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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).