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Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*

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ABSTRACT

Given their medical importance, mosquitoes have been studied as vectors of parasites since the late 1800's. However, there are still many gaps concerning some aspects of their biology, such as embryogenesis. The embryonic desiccation resistance (EDR), already described in *Aedes* and *Anopheles gambiae* mosquitoes, is a peculiar trait. Freshly laid eggs are susceptible to water loss, a condition that can impair their viability. EDR is acquired during embryogenesis through the formation of the serosal cuticle (SC), protecting eggs from desiccation. Nevertheless, conservation of both traits (SC presence and EDR acquisition) throughout mosquito evolution is unknown. Comparative physiological studies with mosquito embryos from different genera, exhibiting distinct evolutionary histories and habits is a feasible approach. In this sense, the process of EDR acquisition of *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus* at 25 °C was evaluated. Completion of embryogenesis occurs in *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* at, respectively 77.4, 51.3 and 34.3 hours after egg laying, *Cx. quinquefasciatus* embryonic development taking less than half the time of *Ae. aegypti*. In all cases, EDR is acquired in correlation with SC formation. For both *Ae. aegypti* and *An. aquasalis*, EDR and SC appear at 21% of total embryonic development, corresponding to the morphological stage of complete germ band elongation/beginning of germ band retraction. Although phylogenetically closer to *Ae. aegypti* than to *An. aquasalis*, *Cx. quinquefasciatus* acquires both EDR and serosal cuticle later, with 35% of total development, when the embryo already progresses to the middle of germ band retraction. EDR confers distinct egg viability in these species. While *Ae. aegypti* eggs demonstrated high viability when left up to 72 hours in a dry environment, those of *An. aquasalis* and *Cx. quinquefasciatus* supported these conditions for only 24 and 5 hours, respectively. Our data suggest that serosa development is at least partially uncoupled from embryo development and that, depending upon the mosquito species, EDR bestows distinct levels of egg viability.

Abbreviations: EDR, embryonic desiccation resistance; HAE, hours after egg laying; SC, serosal cuticle.

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1. Introduction

Mosquitoes (Culicidae) are insects of the order Diptera that have great medical importance, since many species are vectors of parasites such as arboviruses and *Plasmodium* (Clements, 1992). Control of many of these diseases affecting thousands of people every year, depends primarily on actions focused on their vectors (Maciel-De-Freitas et al., 2012; Who, 2013). Although mosquito vectors have been studied since the late 1800's (Christophers, 1960) and despite their public health importance, many gaps still remain concerning the biology of these insects. In this sense, embryogenesis is the least known stage of the mosquito life cycle.

Mosquitoes oviposit in water, and freshly laid eggs are prone to water loss. *Aedes* and *Anopheles gambiae* eggs are known to acquire embryonic desiccation resistance (EDR) in the course of embryogenesis. This trait protects developing embryos from losing water, therefore enabling the egg to survive under dry conditions (Beckel, 1958; Goltsev et al., 2009; Harwood and Horsfall, 1959; Judson and Hokama, 1965; Rezende et al., 2008; Telford, 1957). The EDR acquisition arises abruptly during *Aedes aegypti* and *An. gambiae* embryogenesis, and since egg darkening occurs many hours earlier, both processes do not seem to be coupled. The serosal cuticle (SC), an extracellular matrix containing chitin, is the structure responsible to confer EDR to mosquitoes (Goltsev et al., 2009; Rezende et al., 2008) as well as the *Tribolium castaneum* beetle (Jacobs et al., 2013), seemingly a primitive trait among insects.

The serosal cuticle is secreted by the serosa, an extraembryonic membrane of most insects (Panfilio, 2008). In early embryogenesis the serosa primordium is first defined at the differentiated blastoderm stage. Afterwards, two events occur in parallel: (i) the germ band (i.e. the embryo per se) extends and then retracts, with the concomitant definition of the body segments and (ii) the serosa envelopes the embryo and secretes the SC (Goltsev et al., 2007, 2009; Handel et al., 2000; Rezende et al., 2008). After the end of germ band retraction, the process of dorsal closure begins when embryo epidermis progresses towards the dorsal midline, closing the body (Monnerat et al., 2002; Rezende et al., 2008; Vital et al., 2010). During dorsal closure, the serosa retracts dorsally forming the dorsal organ, finally degenerating (Clements, 1992; Goltsev et al., 2009; Panfilio, 2008; Raminani and Cupp, 1978).

The SC confers a very effective EDR for *Ae. aegypti* eggs, enabling them to survive under dry conditions for months, or even a year (Christophers, 1960; Rezende et al., 2008). This trait is implicated in significant ecological issues, such as the mosquito capacity to continue its life cycle after drought periods (Christophers, 1960) and disperse to new locations (Brown et al., 2011). However, there are still gaps in the knowledge related to this process, e.g. what are the biochemical components of the SC? Which enzymes are needed for SC formation? To what extent are the presence of SC and the EDR trait conserved among mosquito evolution? Could eggs from other species survive in dry conditions for long periods, as described for *Ae. aegypti*? Therefore, we adopted a comparative approach to investigate EDR in mosquitoes. The species *Ae. aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*, belonging to three different genera with distinct evolutionary histories and ecological traits (Christophers, 1960; Clements, 1992; Farajollahi et al., 2011; Reidenbach et al., 2009; Simonsen and Mwakitalu, 2013; Sinka et al., 2010), were studied simultaneously. To our knowledge, this is the first inclusion of Culex eggs in evaluations of the presence of a serosal cuticle and acquisition of EDR.

The objectives of the present study are to determine the time needed for completion of embryogenesis of *An. aquasalis* and *Cx. quinquefasciatus*, to identify SC formation in both species in correlation with embryo morphogenesis and, finally, to investigate the viability of mosquito late embryos when eggs are exposed to dry conditions. In all cases, the model system *Ae. aegypti* was used as a reference species with known SC-driven EDR (Rezende et al., 2008).

2. Methods

2.1. Mosquitoes

Mosquitoes from stable colonies maintained in the Laboratório de Fisiologia e Controle de Artrópodes Vetores, IOC, Fiocruz, Rio de Janeiro, RJ, Brazil, were employed: the *Ae. aegypti* Rockefeller strain (Kuno, 2010) as well as *An. aquasalis* and *Cx. quinquefasciatus* cultivated in the laboratory for respectively 18 and 14 years (Belinato et al., 2013; de Carvalho et al., 2002). *Ae. aegypti* and *Cx. quinquefasciatus* larvae were reared in dechlorinated water and fed with crushed cat food (Friskies®, "Peixes – Sensações marinhas", Purina, Camaquã, RS, Brazil); *An. aquasalis* larvae were reared in brackish dechlorinated water (2 mg of marine salt/mL of dechlorinated water) and fed with powdered fish food (TetraMin®, Tetramarine Saltwater Granules, Tetra GmbH, Germany). In all cases, adults were kept at 26 °C and 70–80% relative humidity and fed ad libitum with 10% sucrose solution. For egg production, females were sugar deprived for 24 h and then blood-fed on anaesthetized guinea pigs.

2.2. Synchronous egg laying

Egg laying was always induced under dark conditions, inside an incubator with precise temperature control at 25 ± 1 °C during 1 hour. Eggs were then kept at 25 °C until reaching the adequate age for the experiments. According to the species, the egg laying stimulus procedure was slightly different: *Ae. aegypti* and *An. aquasalis* females, 3–4 days after blood feeding, were anaesthetized in ice for one minute, transferred to upside down Petri dishes (8.5 cm diameter) where the lid became the base and internally covered with Whatman No. 1 filter paper. After insect revival, the filter paper was wet with dechlorinated water for *Ae. aegypti* and with brackish dechlorinated water for *An. aquasalis*, thus stimulating egg laying. *Cx. quinquefasciatus* females were anaesthetized in ice only 5–6 days after blood meal and transferred to 8.5 cm diameter Petri dishes (not upside down) without filter paper. After insect revival, dechlorinated water was added with the aid of a micropipette through a small hole in the lid until the mosquitoes were pressed against the lid, this procedure immediately prompting egg laying (details in Rezende et al., 2008).

2.3. Defining the end point of embryonic development

The definition of embryonic development completion for *An. aquasalis* and *Cx. quinquefasciatus* at 25 °C was performed as previously described for *Ae. aegypti* (Farnesi et al., 2009), with few modifications. Briefly, 2 hours before the putative eclosion of the first larva (empirically determined) *An. aquasalis* and *Cx. quinquefasciatus* eggs were flooded with, respectively, brackish or dechlorinated water, and egg hatching was evaluated at 30-min intervals. For both species the end of embryogenesis was defined as the time required for eclosion of 50% of total larvae. For *An. aquasalis* three independent experiments were undertaken where each experiment consisted of three replicates of 50 eggs each (total of 450 eggs). For *Cx. quinquefasciatus*, four independent experiments were performed (total of 600 eggs). The percentage of hatching was normalized by viability controls (batch of eggs with total hatching recorded 24 hours after the previously defined end of embryogenesis).

2.4. Analysis of embryonic desiccation resistance (EDR) acquisition

The EDR acquisition evaluation was performed as described previously (Rezende et al., 2008), based on cryobiology-derived protocols (Valencia et al., 1996a,b). At distinct embryogenesis time points, replicates consisting of 40 or 50 eggs, obtained from synchronous egg laying, were placed onto 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 intact eggs were counted under a stereomicroscope. Three independent experiments were performed.

2.5. Bleaching procedures to identify serosal cuticle

Bleach (NaOCl) digestion removes the egg chorion while leaving the SC intact. This procedure has already been successfully employed for *Ae. aegypti* (Rezende et al., 2008) and *An. gambiae* (Goltsev et al., 2009). Eggs immediately before and immediately after EDR acquisition were treated with 50% NaOCl (containing approximately 6% active chlorine at final concentration) for 3–20 min and viewed under a stereomicroscope Stereo Discovery V.12 (Zeiss) coupled with a digital image acquisition system.

2.6. Analysis of embryo morphology after EDR acquisition

Synchronized eggs were fixed and clarified according to a methodology described elsewhere (Trpis, 1970). The embryonic morphology of pools of 100 eggs immediately after EDR acquisition was observed. Images were obtained with the same system described above in item 2.5. Embryonic stages were identified according to Monnerat et al. (2002), Rosay (1959), Vital et al. (2010).

2.7. Analysis of egg viability after exposure of late embryos to dry conditions

Synchronized eggs were transferred from wet to dry conditions at approximately 80% of embryogenesis (i.e. 62, 41 and 27 HAE, respectively, for *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*). In each case, groups of 150 eggs were kept dry for varying periods: 5, 10, 24, 48 or 72 hours, comprising 750 eggs in all. Egg viability was then quantified through L1 larvae counting, after employing yeast extract solution (150 mg/mL) as a hatching stimulus (Farnesi et al., 2009). In some experimental conditions, the total test interval ("wet plus dry") remained below the embryogenesis completion period. In these cases eggs were returned to a moist Whatman No.1 filter paper up to that period (see Fig. 5 for details). For each experimental condition a parallel control sample with 150 eggs was maintained in moist filter paper up to the embryogenesis completion period, when yeast extract solution was applied. Each experiment was performed as independent triplicates inside an incubator at 25 ± 1 °C. Relative humidity inside the incubator ranged from 20% to 55%.

2.8. Statistical analysis

For all experiments, mean and standard error were calculated. One way analysis of variance (ANOVA) ($P < 0.05$) followed by Tukey's Multiple Comparison Test was used in the experiment of abrupt acquisition of desiccation resistance and the experiment of egg viability under dry conditions. The results of these analyses are shown in Figs. 2 and 5.

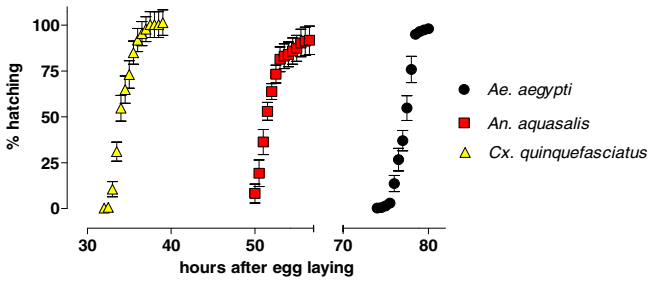

Fig. 1. The time course of mosquito embryonic development at 25 °C varies among species. Cumulative L1 hatching of *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus* is depicted. Average and standard error of each time point, normalized by viability controls (see Methods), are shown. A total of 360 eggs for *Ae. aegypti*, 450 eggs for *An. aquasalis* and 600 eggs for *Cx. quinquefasciatus* were evaluated.

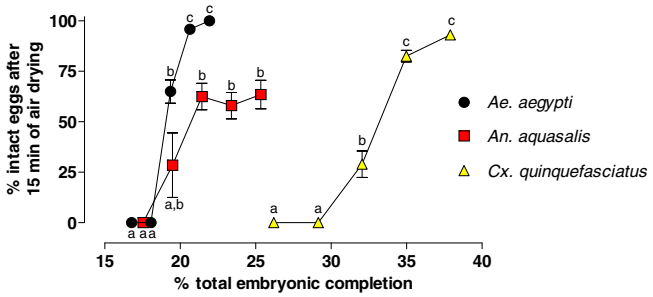

Fig. 2. Abrupt acquisition of desiccation resistance during mosquito embryogenesis. Pools of synchronized mosquito eggs were air-dried for 15 min at different embryonic ages. The percentage of intact eggs (i.e. that did not shrink) was then recorded. The rate of intact eggs in the *y*-axis is expressed relative to the percentage of total embryonic development for each species at 25 °C (in the *x*-axis). Data represent mean and standard errors. A total of 600 eggs for *Ae. aegypti*, 750 eggs for *An. aquasalis* and 750 eggs for *Cx. quinquefasciatus* were evaluated. For each species, values followed by different lowercase letters were significantly different according to Tukey's Multiple Comparison Test.

3. Results

3.1. The total embryo developmental time differs among mosquito species

The three species were tested at 25 °C, temperature previously associated with the highest viability of *Ae. aegypti* embryos, around 96% (Farnesi et al., 2009). *Ae. aegypti* exhibited the longest embryogenesis period, lasting 77.4 hours after egg laying (HAE). *An. aquasalis* presented the intermediate value of 51.3 HAE, and *Cx. quinquefasciatus* was the fastest, its embryonic development being completed after 34.2 HAE (Fig. 1 and Table 1). Given that embryo development of each species requires a distinct time period for completion, it would not be possible to directly compare their physiological processes in 'hours after egg laying'. We then used percentages relative to total embryogenesis for each of the three species. Therefore, in the following results, 100% of embryonic development means 77.4, 51.3 and 34.2 HAE, respectively, for *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*.

3.2. EDR acquisition in An. aquasalis and Cx. quinquefasciatus is related to SC formation

An. aquasalis and *Cx. quinquefasciatus* eggs were air-dried for 15 min (see Methods) in order to investigate if, and when, EDR occurs in these species. The same methodology has been previously adopted to detect EDR in *Ae. aegypti* eggs, at 28 °C (Rezende et al., 2008). In all cases EDR acquisition was confirmed (Fig. 2).

Table 1
Parameters related to mosquito embryonic development at 25 °C.

Species	Hours after egg laying			% Viability[d]
	Completion of embryogenesis[b]	First L1[c]	Last L1[c]	
Ae. aegypti[a]	77.4 ± 0.8	74.0	79.0	96.0 ± 2.0
An. aquasalis	51.3 ± 0.8	50.0	56.5	55.7 ± 17.4
Cx. quinquefasciatus	34.2 ± 0.8	32.0	39.0	66.9 ± 21.4

[a] *Aedes aegypti* data were obtained from Farnesi et al. (2009).
[b] Mean hatching of 50% of larvae specimens.
[c] L1: first instar larvae. The first and last L1 hatching were recorded among 12 replicates.
[d] Values are average ± standard deviation of viability controls (see Methods for details).

Table 2
Time frames associated with mosquito embryonic desiccation resistance (EDR) acquisition at 25 °C.

Species	Period of EDR acquisition	
	HAE[a]	%[b]
Ae. aegypti	14–16	18.1–20.7
An. aquasalis	9–11	17.5–21.4
Cx. quinquefasciatus	10–12	29.1–35.0

[a] HAE: hours after egg laying.
[b] Percentage of total embryonic development time (see Table 1).

In *Ae. aegypti* eggs, EDR is acquired between 18.1% (14 HAE) and 20.7% (16 HAE) of embryogenesis, all eggs younger than 14 HAE shrinking when air-dried for 15 min. In contrast, 16 HAE or older eggs remain intact under the same conditions. *An. aquasalis* exhibits a similar profile, with EDR acquisition at approximately 21% of total embryogenesis. In *Cx. quinquefasciatus*, EDR is attained later, at 35% of embryo development, which in this species corresponds to 12 HAE (Table 2).

Similar to the detection of a serosal cuticle in *Ae. aegypti* (Rezende et al., 2008) and *An. gambiae* (Goltsev et al., 2009) bleach digestion confirmed the formation of this structure in both *An. aquasalis* and *Cx. quinquefasciatus*, eggs completely destroyed before, but not after, EDR is acquired, indicating SC synthesis during this period (Fig. 3). Interestingly, SC of *Cx. quinquefasciatus* directly manipulated with an entomological needle displayed a fragile and gelatinous texture in contrast to that from *An. aquasalis* and *Ae. aegypti*, more rigid to the touch.

3.3. Embryo morphology when EDR is acquired

Embryo morphology at the moment of EDR acquisition, at 25 °C, was also evaluated for the three species, according to the descriptions available in Rosay (1959), Monnerat et al. (2002) and Vital et al. (2010) (Fig. 4). This corresponded to 21% of total embryo development time for both *Ae. aegypti* and *An. aquasalis* and 35% for *Cx. quinquefasciatus*. At that time, *Ae. aegypti* and *An. aquasalis* embryos were at the maximum germ band extension/beginning of germ band retraction stage while those of *Cx. quinquefasciatus* were in the middle of germ band retraction. The morphology analysis of earlier *Cx. quinquefasciatus* embryos confirmed germ band retraction and not germ band extension at 35% of development (data not shown). It is important to mention that immediately before EDR acquisition (i.e. 18.1%, 17.5% and 29.1% of total embryonic development for *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*), the embryos are completely surrounded by serosa cells (data not shown), but the SC is not formed yet.

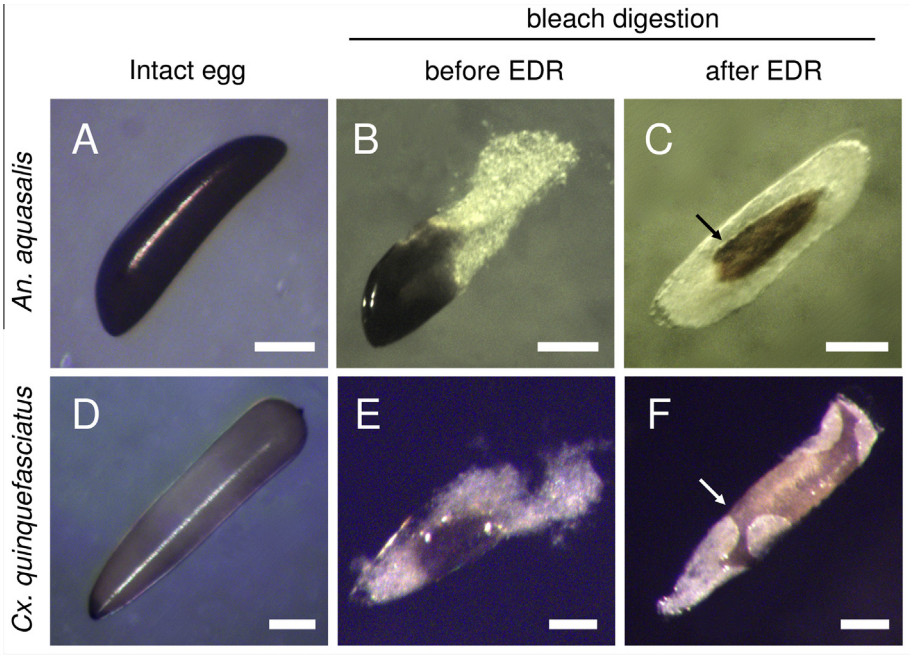

Fig. 3. Acquisition of EDR in *An. aquasalis* and *Cx. quinquefasciatus* is related to serosal cuticle formation. The serosal cuticle presence was determined by bleach digestion. (A and D) Intact eggs; (B, C, E, F) eggs after bleach digestion. (B and E) Eggs before EDR acquisition. (C and F) Eggs after EDR acquisition possess a serosal cuticle that resists to bleach digestion. Arrows: remaining endochorion fragments. Bars = 100 μm.

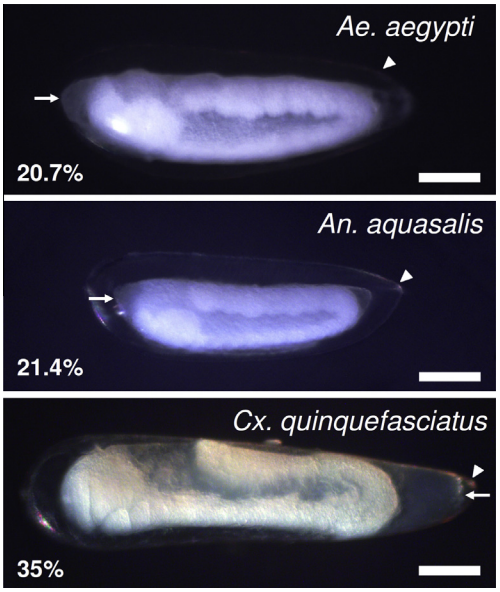

Fig. 4. Embryo morphology at the onset of desiccation resistance acquisition. When EDR is acquired *Ae. aegypti* and *An. aquasalis* embryos are at the end of germ band extension/beginning of germ band retraction while those of *Cx. quinquefasciatus* are in the middle of germ band retraction. Images were obtained after endochorion clarification and embryo fixation (see Methods). Percentages are related to total embryonic development at 25 °C. Arrowheads: clarified endochorion. Arrows: Serosal cuticle. Bars = 100 μm.

3.4. The extent of EDR varies among mosquito species

In order to estimate the physiological relevance of EDR, a viability assay was performed. Eggs at 80% of embryogenesis were transferred from wet to dry conditions for varying periods of time, ranging from 5 to 72 hours (Fig. 5, see item 2.7 for details). For *Ae. aegypti* eggs, viability remained above 86% throughout the experiment. *An. aquasalis* viability was inversely proportional to the time spent under dry conditions, eggs exposed for 72 hours not hatching at all. Viability of *Cx. quinquefasciatus* eggs was only 75% after 5 hours under dry conditions, and no hatching was observed after exposure for longer periods of time.

4. Discussion

We aimed to study the generality of EDR acquisition in different mosquito species of medical importance. Representatives of three important vector genera were chosen: *Aedes*, *Anopheles* and *Culex*. Chemical control of vector mosquitoes is mainly directed against larval or adult stages. There has been little focus on mosquito eggs, despite the potential of being a relevant control target (Beament, 1989). *Ae. aegypti* eggs can remain viable under dry conditions for months due to the EDR phenomenon and SC formation (Christophers, 1960; Rezende et al., 2008). Nevertheless, there was a lack of data regarding the egg viability on dry for relevant physiological periods (i.e. for hours or days during embryogenesis and after its end) in other genera (such as *Culex* and *Anopheles*).

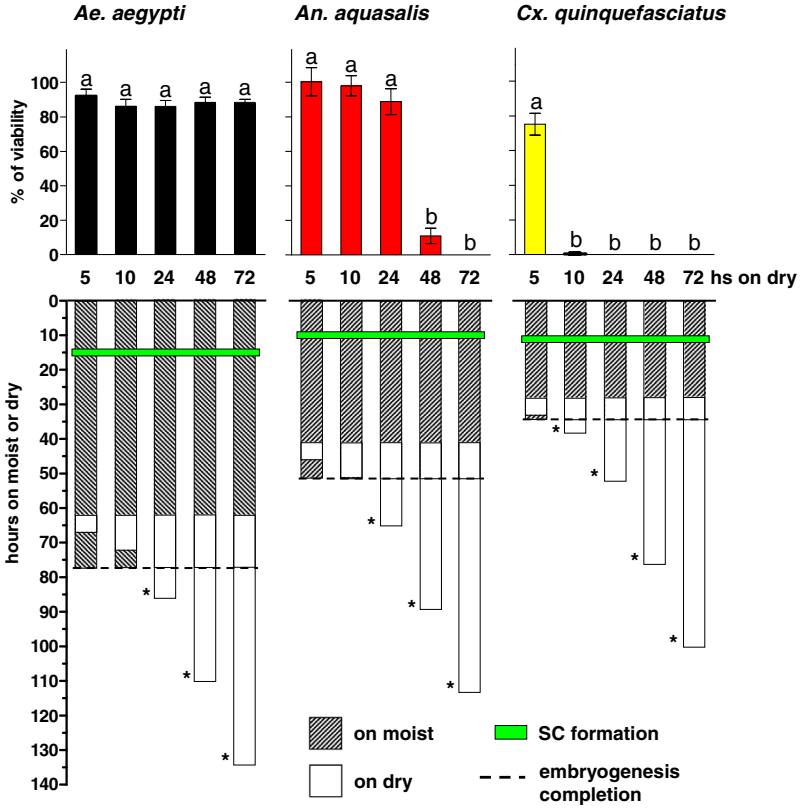

Fig. 5. Egg viability under dry conditions at the end of embryogenesis varies among mosquito species. Pools of synchronized eggs, with 80% of embryogenesis completed, were transferred to dry conditions for 5, 10, 24, 48 or 72 h at 25 °C, as indicated in the lower panels. Hatching of samples was then evaluated (see item 2.7 for details). Data are expressed as percent viability, normalized from control samples, kept under moist conditions throughout development. Green stripe: period comprising SC formation (see Table 2). Dashed line: end of embryogenesis. Hatching stimuli with yeast extract solution was performed at the end of embryogenesis, or when indicated with an asterisk. Each bar represents mean and standard deviation of 450 eggs (triplicates of 150 eggs each). *Aedes aegypti* was used as a positive control of desiccation resistance. For each species, values followed by different lowercase letters were significantly different according to Tukey's Multiple Comparison Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At 25 °C, *Ae. aegypti* embryos finish their development at 77.4 HAE. At this temperature, a great variation is noted among *Aedes* species: *Ae. dorsalis*, *Ae. nigromaculis*, *Ae. albopictus*, *Ae. vexans*, *Ae. sticticus* and *Ae. squamiger* embryonic development terminates, respectively, after 75.7, 75.7, 80.8, 91, 152 and 270 HAE according to estimative from literature data (Moretti and Larsen, 1973; Telford, 1957; Trpis et al., 1973) and Vargas (unpublished results for *Ae. albopictus*). Similar periods for embryogenesis completion are apparent in *An. aquasalis* (51.3 HAE), *An. gambiae* (50 HAE) (Goltsev et al., 2009) and *Anopheles quadrimaculatus* (55.9 HAE) (Farnesi et al., unpublished results), unlike *Cx. quinquefasciatus* (34.2 HAE) and *Culex tarsalis* (46.3 HAE) (Rosay, 1959). These data indicate that it is not feasible to estimate the total embryonic development time of a given mosquito species based solely on its genus. Several aspects, such as ecological niche, geographical distribution and even the number of generations per year can influence the definition of this trait (Gillooly and Dodson, 2000). For example, embryos of *Ae. aegypti*, a multivoltine species, with several generations each year, develop twice as fast as the univoltine *Ae. sticticus* (Christophers, 1960; Trpis et al., 1973). *Cx. quinquefasciatus* exhibits a predominant tropical distribution and also tends to reach the end of embryogenesis faster than *Cx. tarsalis*, present in temperate regions (Wrbu, 2013a; Wrbu, 2013b).

The present study revealed, based on cryobiology-derived protocols from Valencia et al. (1996a) and Valencia et al. (1996b) that *An. aquasalis* and *Cx. quinquefasciatus* eggs also develop resistance to desiccation in the course of embryogenesis. This is due to the formation of the SC, a layer secreted by serosa cells, as detected for *Ae. aegypti* (Rezende et al., 2008), *An. gambiae* (Goltsev et al., 2009) and the *Tribolium castaneum* beetle (Jacobs et al., 2013). EDR arises at an equivalent development moment for both *Ae. aegypti* and *An. aquasalis*, at around 21% of embryogenesis, also corresponding to the same morphogenetic stage (end of germ band extension/beginning of germ band retraction). In *Cx. quinquefasciatus*, EDR appears later, at 35% of embryogenesis and corresponds to a more advanced stage (middle of germ band retraction). Our data suggest that serosa development (and the consequent EDR acquisition) is at least partially dissociated from embryo development, since both developmental programs occur independently in the three mosquito species studied. Uncoupling between embryonic and extraembryonic insect development is corroborated by the normal development of the *Tribolium castaneum* beetle lacking a serosa, a feat accomplished by silencing the transcription factor *Zerknüllt 1* by RNAi in parental specimens (van der Zee et al., 2005).

The viability after exposure to dry conditions at the end of embryogenesis was evaluated in order to assess the physiological relevance of the EDR for different mosquitoes. As expected, *Ae. aegypti* embryos survived drought up to 72 h, the longest period tested, and would survive for months, as described before (Christophers, 1960; Clements, 1992; Rezende et al., 2008). In our experimental conditions, *An. aquasalis* eggs attained high viability rates up to 14 h after the end of embryogenesis (24 h in a dry environment) while viability of *Cx. quinquefasciatus* eggs dropped to almost zero after only 5 h under the same conditions. It could be argued that the low viability of *An. aquasalis* and mainly of *Cx. quinquefasciatus* after the end of embryogenesis is not due to desiccation but rather to eventual metabolic needs of the pharate larvae inside the egg (i.e. larvae could have died from starvation rather than drying). However, *An. gambiae* larvae, if kept in humid microclimates, can hatch up to ~10 days after the end of embryogenesis (Beier et al., 1990; Shililu et al., 2004), for other *Anopheles* species this period possibly being even longer (Clements, 1992). We are not aware of any similar study performed with *Culex* eggs, and further experiments are necessary to completely discard the starving hypothesis for *Cx. quinquefasciatus*. In any case, the *Anopheles* "mild" EDR is probably of ecological relevance in the maintenance of egg viability during dry seasons, for example in sub-Saharan Africa (Beier et al., 1990; Goltsev et al., 2009; Shililu et al., 2004), as adult desiccation resistance is ecologically relevant for the *An. gambiae* complex (Gray and Bradley, 2005).

According to Beckel (1958), blockage of water passage out of the mosquito eggshell is not derived from the SC alone. It is a consequence of the interaction between SC and endochorion and would be driven by the process of sclerotization/melanization (Goltsev et al., 2009; Hopkins and Kramer, 1992). Therefore, the distinct EDR degrees observed in the three species evaluated might be due to many reasons: differences in the endochorion or SC thickness, degree of endochorion sclerotization/melanization or variations in the SC biochemical components. In any case, it is worth mentioning that in lepidopteran *Manduca sexta* eggs, there is a tradeoff between water loss and respiratory gases exchange (i.e. an increase in water retention decreases the capacity of the embryo to exchange gases) (Woods, 2010). We are unaware of any study relating water loss and gas exchange in developing mosquito embryos.

An. aquasalis belongs to the Anophelinae subfamily, while both *Ae. aegypti* and *Cx. quinquefasciatus* belong to the Culicinae subfamily. Their last common ancestor took place ~217 million years ago, while the split between *Aedes* and *Culex* lineages occurred ~204 million years ago (Reidenbach et al., 2009). Therefore, it could be inferred that the delayed SC formation and EDR acquisition observed in *Cx. quinquefasciatus* would be a derived trait, not shared with the *Aedes* and *Anopheles* genera. Moreover, there is a high divergence related to the degree of egg viability under dry conditions among the three species. In this regard, it is interesting that the two extremes of viability in dry conditions (i.e. "high" for *Ae. aegypti* and "low" for *Cx. quinquefasciatus*) occurs in the two species more closely related, while the more distant *An. aquasalis* possesses a "mild" viability.

Given that lower dipterans (including mosquitoes) are essentially aquatic insects (Wiegmann et al., 2011) and the existence of SC and EDR are ancestral insect traits (Jacobs et al., 2013), it could be hypothesized that in the course of evolution generating *Cx. quinquefasciatus*, a waterproofing SC was not under an intense selection pressure. Eggs of *Cx. quinquefasciatus* are laid in water rich in organic matter or even polluted (Clements, 1992; Simonsen and Mwakitalu, 2013) that has lower oxygen content and possesses a myriad of microbes, some of which can be pathogenic. The serosa is capable of eliciting an immune response against pathogens (Gorman et al., 2004; Jacobs and van der Zee, 2013), possibly acting as an immune tissue to protect the developing embryo. Assuming that the weaker EDR is due to a weaker SC, the *Cx. quinquefasciatus* serosa could deviate a significant fraction of the energy employed to synthesize its cuticle to elicit a better immune response. In addition, in this case a weaker EDR would be beneficial, since it would permit a more efficient gas exchange thus allowing a faster development.

Meanwhile, in the course of evolution that originated the *Ae. aegypti* species, a mosquito whose larvae develop in clean water, opposite strategies may have occurred, with a decrease in the gas exchange rate due to an increase of EDR level leading to a longer period of embryonic development completion.

5. Conclusions

This work extends knowledge on the processes of EDR acquisition and serosal cuticle formation in mosquito eggs. Although in all cases the production of a serosal cuticle inhibits embryo water loss, their degree of viability under dry conditions varies enormously depending upon the species. We are currently studying the nature of these differences in *Ae. aegypti*, *An. aquasalis* and *Cx. quinquefasciatus*.

Competing interests

The authors declare that they have no competing interests.

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