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Physical features and chitin content of eggs from the mosquito vectors *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*: Connection with distinct levels of resistance to desiccation

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ABSTRACT

Mosquito eggs are laid in water but freshly laid eggs are susceptible to dehydration, if their surroundings dry out at the first hours of development. During embryogenesis of different mosquito vectors the serosal cuticle, an extracellular matrix, is produced; it wraps the whole embryo and becomes part of the eggshell. This cuticle is an essential component of the egg resistance to desiccation (ERD). However, ERD is variable among species, sustaining egg viability for different periods of time. While *Aedes aegypti* eggs can survive for months in a dry environment (high ERD), those of *Anopheles aquasalis* and *Culex quinquefasciatus* in the same condition last, respectively, for one day (medium ERD) or a few hours (low ERD). Resistance to desiccation is determined by the rate of water loss, dehydration tolerance and total amount of water of a given organism. The ERD variability observed among mosquitoes probably derives from diverse traits. We quantified several attributes of whole eggs, potentially correlated with the rate of water loss: length, width, area, volume, area/volume ratio and weight. In addition, some eggshell aspects were also evaluated, such as absolute and relative weight, weight/area relationship (herein called surface density) and chitin content. Presence of chitin specifically in the serosal cuticle as well as aspects of endochorion external surface were also investigated. Three features could be related to differences on ERD levels: chitin content, directly related to ERD, the increase in the egg volume during embryogenesis and the eggshell surface density, which were both inversely related to ERD. Although data suggest that the amount of chitin in the eggshell is relevant for egg impermeability, the participation of other yet unidentified eggshell attributes must be considered in order to account for the differences in the ERD levels observed among *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*.

1. Introduction

Mosquitoes transmit pathogens that cause diverse human diseases and hence vector control has an important role to block their propagation (WHO, 2013). This is particularly relevant when no vaccines or specific drugs are available, as is the case for dengue and chikungunya viruses (Teixeira et al., 2015).

Although eggs and embryos have the potential to be a suitable control target (Beament, 1989), the egg is the least known life stage in mosquitoes. According to Hinton (1981), insect eggs can be

Abbreviations: ERD, egg resistance to desiccation.

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classified in three groups, depending on external requirements: (i) those that only necessitate oxygen, (ii) eggs that need oxygen and water and (iii) eggs requiring oxygen, water and nutrients. Mosquito eggs belong to the second group and are laid on or near the water surface. Freshly laid eggs increase in size and weight due to water uptake and are susceptible to water loss under dry conditions (Gander, 1958; Kliewer, 1961; Clements, 1992; Rezende et al., 2008).

The eggshell protects the developing embryo from biotic and abiotic stresses, and helps to maintain its water balance. Mosquito eggshell is comprised of three layers: exochorion, endochorion and serosal cuticle (Clements, 1992). Both the exochorion and endochorion are present when mosquito eggs are laid (Monnerat et al., 1999), since they are produced by female follicle cells in the ovaries during choriogenesis (Clements, 1992; Chapman, 1998) (Fig. 1A). The innermost serosal cuticle in turn is an extracellular matrix produced during the first third of mosquito embryogenesis by the extraembryonic serosa, after it completely wraps the embryo (Rezende et al., 2008; Vargas et al., 2014) (Fig. 1B and C).

Mosquito eggs desiccate and die in arid conditions before serosal cuticle formation. This cuticle therefore increases eggshell impermeability (Fig. 1C), allowing eggs to remain viable for many hours, if exposed to a dry environment during embryogenesis (Rezende et al., 2008; Goltsev et al., 2009). Apart from these commonalities, differences in the levels of resistance to desiccation among eggs from distinct mosquitoes species have been described. At the end of embryogenesis *Culex quinquefasciatus*, *Anopheles aquasalis* and *Aedes aegypti* eggs present, respectively, low, medium and high levels of resistance to desiccation since they can survive outside water for, respectively, 5, 24 and at least 72 h (Vargas et al., 2014) (Fig. 1D).

Desiccation resistance is defined as the capacity of any organism to withstand in an arid environment without loss of viability. Organisms that survive drought for longer periods are considered to bear a higher desiccation resistance. Three factors are related to desiccation resistance: rate of water loss, dehydration tolerance (the minimum body water content prior to death) and the whole water content of an organism (Hadley, 1994, Gibbs et al., 1997, Gray and Bradley, 2005).

The egg resistance to desiccation (ERD) phenomenon was previously named 'embryonic desiccation resistance', or 'EDR' (Rezende et al., 2008; Goltsev et al., 2009; Vargas et al., 2014). We revised this term due to two conceptual inaccuracies: (i) the ability to withstand desiccation is not a feature of the embryo *per se*, but rather of the egg as a whole; (ii) resistance to desiccation is present in all life stages of any organism; it is not exclusive of embryos, as 'EDR' might suggest.

The ERD differences observed in the three mosquitoes at the end of embryogenesis (Vargas et al., 2014 and Fig. 1D) can be related to particular factors of each species, like egg size, structure of the three eggshell layers and of the larval cuticle, as well as to variations in presence or amount of metabolites such as glycerol, trehalose, glycogen or triacylglycerols inside the egg (Sota and Mogi, 1992; Hadley, 1994; Sawabe and Mogi, 1999; Gray and Bradley, 2005).

As an attempt to unravel the nature of the distinct ERD levels, we compared several physical aspects of eggs and eggshells of *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*. In addition, chitin presence in the serosal cuticle and eggshell chitin content were also evaluated. Interesting and significant differences have been observed in most of the studied features, and some of them could be related to the different degrees of ERD presented by the three mosquito species.

2. Material and methods

2.1. Mosquitoes rearing, synchronous egg laying and exochorion removal

Ae. aegypti, *An. aquasalis*, and *Cx. quinquefasciatus* mosquitoes from colonies of the Laboratório de Fisiologia e Controle de Artrópodes Vetores, IOC, Fiocruz, Rio de Janeiro, RJ, Brazil were reared as previously described (Vargas et al., 2014). Briefly, immature mosquitoes developed at 26 ± 1 °C and were fed with fish or cat food. Adult mosquitoes were kept in cages at 26 ± 1 °C and fed *ad libitum* with a 10% sucrose solution. Blood meals required for egg produc-

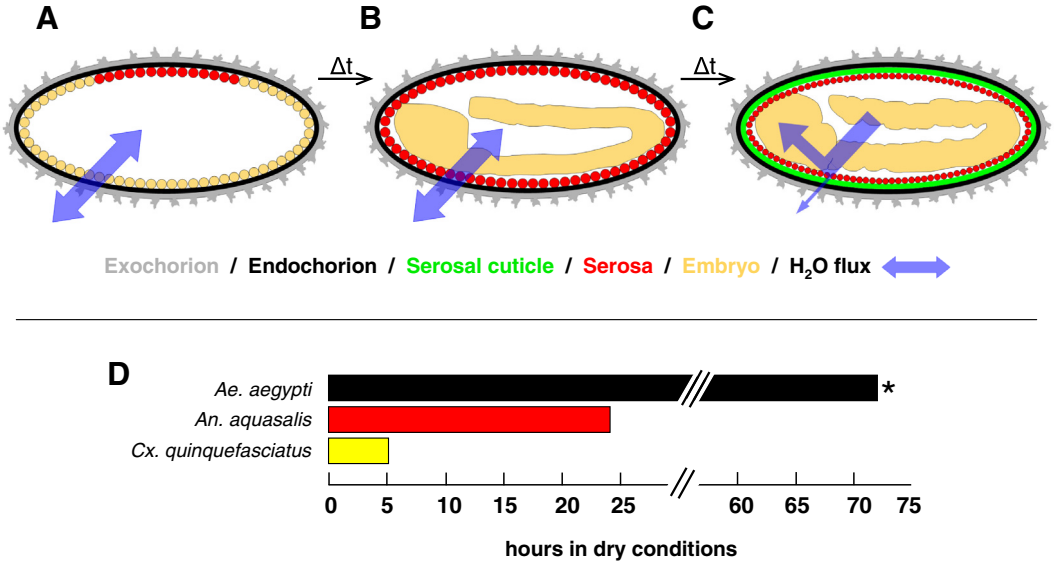

Fig. 1. Mosquito eggshell layers and egg resistance to desiccation. (A) Immediately after oviposition, eggshells are comprised of maternally produced exochorion and endochorion. (B) During embryogenesis, serosal cells surround the embryo and, subsequently, (C) secrete the serosal cuticle that considerably decreases water flow. (D) *Aedes aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* eggs were transferred from water to dry conditions (20–55% relative air humidity) from 80% of complete embryogenesis on. Bars indicate the longest periods of time that eggs keep high viability. Adapted from Rezende et al. (2008), Vargas et al. (2014). *'At least', in this case: the ability of *Ae. aegypti* eggs to preserve high viability under dry conditions for periods much longer than 72 h is well-known (Kliewer, 1961; Christophers, 1960).

tion were performed on anesthetized guinea pigs (Hawk and Leary, 1995).

The method of synchronous egg laying was adapted from previous reports (Valencia et al., 1996): depending on the species, oviposition was forced three to six days after blood meal, when mosquito females were placed in tubes and anesthetized in ice for a few minutes. *Ae. aegypti* and *An. aquasalis* females were quickly transferred to an upside down Petri dish (90 or 150 mm diameter) with a Whatman No. 1 filter paper disk covering the lid (serving as base). After mosquitoes recovery, the paper was moistened with water, which served as an egg laying stimulus. Anesthetized *Cx. quinquefasciatus* females were transferred to head up Petri dishes (not upside down). After mosquitoes retrieval, the egg laying stimulus consisted in addition of water until females were pressed against the lid. All egg laying procedures were performed in the dark during one hour inside a Biological Oxygen Demand incubator at 25 ± 1 °C. In all cases, eggs were kept moist and allowed to develop at this temperature, until being employed on the assays. Other details are described elsewhere (Rezende et al., 2008; Vargas et al., 2014).

For all experiments described below, exochorion was removed with a 50% NaOCl (5–6% active chlorine) treatment for 1 min followed by a thorough wash with dechlorinated water. The only exceptions were the analysis of exochorion role in ERD (Section 2.2) and the serosal cuticle isolation (Section 2.6).

Throughout this work the term 'eggshell' is employed to refer to the ensemble of endochorion and the innermost serosal cuticle (deprived of exochorion).

2.2. Analysis of exochorion role in the Egg Resistance Desiccation

Evaluation of ERD increase related to serosal cuticle formation was performed as previously described (Rezende et al., 2008; Vargas et al., 2014). At distinct embryogenesis time points, replicates consisting of 50 synchronized eggs were placed on a polycarbonate filter (25 mm diameter, 8 μm pore, Poretics Corporation), deposited on a drop of dechlorinated water. Each filter was then blotted on a Whatman No. 1 filter paper to remove all water, and eggs were air-dried for 15 min. Afterwards, the shrunken or unaltered eggs were counted under a stereomicroscope. For each time point two pools of eggs were analyzed: one consisting of intact eggs (with exochorion) and the other of eggs deprived of exochorion, removed as described above. At least two independent experiments were performed for each species and condition.

2.3. Measurement of egg length, width, area and volume

For each experiment, the length and width of at least 16 eggs at 20 or 80% complete embryogenesis were measured. *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* embryogenesis are completed, respectively, at 77, 51 and 34 h after egg laying (Vargas et al., 2014). For each species two independent experiments were performed. Eggs were viewed under a stereomicroscope Stereo Discovery V.12 (Zeiss) coupled with a digital image acquisition system. Egg length and width were obtained with the aid of the Image J software.

Eggs volume and surface area were calculated based on length and width values obtained above. Eggs were treated as prolate spheroids, i.e., tridimensional ellipsis with polar axis diameter higher than the equatorial axis one (Supplementary File 1), as previously considered for mosquito (Sota and Mogi, 1992) and beetle (Gauvin et al., 2001) eggs. Equation of the prolate spheroid surface area is $2{\cdot}\pi a^2{\cdot}(1 + b/ae{\cdot}\sin^{-1}{\cdot}e)$, where "$a$" is half the egg width, "b" is half the egg length and "e" is the eccentricity of the spheroid, calculated through the formulae: $e^2 = 1 - a^2/b^2$. The formulae of the prolate spheroid volume is $4/3{\cdot}\pi{\cdot}a^2{\cdot}b^2$.

2.4. Weighing of whole eggs and eggshells

In all cases eggs were manipulated and measured at late embryogenesis (88–96% complete development). After exochorion removal, pools of eggs were blotted and dried on a filter paper, counted and weighed with the aid of a Denver Instrument balance (APX-200, 0.1 mg of precision) and replaced under moistened conditions until embryogenesis completion. Larval hatching of the three species was stimulated with 150 mg/mL yeast extract solution (Farnesi et al., 2009). The eggshells were then removed from the moist, dried on a filter paper, counted and weighed. At least three independent weightings were performed for each species, each with a minimum of 391 eggs or 301 eggshells. For *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*, a total of 4659, 2675 and 1287 eggs and 1250, 1272 and 979 eggshells were weighed, respectively.

2.5. Endochorion surface analysis with scanning electron microscopy (SEM)

Eggshells obtained as described on Section 2.4 were fixed for one hour at 25 °C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 and washed three times with the same buffer. Samples were post-fixed for 1 h in 1% osmium tetroxide with 0.8% potassium ferrocyanide and 2.5 mM $CaCl_2$ at 25 °C. The eggshells were dehydrated in a crescent acetone series, dried by the critical point method with CO_2, mounted on aluminum stubs and coated with a 20 nm gold layer. For each species, analysis employed at least 10 eggshells in a Jeol JSM6390LV scanning electron microscope (Tokyo, Japan) at Plataforma de Microscopia Eletrônica, Fiocruz.

2.6. Serosal cuticle isolation and chitin detection

Serosal cuticle was isolated from eggshells after larvae hatching as described elsewhere (Rezende et al., 2008): complete exochorion and endochorion digestion was accomplished with 30% NaOCl (approximately 3–3.6% active chlorine) for 15–40 min, depending on the species. The whole process was monitored under a stereomicroscope. The resulting serosal cuticles were washed three times with distilled water and three times with phosphate buffered saline containing 20 mg/mL of bovine serum albumin (PBS-BSA).

For chitin detection the isolated serosal cuticles were incubated with 5 μg/mL WGA-FITC (i.e., the lectin Wheat Germ Agglutinin conjugated with fluorescein isothiocyanate, EY Laboratories) in PBS-BSA solution for one hour in the dark at room temperature. After extensive washing in PBS-BSA the serosal cuticles were mounted and analyzed under DIC and fluorescence microscopy in an Axio Imager A.2 Zeiss coupled with an AxioCam MRc 5 and AxioVision Rel. 4.8 software. All images were acquired under the same microscopy and software parameters. Three independent experiments were performed for each species.

2.7. Chitin content in eggshells

Chitin quantification was done using eggshells obtained in the weighing experiments described on Section 2.4. Chitin was quantified through its glucosamine derivatives obtained after deacetylation, depolymerization and deamination of the N-acetylglucosamine polymer (Lehmann and White, 1975; Zhang and Zhu, 2006; Farnesi et al., 2012). Briefly, triturated eggshells underwent an alkaline digestion that deacetylates chitin, converting it into chitosan, a glucosamine polymer. Chitosan is then depolymerized yielding glucosamine that is deaminated generating soluble aldehydes. After addition of $NH_4SO_3NH_2$, MBTH (3-methyl-2-

benzothiazolone hydrazone hydrochloride hydrate, Sigma #129739) and $FeCl_3 \cdot 6H_2O$ the product is measured spectrophotometrically at 650 nm. Chitin amount is expressed in glucosamine units, according to a standard curve obtained with commercial glucosamine (Sigma #G4875).

The chitin content is normalized per eggshell weight or per eggshell surface area. For both approaches, three and two independent experiments were performed, respectively, for *An. aquasalis* (total of 1272 eggshells) and *Cx. quinquefasciatus* (total of 678 eggshells). For *Ae. aegypti*, normalization per eggshell weight employed three experiments (1250 eggshells) while normalization per eggshell surface area used four experiments (1400 eggshells).

2.8. Statistical analysis

Unless stated, each assay was evaluated in triplicates and repeated three times. For all experiments, mean and standard deviations were calculated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test is indicated when used, ($P < 0.05$). All statistical analyzes were made using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Exochorion does not influence ERD

In order to evaluate if the exochorion is relevant for ERD, the air-drying assay, employed to detect serosal cuticle formation (Rezende et al., 2008; Vargas et al., 2014) was performed. This assay was done in the three species with intact eggs (with exochorion) and with eggs deprived of exochorion at distinct developmental periods, prior to and after serosal cuticle formation (Fig. 2). In no case the exochorion removal influenced the timing or the intensity of egg shrinkage.

3.2. Egg length, width and related measurements varies among species

There are significant differences among the three species on all parameters evaluated ($P < 0.05$) (Fig. 3). *Cx. quinquefasciatus* eggs are the longest ones, followed by *Ae. aegypti* and *An. aquasalis*. *Ae. aegypti* are the widest, followed by *Cx. quinquefasciatus* and *An. aquasalis*; the same is true when total egg volume is considered. Accordingly, *An. aquasalis* eggs have the smallest egg surface area, while both *Ae. aegypti* and *Cx. quinquefasciatus* present similar values for this attribute. Since *An. aquasalis* are the smallest eggs, they present the higher ratio surface area/volume.

These parameters were also compared between 20 and 80% of total embryo development (Table 1). Swelling of eggs during embryogenesis was noted in all species, resulting in increments in all dimensions. This expansion was particularly prominent in *Cx. quinquefasciatus* eggs, that increased its volume almost 17%. In comparison, *Ae. aegypti* eggs volume increased 5 times less while *An. aquasalis* eggs swelled by intermediate amounts.

3.3. Egg and eggshell weight vary among species

Eggs and eggshells were weighed respectively at the end of embryogenesis and after larval hatching (Fig. 4). In both cases *Cx. quinquefasciatus* specimens are the heaviest. *An. aquasalis* eggs are the lightest while eggshell weight of this species is equivalent to *Ae. aegypti*. Comparison of both values enabled to verify that *An. aquasalis* eggshell accounts for about 40% of the whole egg weight. This rate corresponds to roughly twice the other two species (Fig. 4, lower left panel). However, when eggshell surface density (weight/area) was considered (Fig. 4, lower right panel), *Cx. quinquefasciatus* presented the higher rate, followed by *An. aquasalis* and *Ae. aegypti*.

3.4. Endochorion surface aspect varies among species

The external endochorion surface of the three species was evaluated through SEM (Fig. 5). Exclusive features were observed: *Ae. aegypti* exhibits polygonal reticulated marks in high relief throughout its surface, *An. aquasalis* possess a lateral groove along its antero-posterior axis, flanking the ventral side of the eggshell (Valle et al., 1999) and *Cx. quinquefasciatus* presents marks of small circles in low relief proximal to the posterior end of the egg (upper panels, Fig. 5). Apart from these peculiarities, *Ae. aegypti* endochorion surface is rough and irregular, while the *An. aquasalis* one is completely smooth; *Cx. quinquefasciatus* also presents a smooth endochorion, with the exception of its posterior end that also shows a rough, irregular surface (middle and lower panels, Fig. 5).

3.5. The serosal cuticle of different mosquitoes contains chitin

Isolated serosal cuticles from the three species were incubated with WGA-FITC to reveal chitin presence. This labeling procedure has been previously employed to identify chitin in *Ae. aegypti*

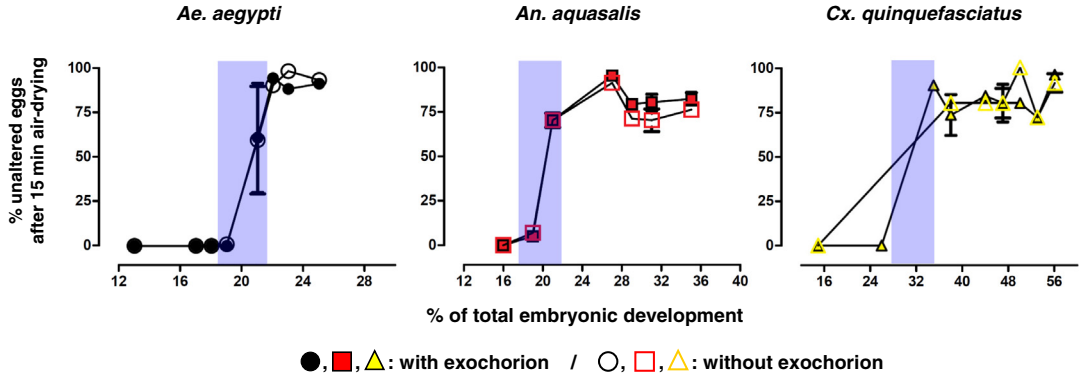

Fig. 2. Abrupt increase in egg impermeability, due to serosal cuticle formation, is not affected by exochorion removal. Pools of synchronized mosquito eggs were air-dried for 15 min at different embryonic ages. The percentage of unaltered eggs (i.e., that did not shrink) was then recorded. The rate of unaltered eggs in the *y*-axis is expressed relative to the percentage of total embryonic development for each species at 25 °C (in the *x*-axis). Blue stripes indicate the period of serosal cuticle formation relative to total embryogenesis (Vargas et al., 2014). Closed symbols: intact eggs, with exochorion. Open symbols: eggs deprived of exochorion. Mean and standard errors are indicated. At least 100 eggs were employed for each time point, species and condition.

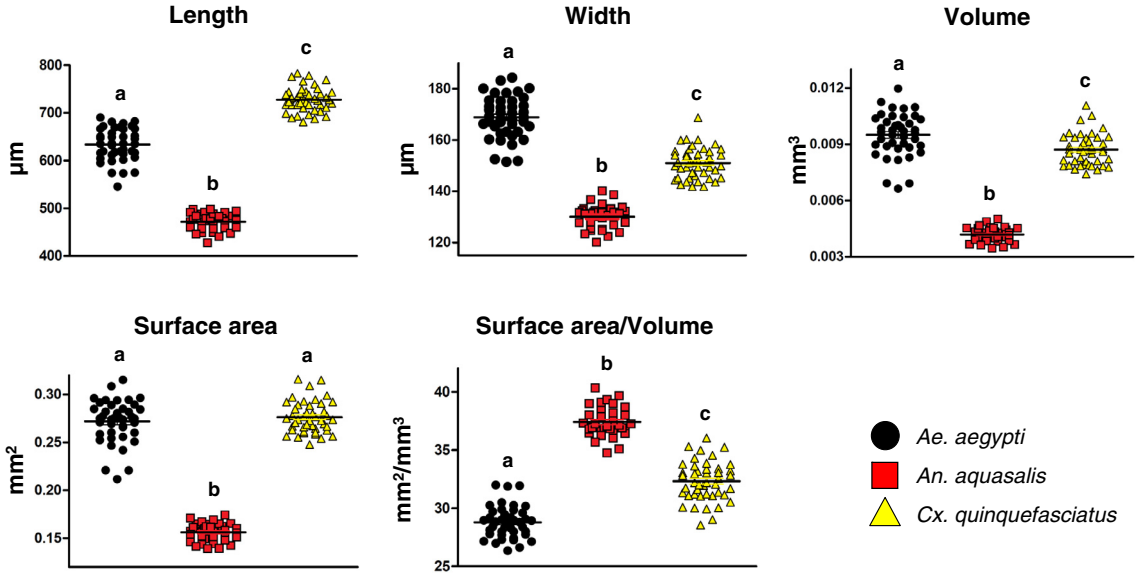

Fig. 3. Egg metric quantities at the end of embryogenesis vary among species. Length and width were measured at 80% of total embryogenesis and then employed to calculate surface area, volume and surface area/volume (see Section 2.3 and Supplementary File 1). Each point represents an individual egg and horizontal lines indicate mean. A total of 41, 33 and 44 eggs were measured for, respectively, *Ae. aegypti*, *An. aquasalis*, and *Cx. quinquefasciatus*. For all parameters, values with distinct lowercase letters were significantly different. Data are presented as suggested by Weissgerber et al. (2015).

Table 1
Egg metric quantities at 20 and 80% of embryo development.

Species	% Embryogenesis[a]	Length (µm)	Width (µm)	Mean area[b]		Mean volume[b]	
				(mm^2)	% Increase	(mm^3)	% Increase
Ae. aegypti	20	621.6 ± 35.7	168.0 ± 6.1	0.265		0.0092	
	80	633.7 ± 34.3	168.9 ± 8.2	0.272	2.5	0.0095	3.2
An. aquasalis	20	460.4 ± 15.0	125.4 ± 4.9	0.147		0.0038	
	80	471.8 ± 18.1	130.1 ± 4.6	0.156	6.4	0.0042	10.2
Cx. quinquefasciatus	20	686.1 ± 15.9	144.1 ± 5.1	0.249		0.0075	
	80	728.0 ± 24.9	151.0 ± 5.9	0.276	11.2	0.0087	16.7

In each case, length and width values represent mean and standard deviation of two independent experiments. A total of 36/41, 37/33 and 40/44 eggs were measured for, respectively. *Ae. aegypti*, *An. aquasalis*, and *Cx. quinquefasciatus* at 20/80% of embryogenesis.
[a] Time of evaluation related to total embryogenesis period (based on Farnesi et al., 2009; Vargas et al., 2014).
[b] Eggs were considered as prolate spheroids (see Supplementary File 1).

serosal and larval cuticles (Rezende et al., 2008; Farnesi et al., 2012) and in *Anopheles gambiae*serosal cuticle (Goltsev et al., 2009). Beyond detection of chitin in all samples (Fig. 6), differences on the serosal cuticle morphology in the three species are evident: those from *Ae. aegypti* and *An. aquasalis* are well-structured while *Cx. quinquefasciatus* ones are fragile and brittle. These differences are also promptly recognized through the direct manipulation with an entomological needle.

3.6. Eggshell chitin content tends to vary among species

Quantification of chitin in the eggshells is presented in Table 2. For each species, values were normalized both per eggshell weight or surface area. Although not significant, *Ae. aegypti* always tended to exhibit the higher values, and *Cx. quinquefasciatus*, the lower amounts.

3.7. Egg volume increase, surface density and chitin content are related with ERD levels

Although many differences were found among the egg attributes evaluated for the three species, most features could not be related with the ERD levels observed for these mosquitoes. The only exceptions, depicted in Fig. 7, are: egg volume increase during embryogenesis, eggshell surface density and eggshell chitin content. While the two first parameters present an inverse relation with ERD levels (Fig. 7A and B), the chitin content is directly related to it (Fig. 7C and D).

4. Discussion

The three mosquito species studied here have a chitinized serosal cuticle that is fundamental for acquisition of egg resistance to desiccation. Two subfamilies are represented: Culicinae, that includes *Ae. aegypti* and *Cx. quinquefasciatus*, and Anophelinae, that encompasses *An. aquasalis*. The last common ancestor of both subfamilies occurred ~217 million years ago while *Aedes* and *Culex* genera divergence started at ~204 million years (Reidenbach et al., 2009). Levels of egg resistance to desiccation vary among *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* (Vargas et al., 2014) (Fig. 1D) and understanding these differences may be relevant.

Mosquitoes frequently lay eggs in temporary water pools and, depending on the species, drought can impair egg viability (Clements, 1992) with a potential consequent impact on their population density and also on the epidemiology of pathogens they

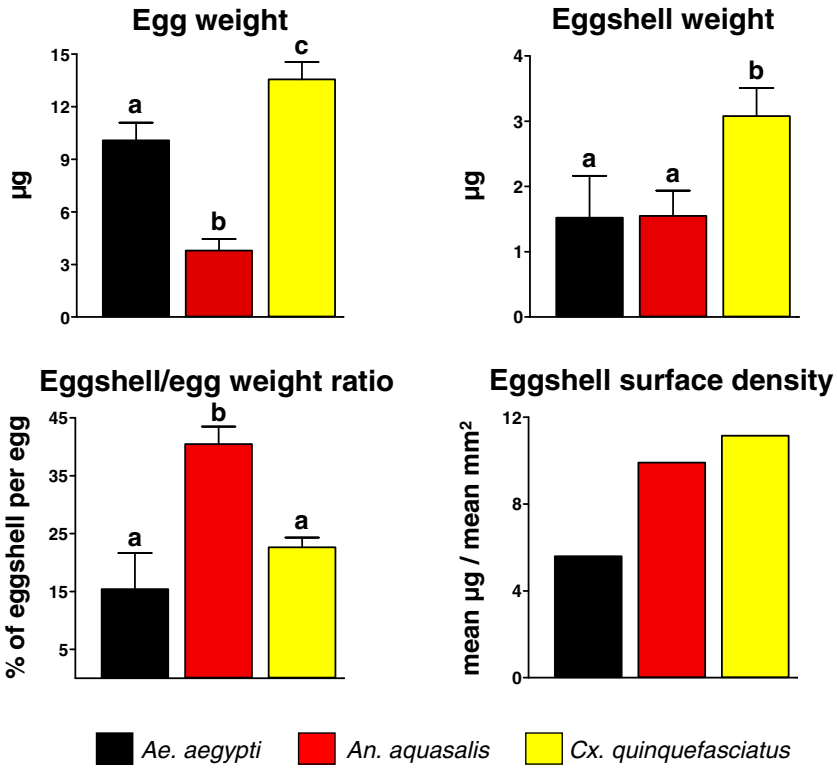

Fig. 4. Egg and eggshell weight as well as related quantities vary among species. Eggs deprived of exochorion were weighed at the end of embryogenesis while eggshells (endochorion and serosal cuticle) were weighed immediately after larval hatching. Except for the lower right panel, bars represent mean and standard deviation of three or more independent experiments, each one containing at least 391 eggs and 301 eggshells. Different lowercase letters indicate statistically significant differences. Eggshell surface density was calculated dividing the mean eggshell weight per mean surface area (Fig. 3, lower left panel).

transmit. Eradication of the *Anopheles arabiensis* infestation in the Northeast Brazil in the 1930s is a historical example of the relevance of water pools regarding control strategies. This species, of the *An. gambiae* complex, was responsible for thousands of malaria deaths in the country (Killeen et al., 2002; Parmakelis et al., 2008). A major component of the eradication campaign was the localization, by larval inspectors, of small bodies of water used as larval habitats and their treatment with the insecticide Paris Green (Killeen et al., 2002).

In some insects it is possible to silence a specific gene in order to evaluate its participation in a given process. This can be done genetically or through dsRNA-mediated RNAi. For example, in the beetle *Tribolium castaneum*, in which the process of gene silencing via dsRNA is easily performed, it is possible to silence the expression of zygotic genes (such as those expressed in the serosa) without affecting egg viability (e.g., Jacobs et al., 2013). Up to date this approach is yet not feasible for mosquito embryos. There do exist a few RNAi studies with mosquitoes aiming the egg but these are directed toward maternal genes that affect early embryogenesis (e.g., Wu et al., 2013) or siRNA directed silencing aiming late zygotic genes (e.g., Clemons et al., 2011). In both approaches, egg viability is compromised and therefore these methods can not be employed to address genes with the potential to affect mosquito egg viability under dehydrating conditions.

Whereas ERD differences among mosquito species or populations can be related to egg attributes (Sota and Mogi, 1992), we measured diverse egg and eggshell physical features in the three mosquito species. Initially, confirmation that the exochorion plays no role in ERD enabled further assays without this layer, that detaches easily from the endochorion (Christophers, 1960; Jarial, 2001).

The values obtained here at the end of embryogenesis for both egg linear dimensions, length and width, were compared to the literature (Table 3). While *Ae. aegypti* measures did not differ from previous reported values, for the other two species we found comparatively higher lengths and lower widths. Besides potential methodological differences among groups, many other factors can account for these variations: genetic background, conditions during immature development, adult female size and blood intake volume.

We found no reports accounting for data on *An. aquasalis* and *Cx. quinquefasciatus* egg surface area or volume. Regarding *Ae. aegypti* egg surface area, Christophers (1960) (p. 142), employing two distinct approaches (camera lucida drawing and plasticine model), found 0.318 and 0.297 mm^2, respectively, while our mean value is 0.272 mm^2. Christophers (1960) (p. 141), also performed direct volume measurements, immersing *Ae. aegypti* eggs in liquids registering values that ranged from 0.0091 to 0.0104 mm^3. These numbers agree with those reported here (mean 0.0095 mm^3), obtained through mathematical formula. Sota and Mogi (1992) registered mean volumes ranging between 0.0178 and 0.0216 mm^3 for *Ae. aegypti* eggs from Gambia, Philippines, Thailand and USA field populations. This corresponds to approximately twice the values here obtained using eggs from the Rockefeller strain.

A direct correlation between ERD and egg volume was found within the genus *Aedes*, when populations from various geographic regions were studied (Sota and Mogi, 1992). In opposition, an *Aedes albopictus* strain selected in the laboratory for increased ERD exhibited eggs with a lower volume than another strain with lower ERD (Sota, 1993). Furthermore, variations in *Ae. albopictus* eggs width and volume depend on the day length to which mothers are exposed (Lacour et al., 2014). In the present work, no relation

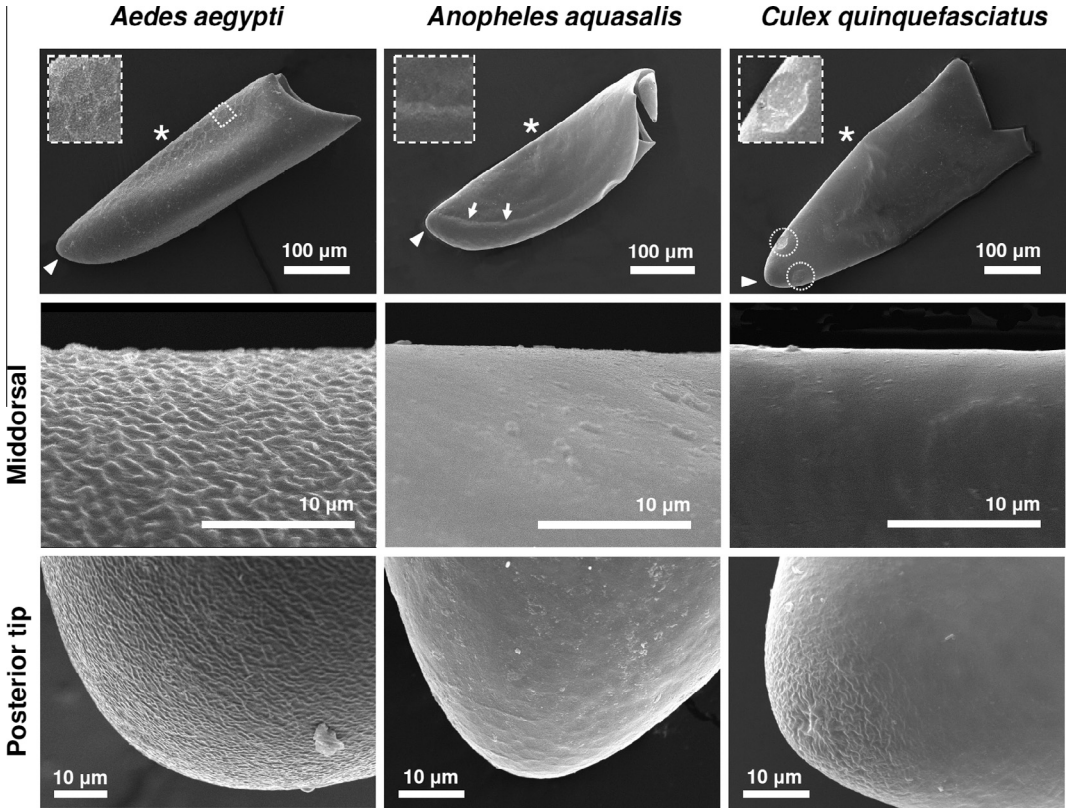

Fig. 5. External endochorion surface aspect varies among mosquito eggs. SEM analysis was performed with isolated eggshells deprived of exochorion and processed after larval hatching. Upper panels: endochorion aspect of whole eggshells. *Ae. aegypti* has high relief polygonal marks (dashed square), *An. aquasalis* posses a lateral sulcus along the antero-posterior axis (arrows) and *Cx. quinquefasciatus* has circular low reliefs (dashed circles), close to the posterior tip. These particularities are highlighted in the insets. Middle panels: middorsal endochorion regions (asterisks on the upper panels). Lower panels: posterior end of the samples (arrowheads in the upper panels). Images are representative of at least 10 specimens of each species.

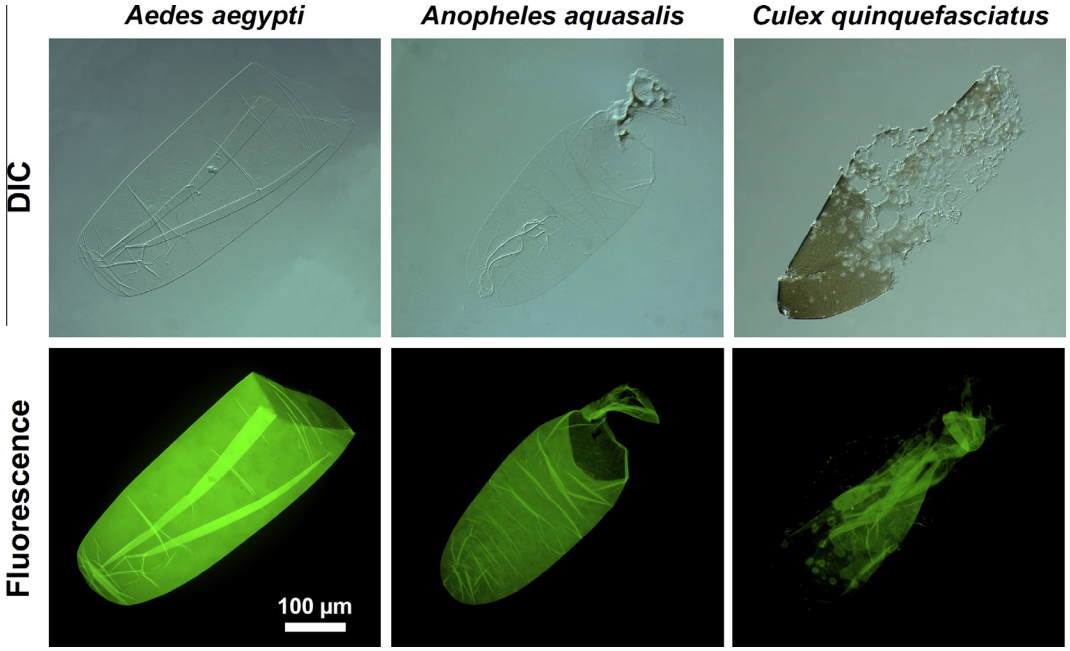

Fig. 6. Chitin is present in the serosal cuticle of mosquitoes. Isolated serosal cuticles were labeled with WGA-FITC. Chitin presence was confirmed in *Ae. aegypti* (Rezende et al., 2008) and first revealed in both *An. aquasalis* and *Cx. quinquefasciatus*. Fluorescence image acquisition was performed under the same conditions for all samples. All images have the same magnification.

was found between egg volume and ERD intensity throughout mosquito genera.

In any biological system it is expected that the higher the surface area/volume ratio, the higher is the water evaporation rate

Table 2
Chitin content in mosquito eggshells.[a]

Species	ng glucosamine/ µg eggshell	ng glucosamine/eggshell surface area (mm^2)
Ae. aegypti	0.674 ± 0.342	3.151 ± 0.528
An. aquasalis	0.317 ± 0.157	2.950 ± 0.931
Cx. quinquefasciatus	0.182 ± 0.037	1.910 ± 0.044

No significant differences in glucosamine content were found among the evaluated samples. The same pools were used for both eggshell weighing and chitin quantification. Measurements of glucosamine/µg of eggshell: three independent experiments were performed for both *Ae. aegypti* and *An. aquasalis* and two for *Cx. quinquefasciatus*. Measurements of glucosamine/eggshell surface area: these values were obtained dividing the glucosamine nanograms values per eggshell number and mean area of each egg. For *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* four, three and two independent experiments were performed, respectively.

[a] As stated in Section 2.1, 'eggshell' refers to the ensemble of endochorion and the innermost serosal cuticle (deprived of exochorion).

(Hadley, 1994). However, we did not find any relationship between ERD and this parameter. Together our data indicate that other factors apart from egg size are relevant to account for the difference in ERD levels presented by *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*.

Ae. aegypti eggs increase in weight and volume during early development due to water uptake (Gander, 1958; Kliewer, 1961). Freshly laid *Ae. aegypti* eggs absorb water gradually during the first 16 h and then keep a steady weight until embryogenesis completion (Kliewer, 1961). It was also verified that arrest of water absorption is concomitant with serosal cuticle formation. Eggs from the three species here evaluated increased in size during embryogenesis, although at different proportions. As suggested by Kliewer (1961), these differences should relate to the distinct dynamics involving serosal cuticle formation. Accordingly, we previously found that while *Ae. aegypti* and *An. aquasalis* serosal cuticle is formed at 21% of embryogenesis, in *Cx. quinquefasciatus* this process occurs later, at 35% of embryogenesis (Vargas et al., 2014). Therefore, only *Cx. quinquefasciatus* eggs would have the potential to keep increasing volume from 20% (when the measurements were made) until 35% of embryogenesis, when the serosal cuticle is formed. An alternative hypothesis relates the higher *Cx. quinquefasciatus* egg expansion to an intrinsic higher water permeability of this species' eggshell. Further investigations are needed to unravel these distinct egg size increase rates during embryogenesis among mosquito species. Notwithstanding, there is a clear inverse correlation between ERD levels and volume increase in the course of embryogenesis (Fig. 7A).

Eggshell thickness is another parameter potentially related to ERD. However, this is difficult to measure in mosquitoes, due to the endochorion sclerotization occurring at early embryogenesis, a process that hampers histological sectioning (Monnerat et al., 1999). The few reports on this issue indicate that endochorion thickness varies among mosquito species: between 3 and 5 µm in *Ae. aegypti* (Clements, 1992; Christophers, 1960) and from 0.6 to 1.2 µm in *Anopheles albitarsis* (Monnerat et al., 1999). There is no information regarding the thickness of mosquito serosal cuticles.

The eggshell contribution to the whole egg weight at the end of embryogenesis was also evaluated. In the present work we found a mean weight of 10 µg for *Ae. aegypti* eggs. For this species, Christophers (1960) and Kliewer (1961) found values of, respectively, 11 and 12 µg. Values for *An. aquasalis* and *Cx. quinquefasciatus* egg weight were not found in the literature. We found a mean eggshell weight for *Ae. aegypti* of 1.5 µg while Christophers (1960) (p. 142) found eggshell weight ranging from 0.79 to 1 µg. In any case, the relative eggshell mass *per se* could not be correlated to the mosquitoes egg ability to resist dry environments.

Meanwhile, eggshell surface density, calculated as the ratio of eggshell weight/area, shows a surprisingly negative correlation with ERD levels (Fig. 7B). We are aware that measuring endochorion and serosal cuticle thickness in these three species will

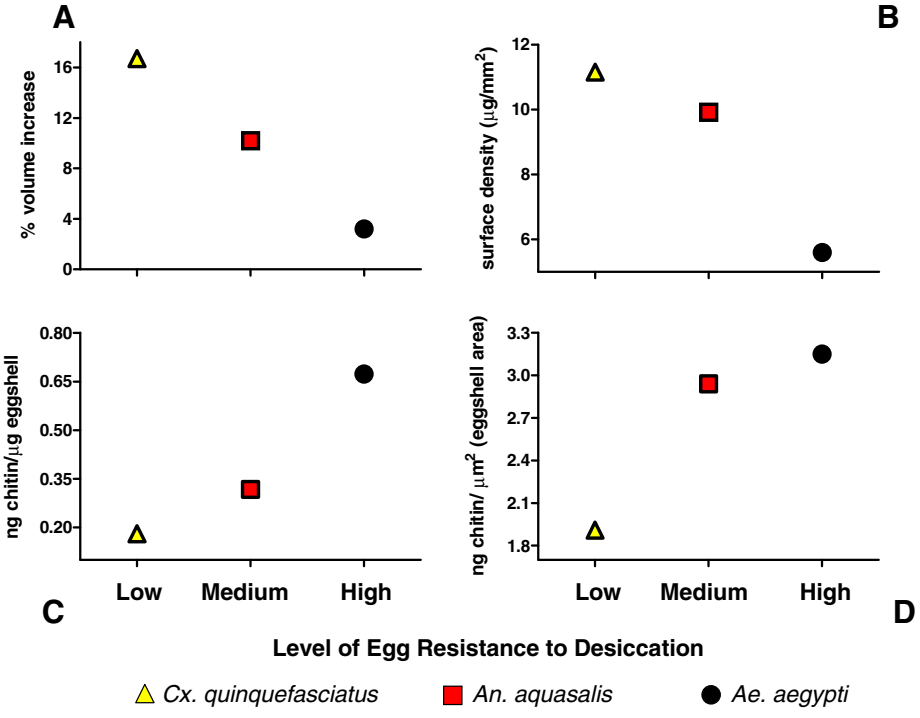

Fig. 7. Mosquito egg swelling in the course of embryogenesis, eggshell surface density and chitin content are related to ERD levels. In all panels ERD levels are classified in increasing categories. (A) Percentage of egg volume increase between 20 and 80% of embryogenesis. (B) Eggshell surface density. (C and D) Chitin content (as glucosamine equivalents) normalized by eggshell weight (C) or area (D).

Table 3
Comparative mosquito egg linear dimension (length and width) ranges reported in the literature.

Species	Length (μm)	Width (μm)	References
Ae. aegypti	545–690	152–184	The present work
	569–765	170–200	Christophers (1960) (p. 139)
	624–704	144–192	Buxton and Hopkins (1927)[a]
An. aquasalis	428–498	120–140	The present work
	403–435	182–219	Maldonado et al. (1997)
	384–449	159–183	Linley et al. (1993)
Cx. quinquefasciatus	681–783	142–169	The present work
	590–659	155–194	Suman et al. (2009)

[a] *Apud* Christophers (1960) (p. 138).

enable to calculate the eggshell volumetric densities (eggshell weight divided by its volume), which could be a more consistent parameter.

SEM analysis has been extensively employed to analyze mosquito exochorion features (e.g., Linley, 1989; Linley et al., 1993; Maldonado et al., 1997; Suman et al., 2009, 2011). In contrast, we found few studies devoted to analyze the endochorion. In the present work, although interesting differences have been detected in the endochorion of the three species (Fig. 5), no correlation to ERD levels could be made. The roughly irregular *Ae. aegypti* endochorion surface with polygonal marks has been observed before (Jarial, 2001) and seems to be ubiquitous in *Aedes* genus (Horsfall et al., 1970). Most likely, these polygonal marks are imprints left by follicular cells that produced the endochorion (Woods et al., 2005). The lateral sulcus present in *An. aquasalis* was also observed in *An. gambiae* (Valle, unpublished results) and probably corresponds to the place where the exochorion float is inserted.

In insects the polysaccharide chitin is present in the larval, pupal and adult integument and also in the peritrophic matrix of the midgut (e.g., Campbell, 1929; Moussian et al., 2005; Arakane et al., 2005; Merzendorfer and Zimoch, 2003; Merzendorfer, 2006). In addition, chitin has been detected in the serosal cuticle of the mosquitoes *Aedes hexodontus*, *Ae. aegypti*, *An. gambiae* and the beetle *T. castaneum* (Beckel, 1958; Rezende et al., 2008; Goltsev et al., 2009; Jacobs et al., 2013). In the present study, this list was expanded to include the serosal cuticle of both *An. aquasalis* and *Cx. quinquefasciatus*.

The lowest chitin content in *Cx. quinquefasciatus* eggshells found here could explain its fragility. Moreover, our data suggest a positive correlation between chitin content and the ERD levels in the three species (Fig. 7C and D). Indeed, recent findings in the beetle *T. castaneum* confirm that chitin presence in the serosal cuticle is relevant to ERD: pRNAi for the *chitin synthase 1* gene (*Tc-chs1*/*Tc-chsA*) results in eggs with a disorganized serosal cuticle. These knocked down eggs are less viable if submitted to low relative humidities when compared to control ones, bearing an intact serosal cuticle (Jacobs et al., 2013). Further evidence shows that chitin metabolism is important for insect ERD. The genes *Knickkopf1* and *Retroactive* are necessary for the proper formation of chitin microfibrils in the extracellular space (Moussian, 2010). Silencing of both genes in *T. castaneum* eggs leads to the formation of a disorganized serosal cuticle (Chaudhari et al., 2015; Jacobs et al., 2015) and lower hatching rates at low humidities, when compared to eggs with a serosal cuticle properly formed (Jacobs et al., 2015).

5. Conclusions

In summary, the data presented here suggest that eggshell chitin content is directly related with increasing ERD levels observed in *Cx. quinquefasciatus*, *An. aquasalis* and *Ae. aegypti*. In contrast, the inverse correlation of mosquito ERD with both egg volume increase during embryogenesis and eggshell surface density suggests that other factors are also relevant for this trait. Further experiments are still necessary to indicate if these correlations lead to causations regarding distinct egg resistance under dry conditions. We are currently investigating if additional eggshell attributes such as its thickness and other biochemical components are relevant for ERD.

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.2015.10.006.

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