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Molecular characterization of vitellogenesis in anautogenous *Culex pipiens pipiens* L. mosquitoes

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Abstract
Mosquito vectors-borne infectious diseases are enormous burden on human health and development worldwide. Disease-transmitting mosquito vector species use a reproductive strategy termed anautogeny that requires a blood meal to initiate egg maturation or oogenesis. Understanding the molecular basis of the regulation of egg development following a blood meal is essential to recognize the developmental biology and life cycle of mosquitoes and pinpoint important targets for control interventions. Vitellogenin (Vtg) is the major yolk protein synthesized during oogenesis and widely characterized in many mosquito species, mainly *Culex pipiens* complex mosquitoes. In this study, we report the differential stage and temporal expression of *vtg* genes in anautogenous *Cx pipiens* complex mosquitoes collected from Egypt following the ingestion of a blood meal using quantative real-time PCR (qRT-PCR) analysis. qRT-PCR analysis of the transcriptional pattern was performed in adult females at different intervals post-blood meal (pbm) and in developmental immatures. The cDNA of *vtg* was detected in *Cx. pipiens* females 24 hours pbm. Sequence analysis of detected *vtg* disclosed high similarity to homologous sequences in other mosquito species, particularly *Cx. quinquefasciatus*. *Vtg* was highly expressed in adult females at 24 h complex, however, in immature stages, only resdiual expression level of *vtg* was observed. In addition, we tested the presence of Vtg protein at time intervals in ovarian tissues of both sugar fed and blood fed females. Protein analysis revealed that two polypeptides of Vtg protein were detected in blood-fed females but not in sugar-fed females.

Keywords: *Culex pipiens*, vitellogenin, vitellogensis, anautogenous, autogenous, mosquitoes

1. Introduction
Mosquito-vector borne diseases (VBDs) like malaria, filariasis, dengue and Zika, are among the most devastating to human health and development in modern times. This is due to the wide geographic distribution and complex multi-stage life cycle of mosquitoes in wide-range of ecological habitats, variable host-seeking and preference behavior, rapid development resistance to almost all insecticide groups, spread of drug resistance in disease pathogens they transmit, the unavailability of effective vaccines against many diseases [1-3]. The situation is further complicated due to the effects of global warming and climate changes. Therefore, there is an urgent need to explore various options for developing novel, effective and sustainable control strategies against major VBDs [4]. *Cx. pipiens* is the most widely distributed mosquito worldwide.

The most efficient mosquito vector species require a blood meal, which is essential for female ovarian development and reproductive cycle to successfully maintain its life cycle [5]. These mosquitoes are called anautogenous, and due to this blood feeding behavior, and therefore are efficient vectors that implicated in the transmission of various-blood-borne disease pathogens. In contrast, mosquitoes species that do not require a blood meal for egg production, are called autogenous mosquitoes and they are poor or non-vectors of diseases. Many species of mosquitoes have been characterized based on the type of reproductive strategy they undertake [6, 7].

Vitellogenesis (process of yolk formation in the oocyte) is a key physiological event in mosquito life and is generally dependent on the availability of a protein-rich blood meal [8]. Blood-feeding initiates a cascade of behavioral, cellular and molecular events in the vitellogenic cycle, an essential phase in mosquito reproduction and the subsequent production

of viable eggs to continue its life cycle and regulates its vectorial capacity for disease transmission. Due to the direct implications of mosquito reproduction for public health and epidemiology of VBDs, the elucidation of the molecular basis of mosquito reproductive physiology, especially vitellogenesis, is critical for the development of novel strategies in vector and disease management [9].

Vitellogenesis takes place through three main stages: pre-vitellogenesis, vitellogenesis and post-vitellogenesis [10]. The main yolk protein precursor genes (YPPs) activated during vitellogenesis are vitellogenin (Vtg), vitellogenic carboxypeptidase (Vcp), vitellogenic cathepsin B (Vcb) and lipophorin (Lp). Out of these, Vtg is the highest in expression and most abundant in mosquito tissues. The gene encoding vitellogenin (Vg), a major YPP in most oviparous animals, is expressed in extraovarian tissues in a sex-, tissue- and stage-specific manner. The cDNA encoding *A. aegypti* Vg has been characterized [11], and its gene cloned [12]. In vitellogenic female insects, the fat body, a powerful metabolic and secretory organ, is engaged in massive production of YPPs for developing oocytes [13]. However, the process of yolk production is disrupted in infected mosquitoes and abundance of the yolk protein precursor (vitellogenin) mRNA is reduced [14]. In the ovary, follicles begin to develop normally; but subsequently, 20–30% undergo follicular apoptosis [15]. A reduction in fecundity (egg production) has been observed in *Plasmodium yoelii nigeriensis* infection of both *A. gambiae* and *A. stephensi* females. The concentration of vitellogenin in hemolymph and ovaries of *A. stephensi* during the first gonotrophic cycle in females infected with *Plasmodium yoelii nigeriensis* was significantly reduced in ovaries of infected females at 24–48 h after the blood meal [16, 17, 18].

In recent years, blood meal-regulated egg development has dominated research in mosquito reproduction. Egg production whether during autogeny or anautogeny requires the synthesis of yolk proteins to supply the developing oocyte. It is therefore of importance to understand the molecular mechanism underlying vitellogenesis and integrate this knowledge in the development of future vector and vector-borne disease control strategies [19]. Because vitellogenin promoters are frequently used to drive transgenes in mosquitoes [20-22], this developmental expression is of practical importance.

In Egypt, Both autogenous and anautogenous *Cx. pipiens* individuals were encountered in the progenies of autogenous or anautogenous female parents. Moreover, more than 75% of field caught females produced mixed progenies. There is no limitation for hybridization between autogenous and anautogenous *Cx. pipiens* mosquitoes for mating due to the lack of premating and post-mating barriers [23, 24]. *Culex pipiens* is a major etiological agent of human and animal diseases: lymphatic filariasis (LF), Rift Valley fever virus (RVFV) and West Nile virus (WNV).

The present work characterized a candidate vitellogenin gene that is potentially involved in vitellogenesis of anautogenous *Culex pipiens pipiens* mosquito. In addition, we investigated the expression of the vitellogenin gene following blood feeding and during the development of immature mosquito development, and analyze the protein composition of the ovarian tissues, major tissue for vitellogenin production, at different feeding status.

2. Experimental procedures

2.1 Mosquito collection

Immature stages of *Cx. pipiens* L. mosquitoes were collected from sewage stations in Giza governorate and transferred into an indoor insectary at 26.5±1 °C, 75-100% RH and a light/dark photoperiod of 16:8 hours. Immature stages were maintained in enamel pans filled with tap water and fed slurry of finely ground fish food Tetramine®. Emerging adults containing both autogenous and anautogenous mosquitoes were provided with a 10% sucrose solution. To select for an anautogenous mosquito line, newly-emerged mosquitoes were placed in individual carton cups and fed on 10% sucrose solution for 6 days at most to allow if any autogenous egg may be laid. During such period, if any autogenous egg rafts were observed, the whole cup was neglected. However, if no egg rafts appeared, adult females were offered a blood meal source to initiate vitellogenesis.

2.2. *In silico* bioinformatics analysis and Vtg primer design

Forward and reverse primer sets gene-specific were designed to amplify and study the putative Vtg transcript, using Invitrogen\Vector NTI Advanced software, version 10. Primers were designed based on an alignment between the two isoforms of the Vtg gene, Vtg-C1 (GenBank accession AY691324), and Vtg-C2 (GenBank accession AY691325) in the closely-related species *Cx. quinquefasciatus*. The used forward and reverse primers are located on the second large exon, 5054 bp in length. The forward primer, VgF spans the fragment from base 3219 to base 3242 and was used in conjunction with two reverse primers, based on the alignment between Vtg-C1 and Vtg-C2. The first reverse primer, VgR1, extends from base 3324 to base 3346 and the second reverse primer, VgR2, extends from base 3375 to base 3408. VgF and VgR1 are assumed to amplify a region of 127 bp expected size, while VgF and VgR2 are assumed to amplify a region of 188 bp expected size (Fig. 1).

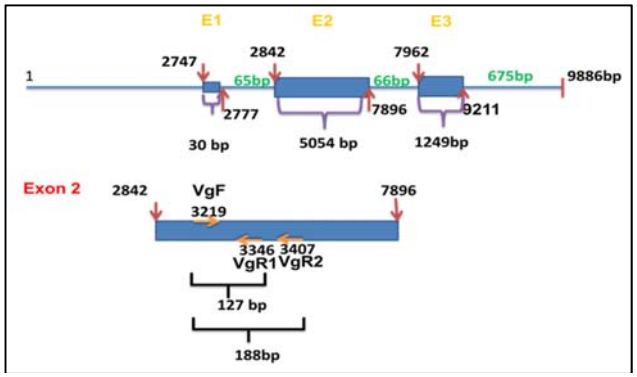


Fig 1: *Culex quinquefasciatus* Vtg gene sequence diagram showing the intron-exon structure and the positions of the designed Vtg-specific primers (VgF, VgR1, VgR2) used for amplification of Vtg homologue(s) from in *Cx. pipiens* collected from Egypt. E: exon region.

2.3. RNA extraction and RT-PCR

Total RNA was extracted from whole female mosquitoes by the Trizol method and according to the manufacturer's instructions (Invitrogen, Cat. No. BSC51M1 Carlsbad, CA, USA). RNA was isolated from aliquots of larvae, pupae,

newly-emerged females, and females at different time points, 24, 48, 72, 96 h post-blood meal (pbm). RNA was isolated from 10 of each mosquito stage per replicate. RNA was quantitated by absorbance at 260 nm. First strand cDNA was generated from the template RNA using RevertAid First Strand cDNA Synthesis (Thermo Scientific, Cat# K1621) in a total 20 µl reaction volume with Oligo (dt) primer, and 5 µg of total RNA template at 25 °C for 5 min, then 42 ^{0}C for 60 min, and finally at 70 °C for 5 min. PCR conditions for Vtg primers were 94 °C for 3 min, followed by 30 cycles at: 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. for reaction normalization, the housekeeping actin gene was amplified using the primers; forward (ATGTTTGAGACCTTCAACTCGC) and reverse (TAACCTTCRTAGATTGGGACG) [25].

2.4. Determination of expression levels using Real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to study differential stage-specific and temporal Vtg gene expression in the studied *Cx. pipiens.* Aliquots of 10 individuals each of larvae, pupae, and adult males and females (0, 24, 48, 72, 96 h pbm) were stored in RNA stabilization solution (RNAlater®, Ambion) at -80 °C until use. qPCR was performed using Stratagene MX3005P integrated QPCR detection and data analysis system (Agilent Technologies) according to the manufacturer's instructions in a 96-well microtiter plates with QuantiTect SYBR green PCR kit (QIAGEN®). Reaction was run in 25 µl, containing 12.5 µl SYBR Green PCR Master Mix, 0.5 µl of each primer set (50 pmol final concentration), 4.5 µl RNase-free water and 7 µl of cDNA templates. Each sample was run in triplicate. qPCR primer sequences were Vtg forward primer: 5'-CCA TCA AGG GWC TGT ACG TCG AG-3'; Vtg reverse primer 1: 5'-ARC TGG TTG TAC TTG GAC TTG AT-3'; Vtg reverse primer 2: 5'-TTC TCC AGT AAC CAG GGG TTC CAT GAC CTT GTA-3'. Conditions of amplification were (I) melting at 94 °C for 5 min; (II) heating at 94 °C for 15 s;. (III) annealing at 60 °C for 30 s; (IV) steps I and II were repeated for 35 cycles; (V) extension at 72 ^{0}C for 30 s [26].

2.5. Protein expression analysis from mosquito ovaries

For protein expression profile analysis, total proteins were extracted from dissected ovaries of the female misquotes as mentioned above. Dissected ovaries were collected from 20 individauls at each time and cracked in cracking buffer as described previously [27]. Protein concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific). Samples were adjusted to 20 mg/ml, and loaded onto 10% polyacrylamide gels stained with 0.1% Coomassie Brillainat Blue R-250, and subsequently destained in solution I (50% methanol, 10% acetic acid) until protein bands were visible.

3. Results

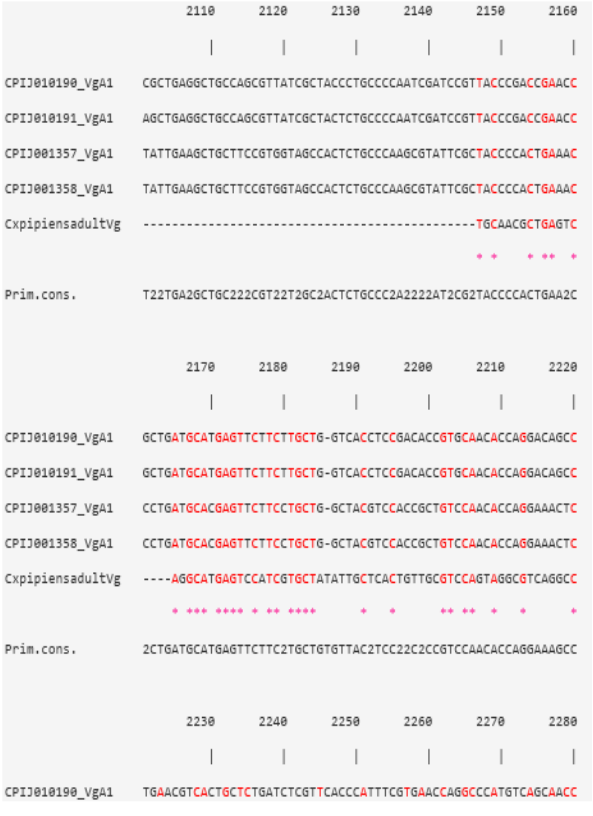
3.1 Sequence and phylogenetic analysis of Vtg

In *Cx. pipiens* adult females blood-meal fed**,** initial amplification of Vtg cDNA transcript using forward and reverse primers produced a Vtg sequence 362 and 155 bp long, respectively (Fig. 2). The two sequence reads in PCR product were assembled into one contig, using a code aligner program (http://www.codoncode.com/aligner/).

Blastn analysis disclosed that multiple sequence alignment of *Cx. pipiens* Vtg at the nucleotide level shared a high degree of similarity to genus *Culex* (Fig. 2). Blast result revealed 95, and 97% identity to *Cx. quinquefasciatus* Vtg-C1 and *Cx. quinquefasciatus* Vtg-A1, respectively. It exposed, as well, 92% identity to both *Cx. tarsalis* Vtg1a and *Cx. tarsalis*Vtg1b.

According to vectorBase resources on *Cx. quinquefasciatus* whole genome (https://www.vectorbase.org/organisms/culex-quinquefasciatus), a comparison of *Cx. pipiens* contig was performed to determine level of similarity, of which five of eleven transcripts were shown to be related to *Cx. quinquefasciatus* vitellogenin, with a high degree of similarity to the sequence contig. One transcript is described as a protein coding vitellogenin convertase gene (CPIJ011701) located on supercontig 3.387, whereas four transcripts are described as proteins that are coding for vitellogenin-A1 precursor genes. Of the four, two transcripts (CPIJ001357 and CPIJ001358) are located on supercontig 3.18, and two (CPIJ010190 and CPIJ010191) are located on supercontig 3.289 (Fig. 2).

The phylogenetic tree of the mosquito vitellogenin family was performed at the nucleotide level. Phylogenetic analysis grouped Vtg sequences from various mosquito species into two branches arisen from one root. One branch gave root for sister groups of *Cx. quinqufaciatus*, *Cx. pipiens*, *Ae. Polynesiensis* (subfamily. culicinae), and *An. albimanus*, *An. stephensi* (subfamily. anophlinae) Vtg sequences. The second root gave branches for sister groups of *Cx. quinquefasciatus*, *Cx. tarsalis*, and *Ae. aegypti* Vtg sequences (Fig. 3).



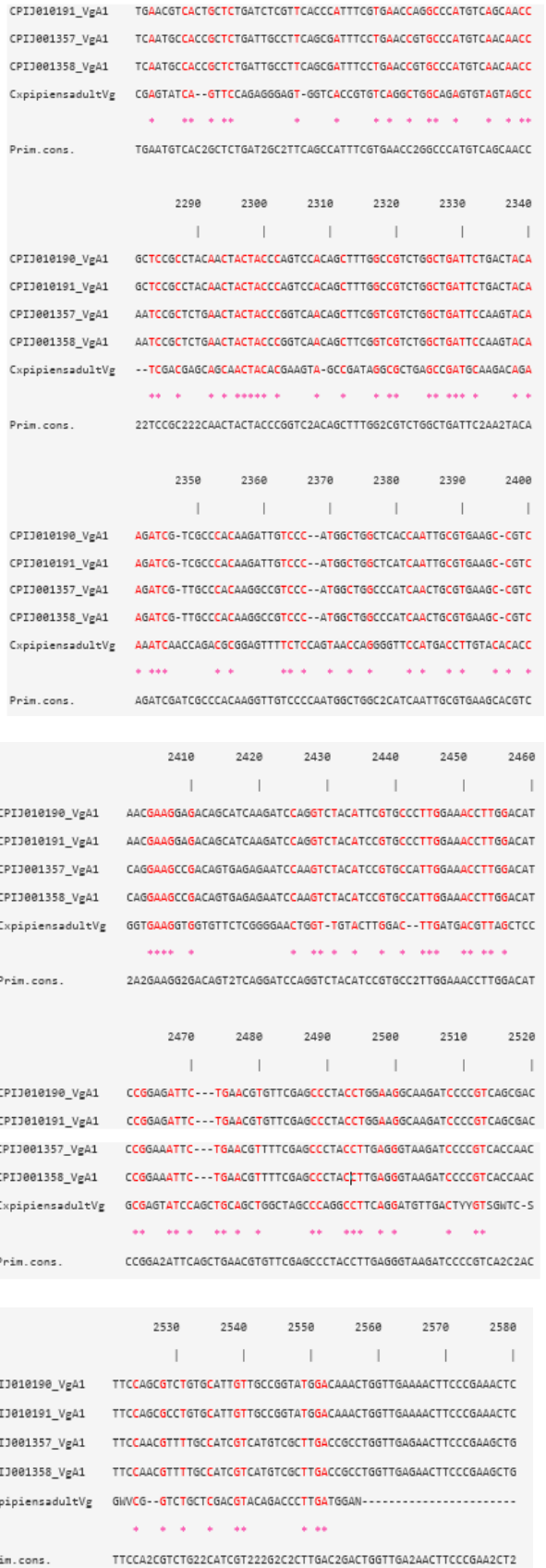


Fig 2: CLUSTAL W multiple alignments at the Vtg nucleotide level of the sequence contig, for *Cx. pipiens* females 48 h PBM, with four Vg-A1 transcripts in *Cx. quinquefasciatus*.

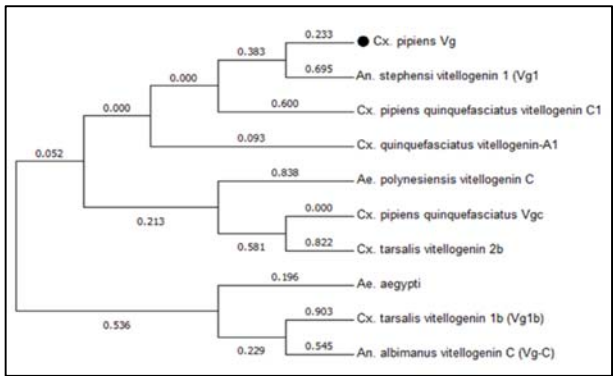


Fig 3: Phylogenetic analysis of Vtg gene transcripts from various species of mosquitoes. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 39 positions in the final dataset.

3.2 Expression profiles of vitellogenin genes

The quantitative characterization of the expression of Vtg gene showed that the fourth larval instars represented the baseline expression for the production of Vtg transcript compared to pupae, adult males, non-blood fed females and blood-fed females tested at 24, 48, 72 and 96 h pbm. All transcript levels were normalized to β-actin expression. Vtg threshold cycle (Ct) value was recorded at 21.78 in the larval stage. Vitellogenin gene was expressed in pupae and males at residual trace levels, 2.27 and 1.61 fold, respectively, when compared to the level observed in larvae (Fig. 4). In adult females, *Vtg* profile was high. For females that have ingested a sugar solution only, Vtg level was ~14 folds compared to larval baseline. As expected, zero h PBM, the first transcript of vitellogenin gene amounted to ~123 folds compared to the larval level (21.78). Twenty four hours PBM Vitellogenin gene expression peaked to ~154 folds then declined to ~50 and ~17 folds, 48 h and 72 h PBM, respectively (Fig. 4).

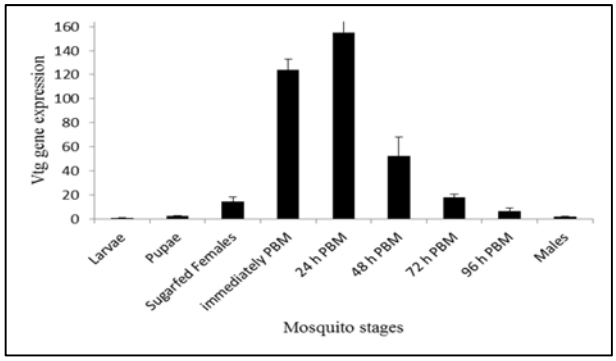


Fig 4: Quantitative expression of *Cx. pipiens* Vtg gene in immatures and adult mosquitoes at different feeding status. Each point represents the average of three replicates. Bars denote to the standard error.

3.3 Determination of vitellogenin protein expression in different mosquito stages

To test whether developmental expression of Vtg leads to the production of protein, total protein from homogenates of dissected ovaries was compared in sugar fed females and in blood fed females tested at interval post-blood meal. The maximum protein content was detected in females fed at 96 h pbm compared to the other tested. The average of total protein

extracted from ovaries in sugar-fed, immediately pbm, females at 24 h pbm, at 48 h pbm, 72 h pbm, and 96 h pbm was 6.9 ± 1.6, 8.75 ± 0.85, 26.15 ± 12.95, 37.95 ± 14.25, 87.9 ± 33.8, and 119.8 ± 51.1, respectively. Electrophoretic separation profile is shown in figure 6. Sugar-fed females reveals lowest bands (5 bands) ranged from 107 to 30 kDa compared to the highest protein bands in females fed 48 h pbm (13 bands) from 225 to 30 kDa. The total number of bands decreased in o h, 24 h, 72 h, and 96 h to 10, 11, 11, and 9 bands, respectively (Fig. 5, Table 1).

Table 1: Protein bands of ovarian tissues dissected from females *Cx. pipiens* at different feeding status

Bands (kDa)	Sugar_Fed	0_h	24_h	48_h	72_h	96_h
225			+			
185			+			
181.6					+	
174.2						+
166.5				+		
165						
151.3					+	
150.3			+			
146.5				+		
141.8			+			
137.3						+
134.5		+				
126.5					+	
123.2				+		
119.5			+			
116.5						
115						
113.2		+				
112.5						+
107.8	+					
102.7		+				
98.6			+			
94.6	+	+				
96.6				+	+	+
80.0						
83			+			
82.5						+
77	+					
78		+				
73						+
69.2				+		
68		+	+			
65	+					+
61		+			+	
59.4				+		
57.3			+			
55					+	+
52				+		
51.8		+				
49						+
47		+				
45.4				+		
44.3			+			
41		+			+	
39.8				+		
36.8				+		
33.6				+		
32.3	+				+	
30.6		+		+		
29.4			+		+	
Total 50	5	11	11	13	11	9

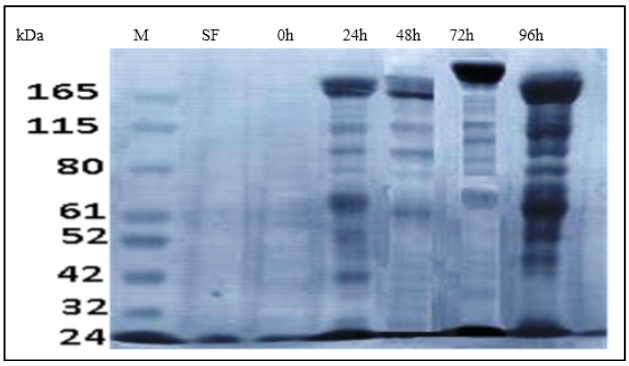


Fig 5: SDS-PAGE of total Coomassie Brilliant Blue stained ovarian protein extract (20 mg/ ml). SF: sugar-fed females, 0: immediately PBM, 24 h PBM, 48 h PBM, 72 h PBM, 96 h PBM, M: protein ladder (KDa).

4. Discussion

Anautogeny is a fundamental process and a major character of mosquitoes that regulate vitellogenesis through intake of protein-rich diets like a blood meal from a wide range of vertebrate hosts (including humans) according to the mosquito species [9]. Therefore, the molecular characterization of Vtg genes regulating vitellogenesis is essential for better understanding of mosquito ovarian development and reproductive biology.

The gene encoding vitellogenin, a major YPP in most oviparous animals, is expressed in extraovarian tissues in a (sex-, tissue) - and stage-specific manner [10]. The cDNA encoding *A. aegypti* Vg has been characterized [11], and its gene cloned [12]. Two distinct members of the vitellogenin gene family, Vg-C1 and Vg-C2, were cloned from *Cx. quinquefasciatus* [28]. Vitellogenin gene sequences may, therefore, serve as ideal molecular markers for inferring phylogenetic relationships among mosquitoes. A BLAST search for the *Cx. pipiens quinquefasciatus* whole genome sequence, four intact Vtg genes were identified, designated as CpVg1a (GenBank accession NZ_AAWU01017720), CpVg1b (GenBank accession NZ_AAWU01017726), CpVg2a and CpVg2b (GenBank accession AAWU01001936) (Chen *et al*., 2010). cDNA encoding the *Ae. aegypti* VgA1 gene has been characterized and its genomic sequence containing 2015 bp of the 5' promoter region cloned [11; 12]. Inferred phylogenetic relationships based on analysis of Vtg sequences suggest that Vg sequenced from *Cx. pipiens* was orthologous to that of Vg-c of *Cx. quinquefaciatus*, and *Ae. polynesiensis* (Fig. 4). Similarly, Vtg from *An. albimanus* and *Toxorhynchites amboinensis* was orthologous to *Ae. aegypti* Vg-C, which in turn, confirms that Vg-C was the ancestral vitellogenin gene [28]. Recently, Da-Silva and co-workers [29] isolated and sequenced 886 bp cDNA fragment of a vitellogenin from *An. aquasalis* females that was similar to that of culicines.

The stringent control of the Vtg gene expression in mosquitoes by blood meal ingestion provides an important model for elucidating hormonal and tissue-specific regulation in the context of complex physiological events surrounding reproduction [10]. The acquisition of blood by a female mosquito causes the fat body to undergo structural changes that triggers a cascade of transcriptional events to synthesize abundant amounts of Vtg in ovaries. In our study, the highest

transcription level was observed in *Cx. pipiens* females at 24 h pbm, which was followed by a gradual decline at 48 and 72 h pbm, thus corroborating the Vtg transcriptional profile in *Ae. aegypti* [30]. Our data are in agreement with recent studies that reported low residual levels of Vtg transcript accumulation prior a female taking a blood meal or in immature mosquitoes in *Cx. tars*alis and *Ae. aegypti* [28, 25]. An important adaptation for anautogeny is that the female then enters a state of cellular and physiological arrest; Vg is not synthesized during a pre-vitellogenic period if a female feeds on flower nectar or sugar-meal solely. This arrest state terminates and vitellogenesis is initiated upon the female ingestion of blood. The vitellogenic period lasts for 48 h, and is differentiated into a synthesis stage and a termination stage [10], which might explain the Vtg transcriptional level in *Cx. pipiens* females, which were sugar-fed after 96 h pbm (Fig. 4). However, in immatures, the presence of traces of Vtg expressed in these stages could be important for survival of the developing embryo.

The present study contributed to initial efforts for establishing the molecular bases involved in *Cx. pipiens* vitellogenesis, which could provide incipient insights to control this important vector, considering this as basic science investigations could benefit mosquito transgenesis technologies.

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