Aedes Aegypti: Morphology the Egg and Embryonic Development

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Research

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Abstract

Background: *Aedes aegypti* (Diptera: *Culicidae)* has great relevance in public health worldwide due to its performance as a vector of arboviruses, which makes the knowledge about its cycle fundamental. It is known that the acquaintance of embryonic kinetics has great potential to assist in the development of new vector control technologies and, consequently, in the control of arboviruses.

Methods: This research proposes to analyze the egg morphology and embryonic development of *Aedes aegypti*. Eggs from the insectary of the Institute of Biomedical Sciences of the University of São Paulo (n = 46) were used. The methodological procedure involved morphological and ultrastructural analyzes using equipment for optical microscopy (light and confocal) and electronic (scanning). Eggs and embryos were observed in initial, intermediate and final thirds of development, kept at a temperature of 28°C, 1°C until collection for processing.

Results: The embryos had different morphological characteristics according to the stage of gestation, in the initial and intermediate stages of development. In the initial third, the presence of primordial epithelium and characteristics suggestive of intense cellular activity were found.

Conclusion: According to the results obtained in the eggs, it is concluded that the great resistance of the chorion in the embryo is a factor that generates difficulties for microscopic analysis of the embryo (mainly in fixation), with a strong protective barrier.

Background

The *Aedes aegypti* has great importance in public health worldwide, due mainly to its involvement in the transmission of arboviruses (Arthropod-borne-viruses) such as Dengue (WHO 2018), *Chikungunya* (Charrel et al. 2014; WHO 2017) and *Zika* (WHO 2018). The simultaneous circulation of its etiological agents in various locations characterizes the epidemiological scenario of these diseases, which increases the risk of spread, the possibility of coinfection and transmission of more than one virus at a time in humans, complicating diagnosis and recovery and the relevance of this vector (Gloria-Soria et al. 2016; Chaves et al. 2018; Carrillo-Hernández et al. 2018).

The lack of an effective vaccine for vast majority of arboviruses (Ferguson et al. 2016), makes clear that there is a need for high levels of vector-directed control (Brazil 2015; Lima-Camara 2016), based on epidemiological and entomological control (WHO 2018; WHO 2017; WHO 2018). Nevertheless, keeping mosquito populations under control is still inefficient. In these cases, eggs are of special importance, as they are easily located, and they are very resistant and can remain viable for more than a year (Brazil 2015), which justifies the fact that the new technologies are destined to destroy eggs or to fail the hatch (Caragata et al. 2019). Therefore, new in-depth knowledge of the characteristics of eggs and the embryonic development would contribute to creating the basis for new control and eradication strategies.

In general, it is known that eggs have an external coating structure called a chorion, with a protective function, allowing gas exchange and minimizing water loss. The chorion is made of distinct layers: the endochorion and exochorion (Clements 1992), these structures are known to have different characteristics between the species and the genus *Aedes*, originating from Gwalior, India (Suman et al. 2011). Inside, embryogenesis occurs after fusion of the female pro-nucleus with the male pro-nucleus, after the oocyte passes through the ovarian tube (Fonseca, Gomes and Araújo 2012).

Previous studies reveal that the external structure of eggs (exochorion) from insects in general, differentiates in several orders such as culicids of medical interest (Obara et al. 2007; Famesi *et al.* 2017; Faull and Williams 2016; Mello et al. 2018; Alencar et al. 2003). Insects, especially those considered to be of medical importance, show phenotypic variation when subjected to environmental changes (Reinhold, Lazzari and Lahondère 2018). Currently, the evolutionary significance of genetic and epigenetic assimilation is widely discussed (Mathews *et al*. 2018). *Drosophila melanogaster* is considered the model insect for the study of embryonic development of insects, (Fonseca, Gomes and Araújo 2012), for which the precarious description about the embryonic morphology of *Aedes aegypti* has been attributed to the problems related to the permeability of the egg, plus the embryonic development has not yet been properly reported. This study was in charge of describing characteristics morphological of *Aedes aegypti* eggs and describing your embryonic development.

Material And Method

Obtaining biological material

Aedes aegypti eggs were made available by the Parasitology Department of the Institute of Biomedical Sciences of the University of São Paulo, USP, collected during the period from November / 2014 to February / 2015 (n=46). For the analyses of the embryonic development, there were synchronization of egg-laying, three groups of eggs of this insect were collected at different times of embryogenesis, without ending/initial, intermediate and final stages of its development. Embryogenesis is considered be completed in approximately 61.6 hours when the temperature is 28 ° C, according to a study by FARNESI *et al*. (2017). All samples were kept at a temperature of 28ºC, and this reason does remain in the original insectary from the laying of eggs until the moment of collection for processing. This pattern was maintained for three different collection times. Each group of samples collected, had its development interrupted when submitted to the tissue fixation procedure. Morphological analysis was performed using resources such as optical microscopy (light and laser scanning confocal) and electronic microscopy (transmission and scanning).

Optical Microscopy: Light

Samples were fixed in a 10% formaldehyde solution. After complete fixation, they were dehydrated in a series of ethanol in increasing concentrations (70 to 100%) and diaphanized in xylol, with subsequent inclusion in histological paraffin. 3µm thick cuts were made in the microtome (Leica, German) and

stained with Hematoxylin-Eosin. The images were obtained through the Nikon Eclipse E-800 light microscope at the Advanced Diagnostic Imaging Center / FMVZ-USP.

Optical Microscopy: Laser Scanning Confocal

The eggs were initially processed with water washing, followed by immersion in 3% sodium hypochlorite until clarification (approximately 30 minutes), with subsequent washing in PBS, 0.02% triton for 5 minutes and again washing in PBS, the material then it was fixed in 3.7% formaldehyde for 20 minutes, after this procedure a new washing sequence was performed with PBS, permeabilization using 1% triton at room temperature and washing with PBS twice. The incubation was carried out in a dark room for 60 minutes and phalloidin (FITC) was used. RNAse was added in the final 30 minutes of incubation. After this treatment, the embryos were again washed with PBS and the nuclei were stained with Propidium Iodide. Fluorescent images were obtained by laser scanning confocal microscopy (Zeiss LSM 510) at the Cellular and Molecular Biology laboratory (BioCeM) of the Institute of Biomedical Sciences-USP.

Transmission Electron Microscopy

The samples were fixed in 2% glutaraldehyde, post-fixed in a 1% osmium tetroxide solution at 4 ° C and a 5% aqueous solution of uranyl acetate at room temperature. Then, the samples were dehydrated in ethanol, increasing concentration, immersed in propylene oxide and soaked in Spurr resin. For light microscopy, semi-thin sections were cut in a Cut Ultra Reichert® ultramicrotome and stained with a 1% toluidine blue solution. The thin 90 nm sections were cut and collected in 200 mesh (Sigma ®) and contrasted with a 4% uranyl acetate solution and a 0.4% aqueous solution of lead citrate. The grids were examined using a transmission electron microscope at the Advanced Center for Diagnostic Imaging, Faculty of Veterinary Medicine and Animal Science (FMVZ) -USP. Specifically, for the analysis of the semi-fine cut, the eggs were subjected to previous processing with the washing in water, followed by immersion in sodium hypochlorite 3% until its clarification (approximately 30 minutes), with subsequent washing in PBS, 0.02 triton % for 5 minutes and again washed in PBS, only then were they fixed in glutaraldehyde giving continuity to the processing.

Scanning Electron Microscopy

The biological material was fixed in a modified Karnowsky solution (5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2), followed by washing in sodium cacodylate buffer, pH 7.2 and posterior fixation in tetroxide osmium (OsO_4) at 1% in 0.2M sodium cacodylate buffer. After carrying out a new series of washing and cleaning controls, the eggs were dehydrated in an increasing series of ethanol to absolute ethanol (50%, 75%, 90% and 100%). After passing at a critical point, the material was mounted on Stubs using double-sided carbon adhesive tape followed by gold-plated coverage, using the sputtering system, and the FEI scanning electron microscope was used to analyze the material. Quanta 250 at the Cell Biology Laboratory of the Butantan Institute. - SP, Capital where they were photomicrographed for further analysis.

Morphometric analysis

Morphometric analysis of the eggs was performed from the measurements of the images obtained in Scanning Electron Microscopy. Linear dimensions of the egg were considered for this analysis:

- Length - distance between the micropyle and the opposite end, Width - largest distance perpendicular to the length,
- Egg index - length to width ratio,
- Diameter of the microcapillary disc - diameter of the anterior structure in the egg

After obtaining measurements were perform analysis of measures of central tendency (mean) and measures of dispersion (standard deviation, maximum and minimum values). Statistical analyzes were performed using the Graph Prism software, 95% Confidence Interval was considered.

Results

Eggs

Macroscopically, eggs of *Aedes aegypti* are whitish in color at the time of oviposition, which quickly becomes black. Some characteristics were identified, such as shiny appearance, tapered extremities, bilateral symmetry, in addition to a flattened surface opposite to a convex surface (Fig. 1A). Morphometrically we observed that the linear dimension of the population of eggs referring to the length was 581.45 ± 39.73 µm and the width of 175.36 ± 11.59. The egg index (length / width ratio) was 3.32 ± 0.26 µm. Concerning the measurement of the micropyle (DM) disk diameter of the evaluated eggs, it remained at 18.75 ± 1.92 µm (Table 1). The results were also compared with those from the studies by Suman et al., (13), Linley (22) and Faull (Fig. 1B).

Table 1
Morphometric parameters related to the linear size of the population of *Aedes aegypti* eggs coming from the municipality of São Paulo-SP in relation to measures of central tendency and dispersion. [*This* Table 1 *should be attached between the 236 and 237 lines*]

	Atributes Morphometric	**Central tendency and dispersion measures**			
		Mean	IC95%	Maximum Value	Minimum Value
Eggs collected in Sao Paulo	Length	581.45 ± 39.73	569.65-593.25	655.20	521.40
	Width	175.36 ± 11.59	171.92–178.80	199.60	156.50
	Length/width	3.32 ± 0.26	3.24–3.40	4.13	2.78
	Diameter	18.75 ± 1.92	18.18–19.32	22.18	14.27

Table 2. Comparative analysis of the morphometric findings of *Aedes aegypti* eggs identified in this study and the results of Suman et al, (2011), Linley (1989), Faull and Williams (2016).

	A. aegypti[a] *	*A. aegypti*[b] **	*A. aegypti*[c] ***	*A. aegypti*[d] ****	*A. aegypti*[e] ****
Length	581.45±39.73	625.65±19.91	670.2±7.2	554.41±36.56	562.62 ± 30.85
Width	175.36±11.59	183.30±11.04	186.3±2.2	167.65±7.05	160.15 ± 9.73
Length/Width	3.32±0.26	-------	3.61± 0.05	3.31±0.18	3.52 ± 0.27
Diameter of micropilar disc	18.75±1.92	-------	-------	33.49 ± 3.9	34.19 ± 5.4

a.Mundim-Pombo et al b.Suman et al, 2011 c. Linley,1989 d. Faull and Wiliams.,2016 e. Faull and Williams, 2016

* Population of São Paulo eggs, **Population of India eggs ***Population of Florida USA eggs **** Population of Cairns-Australia eggs ****Population of Charters Towers eggs - Australia

Ultrastructural SEM showed that the extremities are characterized by poles, the anterior pole, where the entire micropylar apparatus (disc/crown of the micropyle, sectors of the disc of the micropyle and micropyle) is located and is slightly prominent when compared to the opposite pole. The microcapillary device maintains a prominent and continuous circle shape (Figs. 1C and 1D), while the posterior pole is more tapered in relation to the opposite side (Figs. 1C and 1E). In the external coating of the eggs, regularity was identified about the aspect of distribution and shape of their cells, most of them maintaining a hexagonal shape (Figs. 1C and 1E).

In the central region of the chorionic cells, larger diameter tubers are identified. The tubers are symmetrically arranged, there are small peripheral tubers arranged in an organized manner around larger central tubers (Fig. 1F, 1G and 1H). Both appear prominently in the exochorion, thus giving the appearance of high relief or texture.

Transmission microscopy was used in a complementary manner to analyze the chorion (Fig. 1I-L), where it was possible to identify the ornamentations of the exochorion and confirm that the impermeable characteristic of this structure prevents the entry of fixers and resin inside the cell in the sample processing, causing the compromise of these cells. Another finding in relation to the exochorion is that it was more electron dense when compared to its adjacent layer, the endocorion.

The impermeability characteristic of chorion drew attention, which made the analyzes related to it, as being extremely difficult and generating several repetitions in the different stages of this study.

Embryo

The embryonic development of *Aedes aegypti* occurs inside eggs of up to 1 mm in length (Fig. 1A), dark in color and capable of adhering firmly to various substrates. In the period that comprises the initial third of the development of the *Aedes aegypti* embryo, it was possible to verify the scarce presence of bristles in the cephalic region and relatively homogeneous structures in terms of their general morphological aspects. Rupture of the chorion (Figs. 2A and 2B), which in turn will open the area outside the egg and release the larva. The identification of this region as being the cephalic region is possible because this region coincides with the micropillar apparatus.

In the histological analysis, elements characteristic of the ornamentation of the exochorion were identified, since surrounding this region are the central (larger) and peripheral tubercles (Fig. 2I and 2J). In the interior of the egg, embryonic cells are identified, and specifically in the initial third of development (5 hours), it was possible to observe in a semi-thin section, the presence of round cells with a peripheral nucleus and several cells presenting two nuclei, a process suggestive of cell division (Fig. 2K, 2M and 2N). Still in the initial stage of embryonic development, after 18 hours from the analysis of the semi-fine cut, folding of the embryo was found, around the egg internally in about 60% of it, a situation called the extension of the germ band and occurs at the moment of gastrulation when the ventral blastoderm undergoes extension (Fig. 2C, 2D and 2E), it is also suggested that the process of cellular activity is maintaining itself intensely after 18 hours of the onset of cell development, as a large number of cells were found in the process of division (Fig. 2G and 2H).

The intermediate phase (Fig. 3A-F) of development brings characteristics such as the increase in the presence of bristles, including palatal brushes, which in the future will allow the larva to perform movements promoting water flow to bring food to be consumed. There is also a separation of the cephalic and thoracic region and loss of homogeneity of the general morphological aspect. At the developing embryo stage, is evident the presence of a membrane surrounding the embryo and, therefore, located between the embryo and the chorion, this extraembryonic membrane is the serous layer (Fig. 3G).

Still at this stage, embryos were analyzed after 25 hours (Fig. 19C) and 30 hours of development (Fig. 3H to 3M). During this period, the process of formation of the abdominal and thoracic segments and structures such as the respiratory siphon and the last abdominal segment in the form of a caudal appendix (Fig. 3L), a spike that will promote the cleft to release the larva at the time of hatching, was observed of the egg, in addition to the presence of bristles in the cephalic region (Fig. 3M).

In the final third of development (Fig. 4A-D) the body of the future larva already has the divisions in the head, thorax and abdominal segments, but the indicator for completion of growth is mainly the presence of the chorion-breaking spike (Fig. 4C-H). This structure persists until the first larval stage (Figs. 4I and

4J), a factor that differentiates the first and second larval stages. In the period of completion of embryogenesis, prominence in the chorion was identified (Fig. 4E), specifically in the anterior region, which indicates that the larva is ready to gain the aquatic environment. The prominence identification site was the one that gave rise to the rupture of the transverse fissure in this region by the chorion-breaking spike (Fig. 4F-H), in the egg after completion of the embryonic development. This region will be the one where the larva will leave the egg. Histologically, the final stage of development (Fig. 4K, 4L, 4Q) shows that segmentation is better defined, as well as the primitive intestine (Fig. 4M), the discreet presence of bristles in the cephalic region is replaced by the abundant presence of these structures (Fig. 4P).

It is known that an important element of the cytoskeleton is actin, and this, in turn, was marked by the reaction with phalloidin, which then made it possible to understand its distribution by cells, while the nucleus was marked by propidium iodide. The images obtained by confocal microscopy made it possible to follow the sequence in embryonic development after 18 hours of development (initial phase) until the moment when the larva is ready to gain the environment (Fig. 5A to 5F). In this analysis it was possible to identify the embryo in a situation of extension of the germ band (Fig. 5B), after 30 hours of development the serous cell layer surrounding the embryo (Fig. 5B) was clear, and at the end of the development it was verified in 40 hours, the process of segmentation and dorsal closure (Fig. 5C) and within 50 hours an embryo was observed whose thoracic and abdominal segmentation was well defined (Fig. 5D), it is possible to observe the moment of the larvae hatching (Fig. 5E) and for end the embryo after 7 days diapause, with its development finished, ready to gain the external environment. In this case, the presence of visible segmentation was found and between the fifth and sixth abdominal segment, it was possible to show the intestine (Fig. 5F).

Discussion

The characteristics of eggs represented by *Aedes aegypti*, evaluated in this study were black or brown in color and measured less than 1.0 mm in length (Service 2003; Bova, Paulson and Paulson 2016). Despite the fact at the time of egg-laying, they had a white color, darkening afterward (Sallum, Barata and Santos 2007). Other characteristics inherent to the egg were: the presence of oval or elliptical outline, with bilateral symmetry (Suman et al. 2011; Bova, Paulson and Paulson 2016; Sallum, Barata and Santos 2007; de Morais et al. 2019), a characteristic identified in this research.

Fresh eggs are susceptible to water loss and this condition can impair its viability (Vargas et al. 2014). Such a statement suggests that there would be greater permeability in eggs with shorter embryo development time, so there would be greater ease in fixing embryonic tissues and obtaining results consistent with the proposed objectives. However, in this research, it was not possible to identify any easier processing of eggs in any of the evaluated phases, all of them were equally laborious since the resistance of the chorion was a constant complicating factor.

The exochorion generally maintain ornamentations that make it possible to identify the species. It is an excellent parameter for comparing species, as it has ability to reveal significant differences (Suman et al.

2011; Service 2003; Bova, Paulson and Paulson 2016; de Morais et al. 2019). In the figures presented (Figs. 1C, 1E, 1F and 1G) the ornamentations on the exochorion maintained the same pattern of organization in the species studied (Isoe et al. 2019). In addition to *Aedes aegypti*, other culicids of the same genus present ornamentation with polygonal chorionic cells in the exochorion of the egg, where a large central tubercle and other smaller and peripheral ones are observed, except for the anterior surface containing the micropylar apparatus (Suman et al. 2011; Faull and Williams 2016). The central tubercles on the external surface of the egg joined the peripheral tubercles employing thick projections forming a line; however, these differ in terms of their dimensions according to the location in the exochorion (Linley 1989; Isoe et al. 2019). In this study, this projection can be seen in Fig. 1.

When rejecting the hypothesis of equality between the two egg populations, it is suggested that a similarity identified in two distinct populations occurred due to chance, and given the above, the hypothesis arises that there may be microevolution in these populations considering these attributes (Wilk-Da-Silva et al. 2018; Louise, Vidal and Suesdek 2015). However, the use of other taxonomic and phylogenetic tools is necessary to deepen the discussion on this evolutionary process. When comparing morphologically, using scanning electron microscopy, eggs of *Aedes aegypti* in relation to eggs of *Aedes albopictus* (Suman et al. 2011; de Morais et al. 2019), it's affirmed that the former are greater concerning the micropyle disc and pore attribute in relation to the latter and the chorion polygonal cells have little capacity to assist in distinguishing between these species alone. Agreeing with Faull and Williams (2016) who expanded the analysis in different Australian cities regarding *Aedes notoscriptus* (Table 2).

Table 2
Comparative analysis of the morphometric findings of *Aedes aegypti* eggs identified in this study and the results of Suman et al, (2011), Linley (1989), Faull and Williams (2016). [*This* Table 2 *should be attached between the 278 and 279 lines*]

	A. aegypti[a] *	*A. aegypti*[b] **	*A. aegypti*[c] ***	*A. aegypti*[d] ****	*A. aegypti*[e] *****
Length	581.45 ± 39.73	625.65 ± 19.91	670.2 ± 7.2	554.41 ± 36.56	562.62 ± 30.85
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Length/Width	3.32 ± 0.26	------	3.61 ± 0.05	3.31 ± 0.18	3.52 ± 0.27
Diameter of micropilar disc	18.75 ± 1.92	------	------	33.49 ± 3.9	34.19 ± 5.4
a.Mundim–Pombo et al b.Suman et al, 2011 c. Linley,1989 d. Faull and Wiliams.,2016 e. Faull and Williams, 2016					
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The length of the eggs of *Aedes Aegypti*, identified dimensions of smaller than as for samples collected in India the Summan, et al. (2011) study. In another analysis, *Aedes aegypti* from Florida USA, presented a length of less than 700 µm (Linley 1989). The length identified in the egg population of São Paulo also reached lower levels, with a maximum value of 612.39 µm and 621.18 µm respectively. Regarding the chorionic coating, the findings were similar (Table 2), although there are mathematical differences in their measurements. Regarding the chorionic coating, the findings were similar (Table 2), although there are mathematical differences in their measurements.

It is known that *Aedes aegypti* eggs from different regions maintain the possibility of undergoing a process of adaptation to the environment (level of pollution, quality of pollutants in the air, water quality, morphological variation in terms of climate and the city), more studies of molecular biology must be carried out to confirm this (Dujardim, 2008).

The identified resistance of the chorion is consistent (Fonseca, Gomes and Araújo 2012) in which he attributes the precarious description of the embryonic morphology of *Aedes aegypti* to problems related to the permeability of the egg. External extraembryonic membrane, the serous one, can be easily seen (Panfilio 2008). This can be seen not only in the histological analysis (Fig. 3G) in which the serous layer was between the chorion and embryonic tissues, but also in the analysis using confocal microscopy (Fig. 5B) in which it was characterized as a distinct group of cells when compared to the others. However, in both images, the serosa was better evident in the intermediate phase of embryonic development.

After the end of the embryonic development, under favorable environmental conditions (Clean water, Temperature of 27 ± 2 ^{o} C and neutral pH) the eggs of *Aedes aegypti* hatch, with 50% of larval hatching (Falcão et al. 2016; Famesi *et al*. 2009). In this study, some eggs with finished embryonic development were analyzed (Fig. 5E and 5F), in which case approximately 40% of the eggs hatched even after immersion in 3% sodium hypochlorite, that is, even when subjected to unfavorable environmental conditions, approximately 1 hour after the beginning of the sample processing, some embryos were still alive and the eggs hatched.

At the time of hatching, the chorion is ruptured due to the larval muscle activity, which increases in volume and consequently increases the pressure exerted from the spike, a specialized structure, then the rupture of the chorion occurs, from a crack in the part corresponding to the cited coating (Sallum, Barata and Santos 2007; Guindo-Coulibaly et al. 2018). This description was verified in this study (Fig. 4E-H) and the spike mentioned was evidenced histologically in Fig. 3M.

Conclusions

Given the above, it is concluded that there is great resistance of the chorion in the embryo is still a factor that generates difficulties for the microscopic analysis of the embryo (mainly in fixation process), with a strong protective barrier, which makes it difficult to use its embryonic cells study. No weaknesses were identified in the egg phase of the biological cycle, which would be extremely important for further research in order to identify new ways of effectively combating this important vector disease.

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee on the Use of Animals of the Faculty of Veterinary Medicine and Animal Science from São Paulo University, under protocol nº 2889/2013.

Consent for publication

The authors are able to share all dates information if necessary.

Availability of data and materials

The data will be available when requested.

Competing interest statement

The authors declare no conflict of interests.

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Authors contributions

Ana Paula Miranda Mundim-Pombo (paulamedvet@gmail.com): concept and design; collect samples; samples preparation; data interpretation; immunofluorescence analysis; scanning electron microscopy analysis and compliance analysis.

Marisol León: helped data interpretation and compliance analysis.

Hianka Jasmyne Costa de Carvalho (hiankacarvalho@usp.br): essay redaction, translation and revision, reference research and discussion development;

Durvanei Augusto Maria (durvanei@usp.br): helped scanning electron microscopy and immunofluorescence analysis.

Maria Angelica Miglino (miglino@usp.br): macroscopic and microscopic description, study supervision, critical revision of the article and approval of article.

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