Effects of diapause and cold acclimation on egg ultrastructure: new insights into the cold hardiness mechanisms of the Asian tiger mosquito *Aedes (Stegomyia) albopictus*

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ABSTRACT: The Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae, SKUSE), is an important threat to public health due to its rapid spread and its potential as a vector. The eggs of *Ae. albopictu*s are the most cold resistant life stage and thus, the cold hardiness of eggs is used to predict the future occurrence of the species in distribution models. However, the mechanism of cold hardiness has yet to be revealed. To address this question, we analyzed the layers of diapausing and cold acclimatized eggs of a temperate population of *Ae. albopictu*s in a full factorial test design using transmission electron microscopy. We reviewed the hypotheses that a thickened wax layer or chorion is the cause of cold hardiness but found no evidence. As a result of the induced diapause, the thickness of the dark endochorion as a layer of high electron density and thus an assumed location for waxes was decreasing. We therefore hypothesized a qualitative alteration of the wax layer due to compaction. Cold acclimation was causing an increase in the thickness of the middle serosa cuticle indicating a detachment of serosa membrane from the endochorion as a potential adaptation strategy to isolate inoculating ice formations in the inter-membranous space. ***Journal of Vector Ecology*** **41 (1): 142-150. 2016.**

Keyword Index: Chorion, cold tolerance, compaction, freeze avoidance, wax layer, winter survival.

INTRODUCTION

Blood-sucking arthropods, especially mosquitoes, pose health risks for humans, domestic animals, and wildlife due to the pathogens they can transmit. The globally invasive Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus* (Skuse) is capable of transmitting at least 22 mainly tropical arboviruses and other parasites under laboratory conditions and is a known vector of pathogens such as those causing dengue and chikungunya fevers (Genchi et al. 2009, Moore and Mitchell 1997, Shroyer 1986). It is therefore an acknowledged vector in many tropical and subtropical regions in the world, and recently also in temperate regions due to its rapid spread in the last decades (Benedict et al. 2007). The barrier for the further distribution of this mosquito is clearly linked to natural temperature thresholds (Thomas et al. 2012). The aquatic larvae and pupae of this mosquito are not able to cope with annual average temperatures below 11° C (Kobayashi et al. 2002).

In contrast, the egg of *Ae. albopictus* is the only life-stage capable of tolerating temperatures as low as -10° C (Hanson and Craig 1995a). However, the triggers and cellular mechanisms responsible for their below-zero degree tolerance are not yet fully understood. In general, there are three different ways for an insect to cope with those low and otherwise lethal temperatures: behavioral adaptation, freeze avoidance, and freeze tolerance (Duman et al. 1991). Estrada-Franco and Craig (1995) claimed that all studies about the adaptation of insect eggs (mainly dipterans) to subzero temperatures carried out so far demonstrated a freeze avoidance strategy, according to the definition of Duman et al. (1991).

The ability of the pharate mosquito larvae within the eggshell to survive suboptimally low temperatures was described as cold hardiness of eggs by Hanson (1991). The cold hardiness of *Ae. albopictus* is triggered by two environmental factors and probably their interaction. Diapausing is a state of reduced metabolism to hibernate as pharate larvae in an egg and is induced by a shortened photoperiod (Wang 1966). In contrast, cold acclimation by incubating eggs at 5 to 0° C can increase the cold hardiness more effectively than diapausing (Hanson and Craig 1994, Mori et al. 1981). The interaction between these two mechanisms is due to cold temperatures that can also induce diapause even though their impact is less than by photoperiodical induction (Imai and Maeda 1976, Mori et al. 1981, Wang 1966).

Many mechanisms of gaining cold hardiness in insects are based on physiological mechanisms as an increase of colligative low molecular weight antifreezes in order to lower the super cooling point (SCP) (Duman et al. 1991, Furusawa et al. 1982, Storey and Storey 1988). However, neither diapause nor cold acclimation or even geographic origin affected the SCP of the eggs of *Ae. albopictus* and, therefore, a biochemical cold hardiness mechanism seems unlikely for the pharate larvae of *Ae. albopictus* (Hanson and Craig 1995b).

At the cellular level, there are two mechanisms by which freezing harms an organism. On the one hand, freezing damages the cytoskeleton and plasma membrane of insects by intracellular ice formation (Duman et al. 1991). On the other hand, cells react with a loss of water and solutes due to the growth of extracellular ice formations (Duman et al. 1991) that consequently lead to cell dehydration and finally osmotic shock with intracellular and membrane damage beyond repair (Duman et al. 1991). As Storey (1997) concluded, extracellular ice formation, desiccation,

or hypersaline stress from the removal of intracellular water and subsequent osmotic stress, all have the same result.

Focusing on desiccation, Hinton (1981) postulated that the chief protection of the embryo against water loss comes from waxes or fatty acids. There are several mosquito species that are able to cope with considerable desiccation, but the most dry resistant genus is *Aedes* (Hinton 1981, Sota and Mogi 1992a, 1992b, Sota et al. 1993). Comparing species, this ability seems to be correlated with the thickness and darkness of the endochorion of the egg in combination with chitin and waxes (Harwood and Horsfall 1959). In *Aedes dorsalis*, dry resistance develops between the 20th and 27th hour after oviposition (Telford 1957). Moreover, dry resistance seems to be generally connected with the development of primary larvae and serosal cuticle, which is secreting the waxy layer (Clements 1993, Hinton 1981, Telford 1957). In *Aedes hexodontus* eggs, there are two changes in water efflux observed during desiccation: the first after hardening and darkening of the chorion and the second after the formation of the serosal cuticle (Beckel 1958).

Some authors postulated that tanning and/or melanization of the outer serosa cuticle in combination with an interaction with lipids cause the dry resistance of insect eggs (Furneaux and McFarlane 1965, Harwood and Horsfall 1959). Recent research supports this postulation by showing that the development of dry resistance in *Ae. aegypti* is associated with the serosal cuticle secretion combined with chitinization (Rezende et al. 2008). Nevertheless, Rezende et al. (2008) discussed that neither the serosa cuticle nor its chitin layer may be solely responsible for the water impermeability, and an additional wax layer as a part of the serosa cuticle would be an important factor for desiccation resistance in *Ae. aegypti* eggs. However, there is only scarce information about mosquito eggshell thicknesses and no information at all regarding the thickness of serosal cuticles (Farnesi et al. 2015). Interestingly, the diapausing eggs of *Ae. albopictus* are not only more cold hardy than the non-diapausing eggs but are more dry resistant as well (Sota and Mogi 1992a).

Following up on this academic debate we hypothesized that the development of the wax layer as well as the sclerotization and chitinization of the outer serosa cuticle in Culicidae, and especially in Aedini, is a crucial mechanism to achieve cold hardiness. Moreover, we predicted a thicker wax layer and stronger sclerotization and chitinization of the outer serosa cuticle in cold hardy eggs of *Ae. albopictus*. To test these hypotheses, we measured the thickness of all layers in the chorion of diapausing and non-diapausing eggs of *Ae. albopictus* that were either cold acclimated or non-cold acclimated.

MATERIALS AND METHODS

Biological material

To compare the ultrastructure of the eggs, we used a temperate population of *Ae. albopictus* from Riccione (Rimini Province, Italy) with a well documented ability of diapausing and cold adaptation (Thomas et al. 2012). This population was cultured to the F_3 generation in conditioning cabinets (MKKL 600, FLOHR instruments, The Netherlands) at 25 ± 1° C and 90 ± 10% humidity. Non-diapausing eggs were obtained by rearing L_1-L_4 larvae, pupae, and adults under a 16:8 h L:D photoperiod, whereas diapausing eggs where obtained by breeding L_1-L_4 larvae, pupae, and adults of *Ae. albopictus* under a 8:16 L:D photoperiod (Thomas et al. 2012). Whereas other studies induced diapause at 21° C (Hanson and Craig 1994, Thomas et al. 2012), we decided to keep the larvae, pupae, and adult females at 25° C (i) to obtain a full crossed two factorial study design which can only be performed straight if only two factors are independently changed, (ii) because it is the optimal temperature with respect to the intrinsic rate of growth (20° C: 0.072; 25° C: 0.140) in order to reduce unwanted side-effects (Delatte et al. 2009), and (iii) diapausing strains of *Ae. albopictus* have a clear photoperiodic response between 11 and 14 h of light a day, as well at 21° C as at 25-27°. Thus, rearing at 21° C has no significant advantage for inducing diapausing when the photoperiod as the main trigger is shifted to 8:16 h L:D as in this experimental setup (Pumpuni et al. 1992). Eggs were dried for ten days at 25 ± 1° C and 90% RH, and thus were fully sclerotized prior to pooling of ca. 50 individuals of both diapause photoperiodic exposure and dividing them in batches of about 25 eggs for cold and non-cold acclimation exposure in 1.5 ml Eppendorf cups. In order to achieve a full crossed factorial study design, half of the cups with eggs produced by non-diapause and diapause mosquitoes were transferred to a temperature controlled box at +3.11 ± 0.05° C for 24 h for cold acclimation, and the other half of the cups remained at 25° C ± 1° C for 24 h.

Fixation of egg material, and transmission and scanning electron microscopy

After inducing the cold acclimation of half of the diapausing and half of the non-diapausing eggs, batches of 10 to 12 eggs of all four states were firmly broached with a scalpel at the anterior and more blunt pole (see Figure 1b) and transferred to a 2% glutaraldehyde in 0.1 M sodium cacodylate trihydrate buffer (pH 7.2). Fixation over 3 h was performed according to Sahlén (1996) and Monnerat et al. (1999) except for an addition of 10% sucrose to increase molarity to a physiological value of 460 mmol (Bender et al. 1984, Richards and Meier 1974).

After washing in 0.1 M sodium cacodylate trihydrate buffer, the samples were post-fixed for 2.5 h with a 1% solution of osmium tetraoxide in 0.1 M sodium cacodylate trihydrate buffer and then rinsed twice in 0.1 M sodium cacodylate trihydrate buffer. Afterwards, samples were dehydrated stepwise with ethanol (15 min at 50%, 70%, 90%, 2 × 100%) and propylenoxide (15 min at 2 × 100%). Before embedding in 100% araldite (40ml aradit CY [Serva 13824], 40 ml DDSA hardener [Serva 20755], 1.4 ml DMP accelerator [Serva 36975]), samples were first infiltrated with 1:1 propylenoxid:agar (w/w) overnight and then transferred twice to 100% agar for 3 h. After hardening of samples at 60° C for 48 h, semi-cuts and ultra-cuts were prepared (Reichert Ultracut S, Leica; Ultracut, Leica). Sections were always performed from the posterior end. In order to produce comparable sections in the level between the anterior and posterior ends of the egg, semi-cuts were performed until the head of the larva was reached, which was easily detectable by larval mouth brushes (see Figure 1c [b]). Ultra-cut sections were then placed on pioloform foils and stained with approximately 5% uranyl acetate (in methanol) for 12 min. After rinsing in increasing bi-distilled methanol solutions, sections were stained with 4% of Raynolds lead citrate for 8 min and washed in 1% sodium hydroxide solution and bi-distilled water.

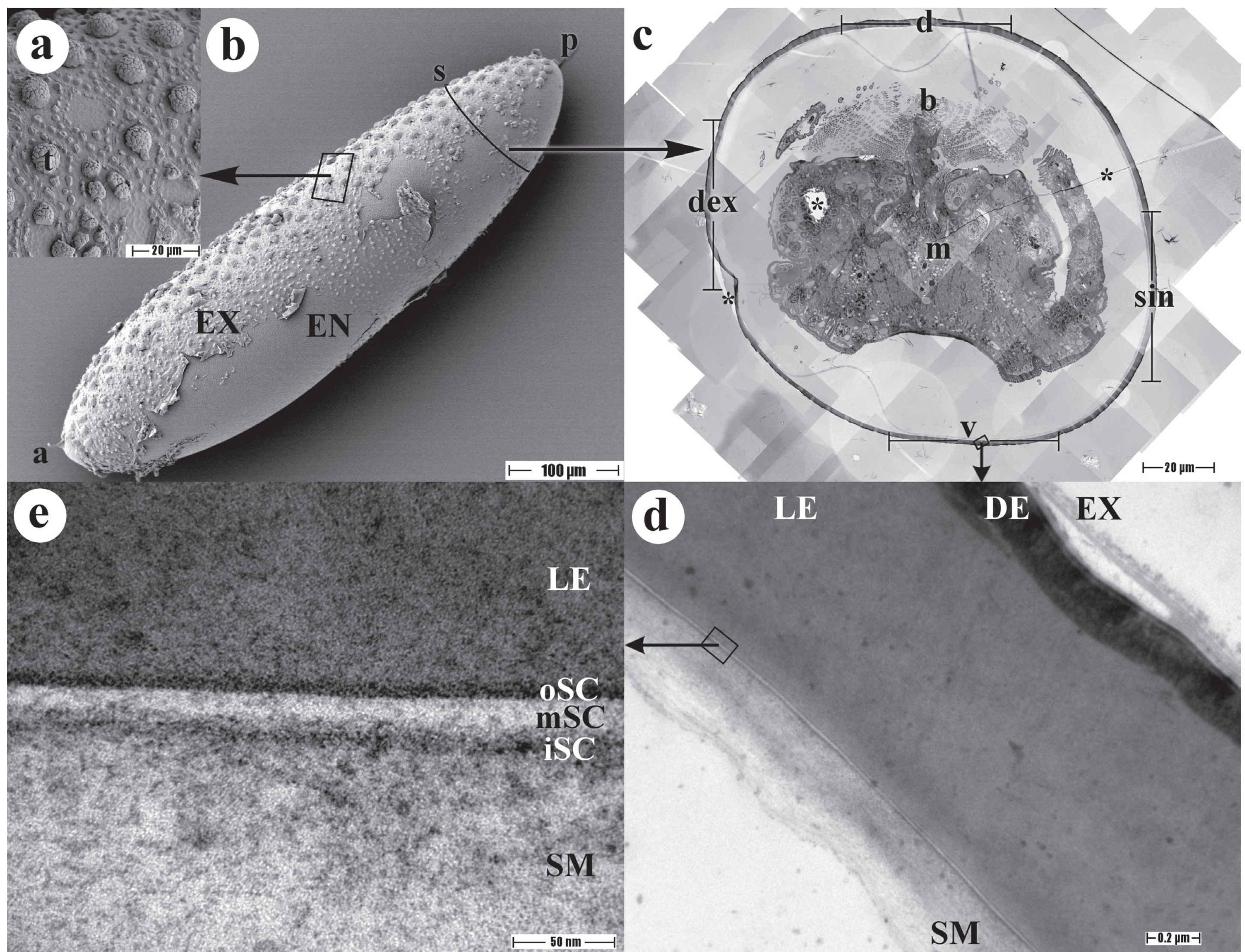

Figure 1. Overview and ultra-cuts of an *Aedes albopictus* egg. a) Detailed scanning electron microscope (SEM) record of the egg surface with exochorion (EX) and attached tubercles (t). b) Due to flaking of the EX exposed endochorion (EN), anterior (a), and posterior (p) pole as well as the cutting section (s) are labeled. c) Transmission electron microscope (TEM) record of the section (s) with mosquito larva (m) and their brushes (b). Dorsal chorion (d), ventral (v), dexter (dex), and sinister (sin) chorion measurement areas are marked, as are artifacts (*). d) TEM record of the whole chorion of the section of the ventral measurement area (see window). EX, dark endochorion (DE), light endochorion (LE), and serosa membrane (SM) are labeled. e) Detailed TEM record of the serosa cuticle (see window). LE, SM, outer serosa cuticle (oSC), middle serosa cuticle (mSC), and inner serosa cuticle (iSC) are labeled.

Each egg layer of five specimens (= biological replicate) of each treatment was measured in triplicate using a transmission electron microscope (Koninklijke Philips N.V., Model CM 12, Emission 1, Filament 28, 80kV, NL) with a digital camera and the attendant software (DigitalMicrograph™, Version 1.71.38). All measurements were taken at four orientations of the egg located at the dorsal, ventral, sinister, and dexter chorion (see Figure 1c). Therefore, each egg layer was measured at five biological replicates, at four positions and in triplicate (n = 60) for each of the four scenarios (n = 240). Due to the 180° dorso-ventral rotation of mosquito embryos during the germ band retraction phase, the developed larvae face the dorsal chorion (Valle et al. 1999). Therefore, readers should keep in mind that the denotation of the orientation differs between the larva and egg shell.

Scanning electron microscope (SEM) images were taken with a Hitachi S-4500 (Hitachi, Tokyo, Japan) by 5kV using air dried eggs of *Ae. albopictus*, placed on a carbon grid, and sputtered for 5 min with gold.

Statistical analyses

All statistics were performed with Prism® (Version 5.01, GraphPad Software Inc., U.S.A.) and Statistica (Version 8.0, Stat Soft Inc., U.S.A.). Prior to statistical analyses, the dataset of layer thickness was checked for outliers (due to artifacts, see Monnerat et al. 1999) by the range of $-\sigma\times3 < \text{mean} < \sigma\times3$ and in total 61 outliers among 1,440 measurements were substituted by group means. The metric datasets describing the ultrastructure of (non-)diapause and (non-)cold acclimated eggs lacked in homogeneity of variances (Cochran's 'gate keeper' test after Box-Cox transformation: p = 0.01) and thus no confirmatory data

analysis in form of an analysis of variance nor reliable generalized linear models could be computed. Therefore, an exploratory data analysis in the form of a principal component analysis (PCA) was computed. In order to determine the number of meaningful eigenvectors of the PCA, non-metric multidimensional scaling analyses (NMDS) were performed with GINKO Multivariate Analysis System (©Unitat de Botànica. Departament de Biologia Vegetal http://biodiver.bio.ub.es/ginkgo/) until Kruskal's Stress-1 was ≤0.1. Furthermore, data clustering was qualitatively compared between PCA and NMDS analyses. Subsequently the main correlations between vectors of depended variables (layers DE, LE, oSC, mSC, iSC, SM) and explanatory variables (cold adaptation, diapausing, orientation, biological replicate) with eigenvectors ≥|-0.25| were then analyzed using Bonferroni's adjusted t-tests.

Terminology of egg layers

It is crucial to clarify the terminology of mosquito egg shell layers because of a controversy that exists in the literature (Clements 1993, Monnerat et al. 1999, Rezende et al. 2008). Unlike Sahlén (1996) or Woods (2010), but according to Clements (1993), Monnerat et al. (1999) and Valle et al. (1999), we define the tubercle, meshwork, and reticulum of an egg as part of the exochorion layer (EX) or outer chorion, followed by a lamina and the thick and smooth endochorion (EN) or inner chorion (misleadingly described by some authors as vitelline envelope or vitelline membrane; see Figures 1d, 2). Due to a lack of comparable studies labeling the inner chorion layers of mosquito eggs, we distinguish between a dark endochorion (DE) and a light endochorion (LE) (Figures 1d, 2). Following the chorion layers are the serosal cuticle (SC) and the thick serosa membrane (SM) (Figure 2). The serosal cuticle is divided into three well-defined thin layers labeled as the outer serosa cuticle (oSC), middle serosal cuticle (mSC), and inner serosal cuticle (iSC) and is probably related to the yellow and white cuticle described for other species (Figure 1e). In studies with crickets, the serosal cuticle was divided into two sublayers: an external and so-called yellow cuticle, which is hydrophobic due to its richness of fatty acids (waxes), and the internal and so-called white cuticle, containing cross-linked chitin (Goltsev et al. 2009).

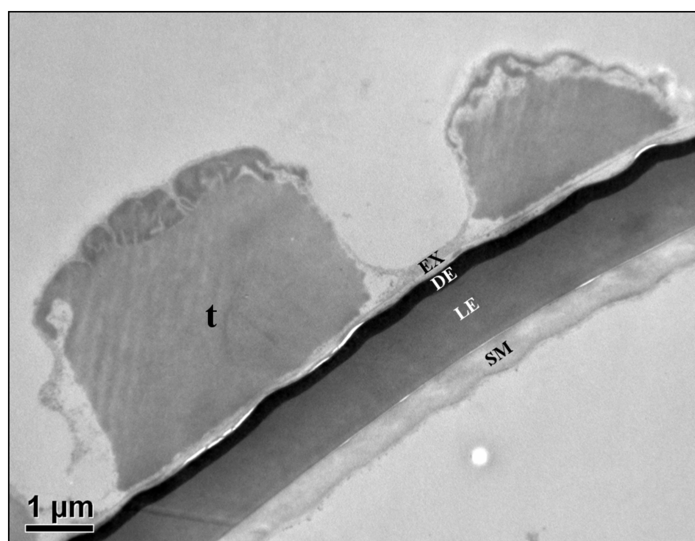

Figure 2. Transmission electron microscope recording of the chorion of an egg of *Aedes albopictus*. large tubercles (t) and lamina on the outside as part of the exochorion (EX), followed by the dark endochorion (DE), light endochorion (LE), and serosa membrane (SM).

RESULTS

Table 1 lists the mean sizes of the six characterized layers in the order from the outer chorion to the inner chorion (DE, LE, oSC, mSC, iSC, and SM) in total of this cross-factorial study design as well as for the appearance and absence of the two mechanisms for increasing cold hardiness (diapausing or non-diapausing, cold acclimation or no cold acclimation), and the results of the measurements for the four different exposure scenarios (non-diapausing and not cold acclimatized, diapausing and not cold acclimatized, non-diapausing and cold acclimatized and diapausing and cold acclimatized).

A four-dimensional configuration is the best solution for structuring the morphometric dataset (NMDS Kruskal's Stress-1 = 0.054) and explains 85.3% of the total variance (Figure 3). PCA component 1 reveals an eigenvalue of 2.19 (36.6% of

Table 1. Metric analysis of the chorion layers depending on pre-exposure. Mean layer sizes [nm] ± SD for the dark endochorion (DE), light endochorion (LE), outer serosal cuticle (oSC), middle serosal cuticle (mSC), inner serosal cuticle (iSC), and serosa membrane in total (n=240) and the effect of the two different cold hardiness inducing mechanisms diapausing (dia) and cold acclimation (acc) (n=120), as well as the layer sizes of the four different scenarios (n=60). Size of grey bars depends on the highest and lowest column value.

	DE	LE	oSC	mSC	iSC	SM
In total	171 ± 42.6	949 ± 139	12.6 ± 4.45	16.6 ± 11.0	16.6 ± 6.92	399 ± 133
non-dia	197 ± 23.4	965 ± 100	12.2 ± 4.42	18.0 ± 7.18	17.9 ± 5.56	387 ± 86.9
dia	146 ± 28.4	934 ± 132	13.0 ± 5.55	15.2 ± 9.26	15.4 ± 6.72	411 ± 120
non-acc	172 ± 43.7	948 ± 131	11.8 ± 4.24	13.6 ± 7.66	15.6 ± 5.31	396 ± 127
acc	171 ± 41.6	951 ± 146	13.3 ± 4.55	19.6 ± 12.7	17.7 ± 8.11	403 ± 140
non-dia, non-acc	203 ± 34.9	976 ± 138	11.3 ± 2.60	13.3 ± 6.37	14.8 ± 4.15	406 ± 153
dia, non-acc	143 ± 27.6	920 ± 119	12.3 ± 5.39	13.8 ± 8.82	16.4 ± 6.21	386 ± 93.9
non-dia, acc	192 ± 41.8	954 ± 150	13.0 ± 3.08	22.5 ± 14.7	20.9 ± 7.81	369 ± 134
dia, acc	149 ± 28.9	948 ± 143	13.7 ± 5.65	16.7 ± 9.54	14.5 ± 7.12	437 ± 138

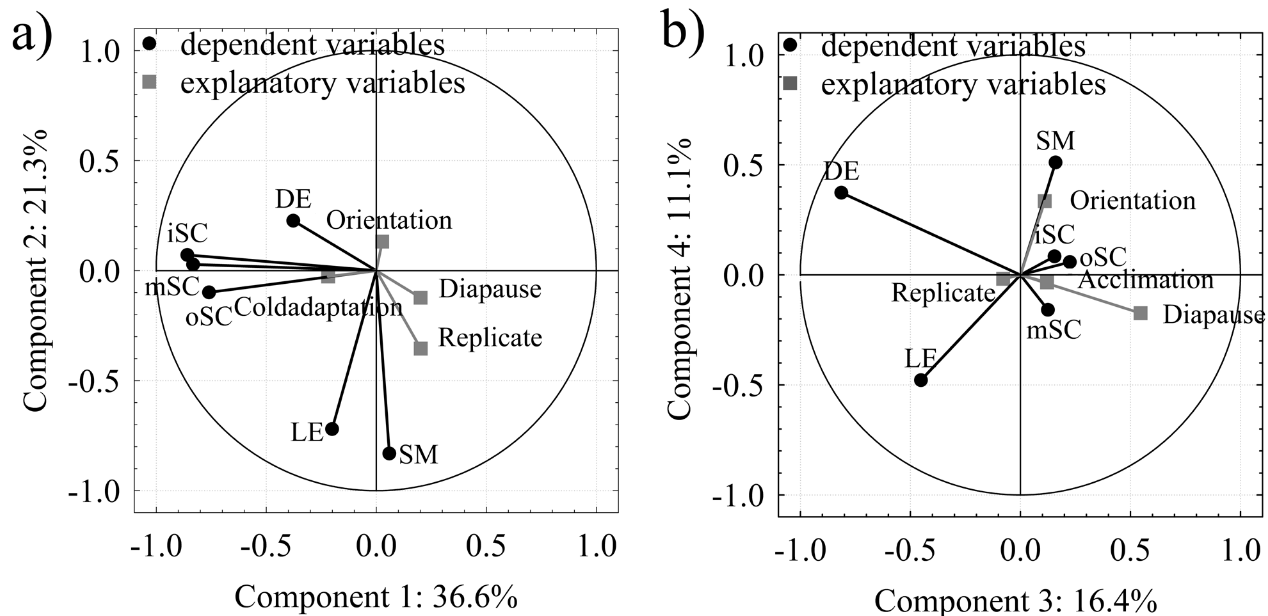

Figure 3. Ordination plots of principal component analysis showing the configuration of the ultra-structural dataset. a) Egg layers in ordination space component 1 (with percentage of variance explained) and component 2; b) Egg layers in ordination space component 3 and component 4. Vectors marked in black with dots are the dark endochorion (DE), light endochorion (LE), outer serosal cuticle (oSC), middle serosal cuticle (mSC), inner serosal cuticle (iSC), and the serosa membrane (SM).

total variance), component 2 of 1.28 (21.3% of total variance), component 3 of 0.98 (16.4% of total variance), and component 4 of 0.67 (11.1% of total variance). Main vectors of dependent variables explaining component 1 are the thin layers oSC, mSC, and iSC, for components 2 LE and SM, for components 3 DE and LE, and for components 4 SM and LE. Dominating vectors of explanatory variables in component 1 are cold adaptation, replicate, and diapause, in component 2 replicate, in component 3 diapause, and in component 4 the orientation.

Correlations with eigenvectors ≥|-0.25| were analyzed more thoroughly (Figure 4). The LE and SM differ significantly between replicates (Bonferroni's corrected t-tests, up to $P \leq 0.001$; Figure 4a). The thickness of LE varies, moreover, significantly between different egg orientations (Bonferroni's corrected t-tests: up to $P \leq 0.001$; Figure 4b). However, SM was not significantly different (Bonferroni's corrected t-tests: $P \geq 0.05$; graph not shown). The dextral and sinistral egg regions are on average 6.01% and 8.23% thicker and the dorsal region is up to 24.3% thicker than the ventral region. Eggs laid by diapausing adults have a thinner DE than those from non-diapause adults (-26.1%, Bonferroni's corrected t-tests: $P \leq 0.001$; see Figure 4c); however, LE (-3.22%) is not significantly thinner (Bonferroni's corrected t-tests: $P \geq 0.05$; graph not shown). Cold acclimated eggs have a significantly thicker mSC than non-cold acclimated eggs (+44.6%, Bonferroni's corrected t-tests $P \leq 0.001$; Figure 4d); however, iSC (+13.4%) and oSC (+12.8%) did not (Bonferroni's corrected t-tests: $P \geq 0.05$; graph not shown). Interactions between cold acclimation and diapausing did not appear to be likely because there was no considerable correlation in the principal component analysis and no interactive synergistically effect of the two mechanisms was detected (Table 1).

DISCUSSION

The low variance of data indicate that a full and not only partial diapause induction was performed at 8:16 h L:D photoperiod and 25° C. Photoperiod and acclimation to low temperature trigger different alterations in the ultrastructure of *Ae. albopictus* eggs and no interaction of the two triggers for cold hardiness was detected. A significant decrease of DE layer in diapausing eggs was detected, whereas a significant increase in mSC layer was measured in response to cold acclimation (exposure to 3° C for 24 h). Furthermore, methodological knowledge of greatest value for follow-up studies has been collected.

The detailed metric analysis of *Ae. albopictus* eggs revealed a high intra-specific variation in ultra-structural morphology, in particular with regard to LE and SM (see Figure 4a). In addition, the thickness of the LE layer varies strongly with the orientation of the eggshell. For example, the dorsal LE is on average 24.3% thicker than the ventral LE. Neglecting these facts may introduce a strong potential bias to TEM examinations of mosquito eggs. The lack in homogeneity of variances in the ultrastructural dataset might be based on the sample size or indicate that an additional explanatory variable is missing in the experimental set-up. We interpret the lack in homogeneity of variances even after Box-Cox-transformation to be a result of artifacts. Most artifacts observed in TEM recordings seem to result from a detachment of the serosa membrane from the endochorion, although the fixation of decapitated eggs was adjusted to the upper end of physiological molarity of mosquito larvae (210-480 mmol) by the addition of 10% sucrose (Bender et al. 1984, Richards and Meier 1974). This detachment may be a result of embryo movement (swelling) during fixation. As quiescent pharate larvae may have an exceptionally high osmolarity in order to decrease the size of

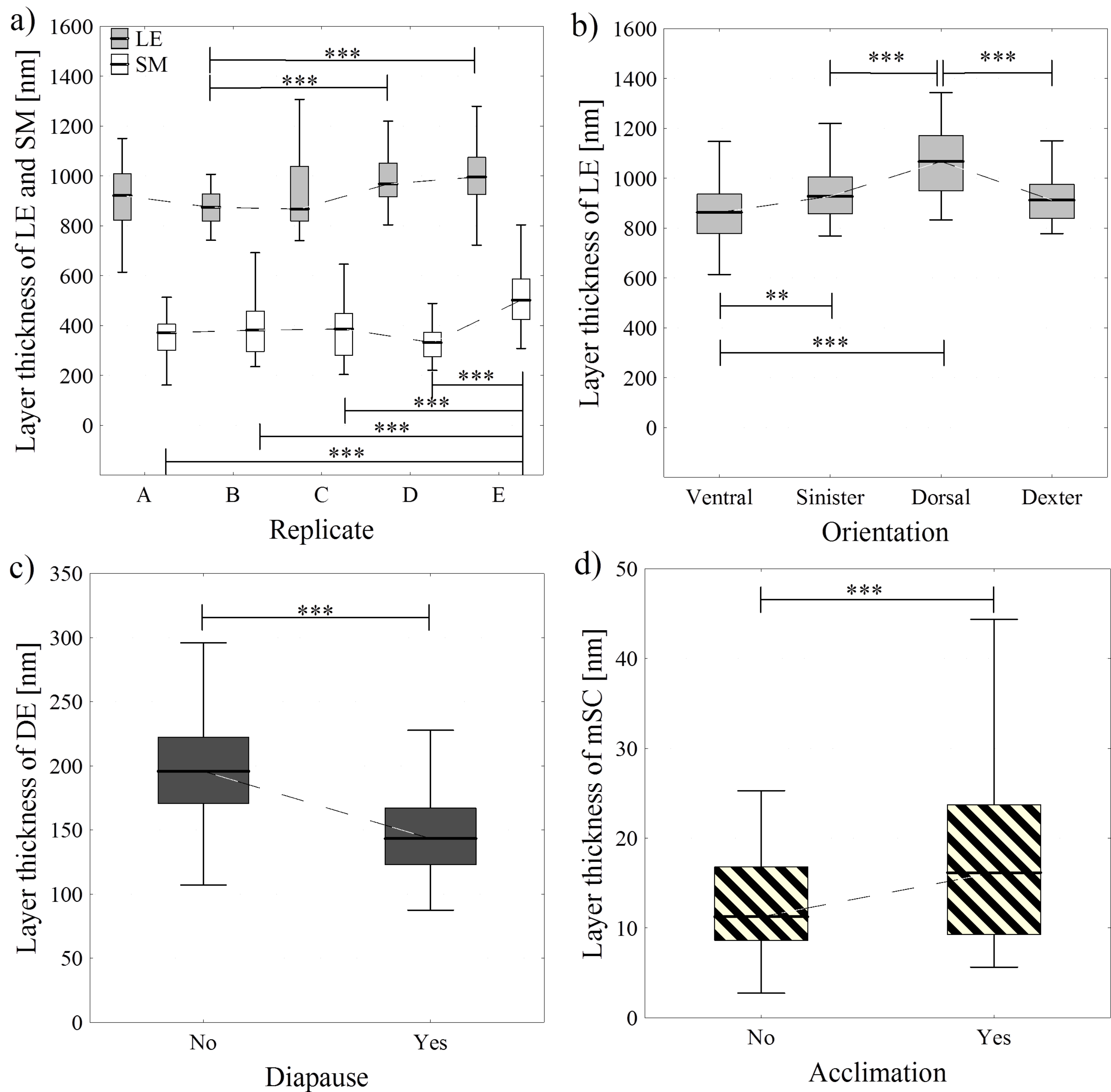

Figure 4. Boxplots (box = median and 25-75% confidence interval, whiskers = non-outlier range) for layer thickness. a) Light endochorion (LE) and serosa membrane (SM) in different replicates, b) LE in different egg regions (orientation), c) Dark endochorion (DE) in response to diapause treatment and d) middle serosal cuticle (mSC) in response to cold acclimation. t-tests with Bonferroni's correction: ** indicates $P < 0.01$; *** indicates $P < 0.001$.

the egg (Hinton 1981), we suggest that the fixation protocol in future studies should be optimized trading off the swelling by using higher molarities of fixation buffer to reach physiological molarities.

Our study does not support the often quoted but not yet reviewed hypothesis that *Ae. albopictus* eggs from cold acclimated and diapausing populations possess a thicker egg shell (Harwood and Horsfall 1959, Sota and Mogi 1992b). In contrast, our data show that diapausing causes a shrinking of the DE layer in *Ae. albopictus* eggs (Figure 4c). The DE may be a possible location for wax and protein accumulation as deduced from its high electron density and its greater thickness compared to the thin oSC and iSC layers (akin to yellow and white serosal cuticle). This electron dense substructure of the endochorion also appeared as distinct layer on the figures of a study with electron scanning images of *Anopheles albitarsis* eggs (Monnerat et al. 1999) but was mentioned neither in that publication nor in the work of Sahlén (1996) with *Aedes* ssp.

Surface hydrocarbons are also supposed to increase the desiccation resistance of diapausing *Ae. albopictus* and do not change in chain length (Urbanski et al. 2010). It was also shown that diapausing induced females produce one third more surface hydrocarbons than non-diapausing ones and that this is caused by the activation of the fatty acyl CoA elongases (Urbanski et al. 2010). It is likely that these surface hydrocarbons (extracted with hexane from the chorion) correspond to the electron dense and considerable DE rather than one of the small SC layers or the SM investigated in our study. Albeit, our study revealed that the layer thickness of the DE even decreased as a result of diapause. Due to the fact that tropical, and thus non-diapausing, eggs have more surface hydrocarbons than diapausing temperate ones (Urbanski et al. 2010), the plane quantity as well as the chain length of hydrocarbons like the suggested waxes seem to be not crucial for desiccation resistance due to diapausing and cold hardiness.

Deep sequencing of whole eggs of *Ae. albopictus* in early developmental stages (embryo), paired-end RNA-sequencing of blood fed and non-blood fed, diapausing and non-diapausing females as well as qRT-PCR of genes of diapause-destined embryos and on pharate 1st instar larvae shed light on the especially lipid metabolism of the diapausing mechanism of the females and the larvae (Huang et al. 2015, Poelchau et al. 2013, Reynolds et al. 2012). Unfortunately, there was no differentiation between the embryo/larvae and the SM cells which are genuinely responsible for the production of the SC layer and thus of utmost importance for developing dry resistance and cold hardiness (Hinton 1981). However, important signals of the thin SM layer may have been subordinated in the noise of signals of the embryo/larvae.

Another study focusing on the plane SM of *Anopheles gambiae* eggs revealed a coincidence in gaining desiccation resistance during ontogenesis and three main GO term enrichments in RNA expression: chitin synthesis and deposition, long chain fatty acid synthesis, and tyrosine metabolism, including catecholamine synthesis that is important for cross-linking chorion proteins (Goltsev et al. 2009). It thus appears that the mode of cold adaptation, as well as diapause, is more of a qualitative alteration of the egg layers than a quantitative one and that a change in configuration, saturation, and thus orientation, as well as cross-linking of proteins and fatty acids, may be the underlying mechanism (Hinton 1981). As proposed by Li and Li (2006), Rezende et al. (2008), and Goltsev et al. (2009), the composition of the chorion regarding additional proteins as well as the sclerotization of the wax layer, should be the subject of further investigations.

As mentioned in the beginning, there are three different ways an insect may cope with subzero temperatures (Duman et al. 1991). The process of freezing always starts with the appearance of an ice seed around a nucleus, either due to the spontaneous crystallization by low temperatures or by inoculating ice formations (Duman et al. 1991). Most of the freeze-avoiding species produce polyols to lower the freezing point as well as promote the ability to supercool the organism effectively (Duman et al. 1991). Polyols can be produced in high molecular concentrations without disrupting the metabolism, as shown by the eggs of the silk moth *Bombyx mori* (Furusawa et al. 1982). However, freeze-avoidant insects can also additionally prevent external inoculating ice formations by creating a cocoon like the silk moth as part of a behavioral adaptation strategy (Duman et al. 1991). Since neither cold acclimation nor diapause change the SCP of *Ae. albopictus* eggs (Hanson and Craig 1995b), the avoidance of inoculating ice formations may be the main challenge for achieving cold hardiness for *Aedes* eggs. Studies show a dramatic decrease of SCP when organisms like chironomids or *Tetracanthella wahlgreni* are in contact with ice, which is why insects that overwinter in moist habitats are either freeze-tolerant or utilize a waterproof encasing structure (Danks 1971, Sømme and Conradi-Larsen 1977, Sømme 1982). Therefore, the shrinking of the DE, perhaps in combination with the cross-linking of proteins and sclerotization, may be interpreted as a compaction of the fatty acids creating a physical barrier against inoculating ice formations.

Critically interpreting our findings, one might also assume that the light and electron poor mSC may not even be a layer of the chorion itself but the inter-membrane room between the white (oSC) and yellow serosal cuticle (iSC). In the process of freezing and in the case of disruption of the DE as a physical barrier by inoculating ice formations, a wider inter-membrane room followed by the iSC and the SM may function as a second barrier. The growing ice lettuce of the inoculating ice formation, e.g., through chorionic hydropyles inside the inter-membrane room, excludes solutes and results in an increasing osmotic potential of the remaining liquid water in the inter-membrane room. This creates a water efflux of the embryo followed by an increasing osmolarity and therefore decreasing SCP of the embryo. Thus, the ice formations remain in the inter-membrane room and their growth slows down as they become larger. In this stage, the embryo may struggle with osmotic stress by these extracellular ice formations but survive the physical damage of intracellular ice formations in a trade-off.

Our detailed metric analysis of *Ae. albopictus* eggs reveals different ultra-structural alterations produced by photoperiod and cold acclimatization and thereby significantly contributes to our better understanding of complex mechanisms involved in the cold hardiness of aedine species. Follow-up studies regarding the rapidity of the development of low-temperature phenotypes in *Ae. albopictus* may help us to better understand the enormous invasion success of this human disease-transmitting mosquito species towards higher latitudes and altitudes worldwide.

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