to explain this kinetic difference is that this protection mechanism against a dried-up environment must be subject to a strong selective pressure, especially in the tropics where the threat of death by desiccation is extreme (Tauber et al., 1986). However *A. albopictus* eggs are laid in outdoor water containers likely to dry up, and most eggs in diapause process are laid during the favorable season when average temperature is still high in temperate area. The additional delay in the formation of serosal cuticle generated by diapause preparation incurs a longer period of sensitivity to desiccation in a context of strong evaporation. Thus the induction of diapause syndrome could have hazardous effects on eggs, even if diapause-programmed eggs later offset this risk notably with a higher quantity of surface lipids on the chorion which increases desiccation resistance (Sota and Mogi, 1992a; Urbanski et al., 2010a). The serosal cuticle is an important structure protecting from desiccation but not the only one (Rezende et al., 2008). The second hypothesis is that tropical strains developed other structural and metabolic mechanisms to improve the egg's waterproof quality, and counterbalance a more rapid but potentially weaker or thinner serosal cuticle. This hypothesis is supported by the presence of a higher quantity of surface hydrocarbons on tropical eggs than temperate eggs (Urbanski et al., 2010a). Temperate strains would favor the production of a stronger or improved serosal cuticle than tropical strains, which requires a prolonged period of formation.

Previous articles provided information about *Aedes* eggs morphology affected by the duration of light exposure. Eggs of the tropical species *A. (Oc.) epactius* reared under SD were wider than those reared under LD. Electron microscopy studies of eggs of the close temperate species *A. (Oc.) atropalpus* able of diapause revealed different and stronger modifications in size and shape: LD eggs were longer and narrower than SD eggs, with changes in the outer chorion structure (Linley and Craig, 1994). However no differentiation of the possible factors, day length and diapause, responsible for these changes was obtained. Our study is thus the first to demonstrate that maternal photoperiod, and not diapause, influences egg volume in an *Aedes* species capable of diapause.

The structure of mosquito eggs is therefore sensitive to several seasonal factors. Indeed, *Anopheles sacharovi* (Favre) and *Anopheles punctipennis* (Say) produce "winter" eggs almost totally covered by exochorion (Theodor, 1925; Fritz and Washino, 1992), and "winter" eggs of *A. sacharovi* possess a small float and are larger than "summer" float-less eggs. In these cases, the morphological differentiation originates in response to temperature fluctuations, and not from the diapause syndrome, as diapause occurs at the larval or adult stages in Anopheles species (Theodor, 1925). The latter are capable of egg quiescence, a process fairly similar to diapause at the molecular level (Poelchau et al., 2013b), however quiescence is by definition an aseasonal state of inactivity (Vinogradova, 2007).

The mechanisms involved in egg structure variability in mosquito are not determined and may be multiple. Concerning the photoperiodic causality, we suspect that a circadian rhythm plays a part in the hormonal production and reserve storage, such as was demonstrated in several insect groups, including mosquitoes (Bloch et al., 2013). Egg production is regulated by hormones which are photophase dependent, as demonstrated in Hemiptera *Rhodnius prolixus* (Vafopoulou et al., 2012). Lipids represent the

Table 3
Effect of maternal photoperiod on development of *Aedes albopictus* embryos of temperate and tropical strains. Results are presented for SD reared mosquitoes. There is one degree of freedom for each parameter. The estimate and *t*-value for LD reared mosquitoes are the same but multiplied by −1. A statistically significant effect on dependent-parameters c_1 or d_1 demonstrates an influence of the maternal photoperiod on the embryonic developmental time.

Strain	Parameter	Serosal cuticle			Segmentation			Ocelli			Egg burster		
		Estimate (±S.E.)	*t*	*p*[a]	Estimate (±S.E.)	*t*	*p*[a]	Estimate (±S.E.)	*t*	*p*[a]	Estimate (±S.E.)	*t*	*p*[a]
Temperate	c_1	9.69 (±0.14)	70.79	**<0.01**	19.14 (±0.10)	184.84	**<0.01**	26.62 (±0.79)	33.82	**<0.01**	36.87 (±0.64)	57.58	**<0.01**
	d_1	1.68 (±0.14)	11.90	**<0.01**	−0.62 (±0.09)	−7.12	**<0.01**	11.30 (±0.70)	16.18	**<0.01**	−0.80 (±0.54)	−1.47	0.14
Tropical	c_1	−0.51 (±0.17)	−3.02	**<0.01**	−1.16 (±0.49)	−2.35	0.02	2.39 (±0.56)	4.28	**<0.01**	3.64 (±0.28)	12.94	**<0.01**
	d_1	−0.31 (±0.11)	−2.96	**<0.01**	0.73 (±0.50)	1.45	0.15	1.46 (±0.45)	3.23	**<0.01**	−0.79 (±0.28)	−2.82	0.01

[a] Significant *p*-values are in italic and boldface.

major energetic source of eggs and are essential for the development of the embryo. Lipid reserve in eggs is provided by the mother (Ziegler and Van Antwerpen, 2006). If that storage is dependent of photoperiod, and is more particularly developed during scotophase, long nights will enhance egg volume. Organism size cannot be explained by the simple sum of mechanisms that regulate the size and number of cells in organs (Nijhout, 2003), but a positive relationship exists with the energy stock and egg size in some species, like the butterfly *Bicyclus anynana* (Geister et al., 2009). A study carried out on a US temperate strain of *A. albopictus* found a lipid reserve more important by 30% in diapause-induced pharate larvae (Reynolds et al., 2012), linked to an increase in egg volume. Unfortunately the lack of tropical strain as control group in Reynolds experiment led them to conclude that the increase in egg volume was a consequence of diapause expression, whereas the presence of the control group in our experiment allowed us to demonstrate the photoperiodic causality.

Nevertheless, we cannot exclude the possibility that this increase in egg size under short day length could be a relic feature of a lost diapause capacity in tropical populations. Indeed, diapause can be quickly counter selected in laboratory (Pumpuni, 1989), or in field populations as described during the species' colonization of Florida (Lounibos et al., 2003). On the contrary, even though Brazil was colonized by tropical strains of *A. albopictus*, in laboratory some populations still show a small but significant decrease in egg hatchability under short photoperiod (Lounibos et al., 2003). In consequence it is possible that tropical populations could be selected to express diapause again. The black cutworm *Agrotis ipsilon* is a good example of persistence of diapausing metabolism: even though larvae and adults of this species are able of diapause elsewhere in the world, Japanese populations migrate from north to south of the island to overwinter. It allows them to avoid diapause initiation, but adults reared under short day length show a delay in the development of their ovarian maturation (Tauber et al., 1986).

We make the hypothesis that short day length is interpreted by females as stressful environmental conditions. If bigger eggs are better adapted to survival in harsh environment, then females will anticipate poor development conditions for its offspring by laying eggs of bigger size. For example, a higher amount of yolk in bigger eggs could increase the survival of embryos. On an interspecific scale, larger eggs have an increased developmental duration (Gillooly and Dodson, 2000), and in Aedes (Stegomyia) species larger eggs are more resistant to desiccation (Sota and Mogi, 1992b). It is unclear how these interspecific observations could be verified inside a species. No modulation of desiccation resistance was observed in eggs of a tropical strain of Kuala Lumpur, Malaysia, reared under different photoperiodic conditions (Urbanski et al., 2010a).

Photoperiodic rearing conditions don't modify maternal wing length; consequently we dismiss a possible indirect effect of maternal size on eggs volume. Previous works on mosquitoes showed that egg length is neither influenced by mosquito wing

size (Shannon and Hadjinicalao, 1941; Pumpuni et al., 1992) nor by maternal larval nutritional regime (Pumpuni et al., 1992). However it must be noted that these studies did not measure egg width, which seems to be the main factor of egg volume variability. Finally, the ecological meaning of photoperiod is questioned in tropical populations. Indeed, annual day length variation is subtle under tropics and less well correlated with environmental events (Tauber et al., 1986).

5. Conclusions

The comparisons of the embryogenesis chronology and egg size demonstrate the existence of photo-induced maternal effects in temperate and tropical populations of *A. albopictus*. This study reveals the major influence of maternally induced diapause on the developmental time of the temperate embryos. It is a helpful tool to explore the many facets and implications of diapause metabolism on the organism. It underlines the need to investigate the physiological consequences of diapause preparation in *A. albopictus* by genomic and proteomic approach. Recently the genome of the yellow fever mosquito *A. aegypti* (Nene et al., 2007) was sequenced and will thus become a reference model for developmental studies (Clemons et al., 2010). Although unable of diapause, it is a closely related species of the Asian tiger mosquito (Reinert et al., 2004) and will provide precious data for comparison. Genomes of an Italian and a Chinese strain of *A. albopictus* are currently sequenced and annotations are expected this year (Bonizzoni et al., 2013). These will help to improve our knowledge on the molecular processes of diapause, already initiated on early diapause preparation in oocytes (Urbanski et al., 2010b), embryonic diapause preparation (Reynolds et al., 2012), diapause initiation and maintenance (Poelchau et al., 2013b) and diapause termination.

Understanding the course of diapause could be useful to develop a new strategy for mosquito population control, by inhibiting diapause and foiling winter survival (Tauber et al., 1986; Hanson et al., 1993). In the light of these elements *A. albopictus* emerges as a fantastic biological model for the study of maternal effects and egg diapause.

6. Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2014.10.008.

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