Developmental biology and evolutionary basis for drought tolerance of the Anopheles gambiae embryo

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A R T I C L E  I N F O

Article history:
Received for publication 18 November 2008
Revised 19 February 2009
Accepted 23 February 2009
Available online 17 March 2009

Keywords:
Mosquito
Anopheles gambiae
Embryo
Extraembryonic membranes
Serosa
Serosal cuticle
Evolution
Microarray
Gene regulatory network
Drought tolerance

A B S T R A C T

During the evolution of the Diptera there is a dramatic modification of the embryonic ectoderm, whereby mosquito embryos contain separate amnion and serosa lineages while higher flies such as Drosophila melanogaster contain a single amnioserosa. Whole-genome transcriptome assays were performed with isolated serosa from Anopheles gambiae embryos. These assays identified a large number of genes implicated in the production of the larval cuticle. In D. melanogaster, these genes are activated just once during embryogenesis, during late stages where they are used for the production of the larval cuticle. Evidence is presented that the serosal cells secrete a dedicated serosal cuticle, which protects A. gambiae embryos from desiccation. Detailed temporal microarray assays of mosquito gene expression profiles revealed that the cuticular genes display biphasic expression during A. gambiae embryogenesis, first in the serosa of early embryos and then again during late stages as seen in D. melanogaster. We discuss how evolutionary modifications in the well-defined dorsal-ventral patterning network led to the wholesale deployment of the cuticle biosynthesis pathway in early embryos of A. gambiae.

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Introduction

The serosa and amnion are extraembryonic membranes (EM) produced during early embryogenesis in most insects (Panfilio, 2008). The serosa is a protective membrane that can enclose the entire embryo. In most cases the amnion and serosa are destroyed before the completion of embryogenesis and do not contribute to a definitive larval organ (Schmidt-Ott, 2005).

The developmental origins of the amnion and serosa are used as a phylogenetic trait (Cobben, 1968), and there is considerable information about the evolutionary origins of these tissues in primitive insects and non-insect hexapods (Machida, 2006). Most taxa, including both hemimetabolous and holometabolous insects, manifest distinct amnion and serosa tissues (Dorn, 1976; Panfilio, 2008). However, higher Dipteran lineages radiating within the Cyclorrhapha taxon, specifically the Schizophora (Rafiqi et al., 2008) as represented by the best-studied insect, Drosophila melanogaster, contain a single extraembryonic membrane, the amnioserosa, in place of separate amnion and serosa membranes (Demerec, 1950; Panfilio, 2008; Schmidt-Ott, 2000). Here, we investigate the evolutionary and physiological basis for this reduction in complexity.

In most insects the amnion and serosa are tightly linked to blastokinesis — a process of spatial reorientation of the embryo during development (Cobben, 1968; Panfilio, 2008). These tissues have also been adapted for a variety of functions in different insects. For example, the amnioserosa of D. melanogaster is required for germ band elongation, retraction (Lamka and Lipshtiz, 1999), and dorsal closure (Scuderi and Letou, 2005). In some species the serosa is involved in water retention, and is primarily responsible for the elaboration of an extracuticular cuticle, the serosal cuticle (Hinton, 1981), which is intimately associated with the inner side of the maternally provided chorion. Recently, we have shown that the secretion of the serosal cuticle coincides with the acquisition of drought tolerance in the mosquito Aedes aegypti, the main dengue vector (Rezende et al., 2008).

The multilayer structure of the serosal cuticle varies dramatically between species (Hinton, 1981), but is generally composed of two types: an external (so-called yellow cuticle in crickets) hydrophobic epicuticle that was hypothesized to be enriched in lipids (Beamont, 1946a,b, 1949; Beckel, 1958; Harwood and Horsfall, 1959; McFarlane, 1960) and an internal multilayer endocuticle containing chitin (so-called white cuticle) (Campbell, 1929; Jahn, 1935; Slifer, 1937b). The
outer layer contains tyrosinase and manifests dopamine-based sclerotization (Furneaux and McFarlane, 1965a,b; McFarlane, 1960).

We previously examined the genetic changes underlying the transition from two EMs in the lower Diptera as represented by *Anopheles gambiae* to one in the higher flies as in *D. melanogaster* (Goltsev et al., 2004, 2007). The expansion of the dorsal ectoderm in *A. gambiae* (as compared with *D. melanogaster*) embryos is due, at least in part, to changes in the regulation of *Sog* expression, while the separation of dorsal cell lineages depends on the localized expression of specialized repressors. In this study we investigate the gene regulatory networks governing the function of the serosa in *A. gambiae*. Transcriptome profiling of the *A. gambiae* serosa, along with microarray analysis of staged embryos, suggests that many components of the larval cuticle gene pathway display biphasic expression during *A. gambiae* embryogenesis. In contrast, there is just a single phase of expression in *D. melanogaster*, during late stages of embryogenesis. The expression of the cuticle genes in the *A. gambiae* serosa is coincident with the timing of embryonic resistance to desiccation. We propose that the failure to deploy the cuticle gene battery in the *D. melanogaster* amnioserosa is due to subtle changes in the dorsal–ventral patterning network.

**Results**

**Serosal cuticle formation coincides with desiccation resistance**

Embryos were treated with Benserazide to inhibit Dopa-decarboxylase, a crucial component of the melanization pathway that contributes to the darkening of the mosquito eggshell shortly after egg laying (Monnerat et al., 1999). The clarified embryos were used for time-lapse microscopy, which identified all of the classical phases of embryogenesis, including cellularization, germ band elongation, retraction, and dorsal closure (see video of mosquito development in Supp. Fig1. mov). The amnion and serosa are established from distinct primordial territories (Goltsev et al., 2007) during early stages of *A. gambiae* embryogenesis (summarized in Fig. 1A). The contractile amnion migrates around the germ band and stretches the serosa until it encapsulates the entire embryo at ~8 h post-fertilization. Both membranes detach from the poles and the serosa becomes tightly associated with the endochorion (Fig. 1D and time-lapse video of mosquito development, Supp. Fig1. mov). From this timepoint mosquito embryos begin to acquire resistance to prolonged periods of desiccation (see Materials and methods for details), and full resistance is acquired at ~14 h after egglaying (Fig. 1B). This phenomenon is similar to results obtained with *A. aegypti* and coincides with the secretion of the serosal cuticle (Rezende et al., 2008). This cuticle is tightly linked to the inner layer of the eggshell, between the serosa and the endochorion, thereby complicating in vivo visualization. At later stages it can be clearly seen as an extra layer using a phenol-based CMCP mounting medium (Fig. 1E). Its presence is also revealed by strong bleach treatment, which dissolves the maternally provided chorion (Slifer, 1945). After 15 h following fertilization *A. gambiae* embryos become resistant to treatment with bleach while 7-h embryos are susceptible to this treatment (see Supp. Fig. 2), as seen for *A. aegypti* embryos (Rezende et al., 2008). Bleach treatment was used to isolate the serosal cuticle and it was found to contain chitin on the basis of fluorescent staining with Calcoflour (see Materials and methods for details) (Fig. 1C), as described for *A. aegypti* (Rezende et al., 2008). There is no auto-fluorescence of isolated serosal cuticles from *A. gambiae* (data not shown).

**Microarray assays identify genes that exhibit serosa-specific expression**

We found that after 9 h post-fertilization serosal cells remain attached to the eggshell after separation from fixed embryos (Figs. 2A–C). This association provided an opportunity to isolate the serosa by simply removing the eggshell, which was performed with 10.5 h embryos. RNA was extracted from isolated eggshells (with serosa) and

![Fig. 1.](image)
dechorionated embryos. The efficacy of the eggshell extraction method was tested by quantitative PCR assays: Chitin Synthase 1 transcripts, used as an internal control of this procedure (Rezende et al., 2008), were readily detected in the eggshell RNA extract, but only background levels were seen in dechorionated embryos (Fig. 2D).

Microarray assays identified 538 probe sets representing 359 genes that are significantly upregulated in the eggshell fraction as compared with dechorionated embryos (Fig. 2E, Supp. Table 2). While these genes represent a variety of processes (see GO-terms enrichment analysis in Supp. Table 1 and manually selected groups in Supp. Table 3), many cluster within biosynthetic pathways controlling chitin synthesis and deposition, tyrosine metabolism and catecholamine synthesis, and long chain fatty acid (presumably — wax) synthesis. All of these pathways have been implicated in the elaboration of the larval cuticle in D. melanogaster (Payre, 2004; Wigglesworth, 1948). Accordingly it appears that they are also used for the production of the chitinized white layer, as well as the tyrosine-mediated cross-linking and synthesis of the yellow waxy layer of the serosal cuticle (Slifer, 1937a) (see in Discussion).

Representative genes from each class were used for *in situ* hybridization assays in *A. gambiae* embryos (Fig. 3). All of the genes that were tested are activated either before or during the deposition of the serosal cuticle and display selective expression in the serosa. Some of the genes are expressed in the presumptive serosa prior to its spreading around the embryo (Figs. 3A, G, M), while others are expressed during (Fig. 3K) or after (Fig. 3F) spreading.

Not all of the genes expected to participate in insect cuticle synthesis were detected by the microarray assays. The mosquito orthologs of *Ddc* (dopa-decarboxylase), *pale* (tyrosine hydroxylase), and *kiv* (chitin synthase 1) (AGAP009091, AGAP006023, and AGAP001748 respectively) fell below the cutoff. Nonetheless in *in situ* hybridization indicates that all three genes are indeed expressed in the serosa, although the genes are activated at somewhat divergent stages. Chitin synthase 1 is expressed in the mature serosa and can be detected by *in situ* hybridization only in isolated eggshells (Fig. 3F). Apparently the failure to identify chitin synthase 1 is due to a flaw in the design of the specific probe sets on the *A. gambiae* microarray (data not shown).

The failure to identify dopa-decarboxylase and tyrosine hydroxylase may be due to their early onset of expression (Figs. 3G, M), prior to the stage when the serosa can be isolated by removal of eggshells.

In addition to the aforementioned biosynthetic pathways we also observed upregulation of several potential estrogen dehydrogenases — members of a family of enzymes (Gilbert, 2004) involved in the maturation of steroid hormones (Supp. Table 3, part2). This observation is consistent with studies suggesting production of ecdysone-like steroids by extraembryonic tissues (Kozlova and Thummel, 2003). Genes involved in programmed cell death (Supp. Table 3, part1) are also expressed in the serosa, probably foreshadowing its destruction ∼28 h after fertilization (as inferred from time-lapse movies). A number of serine peptidases and serine protease inhibitors are also upregulated in the serosa (Supp. Table 3, part2). These genes have been implicated in innate immunity by inducing an enzymatic cascade leading to nuclear import of rel — family transcription factors (Reichhart, 2005). Finally, a large number of enzymes potentially involved in detoxification such as cytochromes, GSTs and active radical scavenging (superoxide dismutases) are also expressed in the serosa (Supp. Table 3, part2).

**Serosal genes show biphasic profiles during mosquito development**

The identification of cuticle gene transcripts in the serosa raises the possibility that these genes might display biphasic expression

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**Fig. 2.** Isolation of serosa cells attached to eggshells for microarray analysis. (A) Fixed 10.5 h embryo inside eggshell on the surface of sticky tape covered slide before extraction. (B) Same field as in A but after embryo extraction from shell. White embryo is seen in the bottom right of the image. (C) An eggshell prepared similarly to B was stained with DAPI to field as in A but after embryo extraction from shell. White embryo is seen in the bottom right of the image. (C) An eggshell prepared similarly to B was stained with DAPI to

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patterns during A. gambiae embryogenesis. Microarray assays were performed on staged A. gambiae embryos, from fertilization to 46 h of development (which is close to hatching at ∼50 h post-fertilization). RNA was extracted from staged embryos roughly every 3 h after fertilization, and then hybridized to the A. gambiae transcriptome microarray. Putative serosal genes were selected from the dataset based on the previous analysis of isolated serosa (see Supp. Table 2). We found that the temporal profiles of mosquito serosal genes generally manifest two peaks — early expression coincides with the synthesis of the serosal cuticle, while late expression probably reflects formation of the larval cuticle during terminal phases of embryogenesis (Figs. 4B–D). The biphasic mode of cuticle gene expression was validated by qPCR assays on selected genes (Fig. 4E). Orthologues of A. gambiae serosal genes were identified in previously published D. melanogaster microarray assays. Approximately 200 gene pairs were compared in A. gambiae (Fig. 4A top) and D. melanogaster (Fig. 4A bottom). Strikingly, most of the genes implicated in the formation of the A. gambiae serosal cuticle are expressed only during advanced periods of D. melanogaster embryogenesis, while most of the genes display biphasic expression in A. gambiae.

Thus, larval cuticle genes are deployed in the early A. gambiae embryo to form the serosal cuticle. D. melanogaster lacks a serosa, has no associated serosal cuticle, and the genes responsible for the formation of the larval cuticle are activated only during late embryogenesis.

Discussion

Cuticle gene battery

Sclerotization, wax and chitin deposition are used at different stages of the insect life cycle for the synthesis of serosal (Campbell, 1929; Jahn, 1935; McFarlane, 1960; Slifer, 1937b), larval (Moussian et al., 2006), pupal and adult cuticles (Wigglesworth, 1948). Sclerotization and chitin are also used for the production of the peritrophic matrix (Shao et al., 2001), tracheal lumen (Araujo et al., 2006), and wound healing (Mace et al., 2005). It is not known whether the same gene batteries are used for all of these processes, or if specialized versions of the cuticle genes are used for different purposes. The tight association of the A. gambiae serosa with the eggshell provided an opportunity to isolate a homogenous tissue at the time when the serosal cuticle is synthesized. A large number of genes encoding components of the larval cuticle were found to exhibit an early wave of expression in the developing serosa. These results strongly suggest that the same gene battery is used for the synthesis of the serosal and larval cuticles.

The absence of the serosa is a phylogenetic hallmark of higher Diptera (Rafiqi et al., 2008; Schmidt-Ott, 2000). Basal insects such as crickets (Slifer, 1937b) and beetles (van der Zee et al., 2005) contain serosal tissues similar to those seen in mosquitoes. Thus, during the evolution of the Diptera there is a transition in the expression of the cuticle gene battery from a biphasic mode (serosa and larval epidermis) to the singular mode (larval only) characteristic of the D. melanogaster embryo. In principle, a limited number of mutations in the DV gene network are sufficient to account for the loss of the serosa and serosal cuticle, as we discuss below.

Evolution of serosa transcription networks

What is the basis for the selective activation of the cuticle genes in the A. gambiae serosa and the loss of this activation in D. melanogaster? The microarray assays using isolated serosa identified a number of regulatory genes (Supp. Table 2) that may be lost in the amnioserosa
of *D. melanogaster*. Included in this list are: AGAP005311 – Anopheles ortholog of *Drosophila* homeobrain, AGAP010623 – X-box binding factor, and AGAP005137 – a lim homeobrain gene. Transcription factors generally belong to relatively low abundance molecules and are therefore underrepresented in a population of upregulated mRNAs. To compensate for this we used a lower cut-off value when comparing serosa vs. embryo transcripts for putative regulatory genes (0.65 rather than 1.0) and identified additional genes including AGAP011625 – mosquito AP-2, AGAP009515 – mosquito Dorsal, and AGAP007327 – a major transcriptional effector of edcsyne signaling Eip74 (Fletcher and Thummel, 1995). *In situ* hybridization assays confirmed that the lim homeobrain gene, *homeobrain* and Dorsal are indeed expressed in the developing serosa of the early *A. gambiae* embryo (Fig. 5). Dorsal is also expressed in the *T. castaneum* serosa (da Fonseca et al., 2008). None of these genes are expressed in the amnioserosa of the *D. melanogaster* embryo (BDGP fly in situ database – data not shown). Previous studies demonstrated that edcsyne signaling (Chavez et al., 2000) is critically involved in the regulation of cuticle synthesis both at larval and late stages of *Drosophila* development. Moreover, cuticle genes such as Ddc and pale (Mace et al., 2005) are induced during wounding of the *D. melanogaster* epidermis. The characterization of minimal cis-regulatory sequences identified grainyhead (*grh*) as a key activator of Ddc (Mace et al., 2005). *Grh* is known to be a critical regulator of proper integument function in *D. melanogaster, C. elegans* and mice (Jane et al., 2005). We found that *grh* exhibits selective expression in the serosa anlagen of *A. gambiae* embryos (Fig. SC), but it is also expressed in the dorsal ectoderm of early *D. melanogaster* embryos (Huang et al., 1995), so it is not sufficient to account for the deployment of the cuticle gene battery in *A. gambiae*. Interestingly, the analysis of the *D. melanogaster* Ddc enhancer raised the possibility that additional regulatory factors such as rel – family genes (exemplified by dorsal and its orthologues in insects) might participate in its activation (Mace et al., 2005).

Many insects (Dorn and Romer, 1976; Imboden and Lanzrein, 1982; Laguex et al., 1979; Scalia and Morgan, 1982) including *D. melanogaster* (Kozlova and Thummel, 2003) manifest a mid-embryonic peak of edcsyne production, yet Eip74EF is not expressed in the *Drosophila* amnioserosa (BDGP in situ database, data not shown). Perhaps *Eip74EF* is needed to activate at least some of the serosal cuticle genes in response to edcsyne. It is possible that a number of mosquito-specific serosal transcription factors such as hunchback, tramtrack, ems (Goltsev et al., 2007), dorsal, AP-2, and the lim homeobrain gene AGAP005137 facilitate serosa-specific Eip74EF induction by EcR (edcsyne receptor). *Dorsal* might act synergistically with *grh* to activate genes such as Ddc and pale that are independent of edcsyne signaling. Thus, the expression of just a few serosa-specific transcription factors, possibly under control of the homeobox gene zen (Goltsev et al., 2007), might be sufficient to account for the deployment of the cuticle gene battery in the *A. gambiae* embryo.

Role of serosa in drought resistance

One of the major factors determining the occurrence of malaria in sub-Saharan Africa is the capacity of *A. gambiae* to actively maintain malaria transmission in the driest areas and months of the year. This drought tolerance is a major adaptation contributing to the apparent continuing expansion in the range of *A. gambiae* (Beier et al., 1990; Onyabe et al., 2003; Toure et al., 1994). So far two major strategies for survival during the dry seasons have been suggested: reproduction in localized moist niches and aridity tolerance of adults (Gray and Bradley, 2004, 2005), larvae and eggs (Beier et al., 1990; Minakawa et al., 2001; Shillulu et al., 2004). There are several factors that might influence embryonic drought resistance, including: the structure of the eggshell, the capacity of the early embryo to retain water, and the ability of older embryos to undergo diapause.

The present study provides insights into the mechanisms of embryonic drought resistance. It has been proposed that crosslinking and melanization of the mosquito eggshell are the major mechanisms for protecting the embryo against desiccation (Li and Li, 2006). However, we believe that it is unlikely that these reactions contribute significantly to drought resistance since the early embryo is highly sensitive to desiccation (Fig. 1B). Resistance is not acquired until 14 h after fertilization, during the time when the serosal cuticle is formed. We also note that there is an early spike of aquaporin gene expression, which might contribute to the ability of the early embryo to retain water (Y.G. unpublished observations). We propose that the serosal cuticle produced by the *A. gambiae* serosa is primarily responsible for the desiccation resistance of the early embryo. Since the same gene battery is used for the serosal and larval cuticle it is possible that mutations affecting embryonic drought tolerance will also impair the resistance of adult mosquitoes. The identity of serosal genes uncovered in our study provides a foundation for population control through the production of transgenic mosquitoes with compromised drought tolerance.

Materials and methods

Mosquito stocks and egg collection

*A. gambiae* population was reared at 27 °C, 75% humidity, with a 12-h light/dark cycle. Adults were maintained on a 10% sucrose solution and females were blood-fed on anesthetized hamsters. For synchronized embryo collection the females were placed in the dark at 27 °C for 1 h inside 15 cm Petri dish lined up with circles of wet Whatman paper. The developmental time was counted starting from the moment Whatman paper was moisturized with water. Mosquito embryo fertilization happens at the moment of egglaying. Due to constraints in experimental setup, after 2 h following the start of collection the eggs were shifted from 27 °C and constantly kept at 25 °C. As our unpublished observations show 2 h incubation at 27 °C augments egglaying effectiveness yet does not change the developmental dynamics of time course as sampled at 25 °C.

Time lapse microscopy of mosquito embryogenesis

Eggs were collected into 500 μM Benserazide (Sigma, St. Louis, MO, Catalog # B-7283) solution in water and glued with double-sided sticky tape to the bottom of Petri dish filled with collection solution. The images were recorded with Nikon microscope fitted with submersible 25× objective. Due to the limit in the size of the view field the images of anterior most part of the embryo were not

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**Fig. 4.** Transition from biphasic (in *A. gambiae*) to singular in (*D. melanogaster*) expression of serosal/cuticular genes. (A) Developmental expression profiles of *A. gambiae* genes enriched in serosa (top), compared to the developmental expression profiles of *D. melanogaster* orthologues of mosquito serosa genes (bottom). Fly data was extracted from (Hooper et al., 2007). Each row is a gene, and each column is a time point (indicated in grey along the diagrams). The intensities of the colors indicate the magnitudes of induction (red) or repression (green) for each gene. Black means no change of expression compared to average expression level across the time course. Mosquito genes (only those that could be matched to *Drosophila* orthologues are shown) are sorted according to enrichment in serosa, *Drosophila* orthologues are sorted according to mosquito sorting. Individual expression profiles of selected genes (picked based on manual analysis of annotations and public databases see more details in Supp. Table 3) involved in (B) Chitin synthesis and deposition, (C) Tyrosine based crosslinking and (D) Wax synthesis. (E) Expression profiles for selected mosquito genes generated from microarray data were independently checked by qPCR. In B, the profile of AGAP007148 was obtained by qPCR (see Materials and methods for details). Red and green lines are AGCHS1a and AgCHS1b, respectively. Bars represent Standard Error of the Mean. *Drosophila* profiles on B, C and D represent expression values from data collected by (Arbeitman et al., 2002) for CG2666 (khv), (Manak et al., 2006) for CG32209 (Serpentine), and (Hooper et al., 2007) for the rest. The corresponding mosquito profiles represent z-transformed (value-average(acrossprofile))/stdev(acrossprofile) microarray data from this study.
SCs were incubated with 1 mg/ml Calcofluor (Sigma, St. Louis, MO, previously designated as Fluorescent Brightener 28, Catalog # F-3543) during an incubation period of 10 min in the dark, thoroughly washed with 25 mM pH 6.2 sodium phosphate buffer and analyzed under a microscope with epifluorescence, dark field or DIC. Calcofluor specificity and presence of chitin in serosal cuticle is further discussed elsewhere (Rezende et al., 2008). 

**Isolation of serosa cuticles by bleach treatment and Calcofluor staining**

Serosal cuticle (SC) was isolated from fully developed embryos. Complete digestion of exo- and endochorion was attained with 50% household bleach (2.6% active sodium hypochlorite) during 25–30 min inside a Falcon cell strainer (70 µm nylon, Becton Dickinson). The remaining SCs, with larvae inside, were then thoroughly washed with 25 mM pH 6.2 sodium phosphate buffer. This last procedure breaks the SC, often at the line of dehiscence (Judson and Hokama, 1965). Isolated SCs were incubated with 1 mg/ml Calcofluor (Sigma, St. Louis, MO, also named Fluorescent Brightener 28, Catalog # F-3543) during 10 min in the dark, thoroughly washed with 25 mM pH 6.2 sodium phosphate buffer and analyzed under a microscope with fluorescence, dark field or DIC. Calcofluor specificity and presence of chitin in serosal cuticle is further discussed elsewhere (Rezende et al., 2008). 

**Analysis of mosquito egg viability in arid conditions**

Synchronized developing eggs obtained as indicated above were transferred from wet to dry conditions at different embryonic ages: 8, 10, 12, 14 and 22 h after egglaying (HAE). In each case, groups of 100 eggs were taken from moisture Whatman No. 1 filter paper and transferred onto dry polycarbonate filters (2.5 cm diameter, 8 µm pore, Poretics Corporation). These filters were then blotted on dry Whatman No. 1 filter paper to remove all water and eggs were left drying by air-exposure for 20 h (e.g. 8 HAE embryos were left on dry from 8 until 28 HAE, 10 HAE embryos were left on dry from 10 until 30 HAE and so on). Following this 20 h period on dry the eggs were placed into a Petri dish filled with water until the end of embryogenesis (~50 HAE). From 50 HAE on, larvae hatching was counted every 30 min, until no more hatching occurred. Viability was scored as percentage of total hatched larvae related with the total amount of eggs, normalized by parallel control samples kept on water throughout the whole embryogenesis. Experiments were performed at least in triplicates inside an incubator at 25 °C with 45–60% relative humidity.

**Real time quantitative PCR (qPCR)**

The cDNA was synthesized with Superscript III (Invitrogen) with the oligo-d(T)15 primer according to manufacturer instructions. A final concentration of 4 ng RNA/µl was employed at the final volume of the reverse transcriptase reaction. Final concentrations above 8 ng RNA/µl inhibits the proportional synthesis of the cDNA pool (data not shown). The qPCR reactions were carried out in ABI Prism 7300 (Applied Biosystems) with the Quantifluor SYBR Green Kit (QIAGEN). The following primers were used.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Primer Sequence</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>AGAP011812</td>
<td>5′-ACGTACTCCTGTGCGCAGATCT 3′</td>
<td>ELOVL, endolysin</td>
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<tr>
<td>AGAP001748</td>
<td>5′-TGGTTCCAGCTGCAGATCT 3′</td>
<td>Chitin Synthase, AGCHS1</td>
</tr>
<tr>
<td>AGAP011936</td>
<td>5′-ACGTACTCCTGTGCGCAGATCT 3′</td>
<td>Serpentine</td>
</tr>
<tr>
<td>AGAP004500</td>
<td>5′-GGATCGCGCGAGATCTGGT 3′</td>
<td>Knickkopf</td>
</tr>
</tbody>
</table>

The primers 5’gamexpRP1 (5′-GCTATGATAAACTCGCTCCCAA 3′) and 3’aequaRP1b (5′-TCATCAGCACCCTGCAGT 3′) were employed to amplify the constitutive gene rp49 (AGAP002122), used as an internal normalizer for qPCR (Gentile et al., 2005). 

**Identification of mosquito serosa genes and statistical analysis**

10.5 h mosquito embryos were collected and fixed as described previously (Goltsev et al., 2004). Following embryo dissection procedure, the empty eggshells remaining on double-sticky tape (Scotch3M) were washed off the tape by Xylenes and equilibrated in ethanol. Total RNA was prepared from fixed eggs, dissected embryos or purified shells using RecoverALL kit (Ambion). Biotin-labeled probes were synthesized using two-step amplification kit (Affymetrix) and hybridized to Plasmodium–Anopheles expression arrays (Affymetrix). Three biological replicates were obtained for every each of the purified shells (serosa) or extracted embryo groups at 10.5 h. Log base 2 expression values all probesets on the array were computed using RMAExpress (Bolstad et al., 2003). Log2Fold values for each probeset were computed by subtracting the average expression value in the whole embryo group from the average expression value in the serosa group. A p-value testing each Log2Fold value against zero was obtained using a moderated two-sample t-test (Smyth, 2005). A false discovery rate (FDR) was obtained by adjusting these p-values by the Benjamini–Hochberg method for correction for multiple hypothesis testing (Benjamini and Hochberg, 1995). Custom built table reassigning the probesets according to most recent Anopheles genome annotation was used for matching of Plasmodium–Anopheles microarray probes to mosquito genes (Supp. Table 2). Probesets matching genes with statistically significant differential expression in serosa were chosen using cut-off values of Log2Fold greater than 1 (two-fold change) plus p-value<0.1. The volcano plot in Fig. 2E highlights the 359 probesets satisfying the criteria. GenMapp2 software (Salomonis et al., 2007) was used to identify GO-terms.
enriched in the population of serosal genes. GoElite (http://www.genmapp.org/go_elite/go_elite.html) software was used to remove redundant nested GO-terms from MAPFFinder results. The microarray data can be downloaded from http://www.ncbi.nlm.nih.gov/geo/, accession number GSE14851.

**Mosquito developmental microarray timecourse**

Total RNA from unfixed staged eggs at sequential (2 h, 4 h, 6 h, 7 h, 8 h, 10 h, 13 h, 16 h, 19 h, 22 h, 25 h, 28 h, 31 h, 34 h, 37 h, 40 h, 43 h, and 46 h) time points was purified initially by Trizol (Invitrogen) and then further by RNAeasy (Qiagen) kit. Biotin-labeled probes were synthesized using regular labeling kit (Affymetrix) and hybridized to Plasmodium–Anopheles expression arrays (Affymetrix). All time points were sampled with three independent biological replicates. RNA-express software was used for processing of raw expression data and estimation of log base 2 expression values (Supp. Table 4). Normalized expression levels depicted in heatmap data can be downloaded from http://www.ncbi.nlm.nih.gov/geo/, accession number GSE15001.

**Heatmap and time point for**

**of raw expression data and estimation of log base 2 expression values (Supp. Table 4). Normalized expression levels depicted in heatmap of raw expression data and estimation of log base 2 expression values (Supp. Table 4). Normalized expression levels depicted in heatmap data can be downloaded from**

**Supplementary data** associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.02.038.

**Appendix A. Supplementary data**

References


**Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.02.038.**