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Vitellogenin gene expression during the development of anautogenous malaria vector, *Anopheles culicifacies* A.

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ABSTRACT

Aim: Anautogenous mosquitoes require a blood meal before they can lay their first batch of eggs. Unluckily, this happens at the consequence of pathogen transmission and the spread of vector-borne diseases. The reproductive success of all oviparous species including insects depends on vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes. Therefore, during the present study, the temporal and spatial expression of the Vg gene has been studied in *Anopheles culicifacies*, which is the major vector in Indian subcontinent.

Methodology: The Dhera strain of *An. culicifacies* Species A was maintained in an insectary at appropriate rearing conditions. The RNA was extracted from the female fat bodies after blood feeding at different time intervals. The temporal and spatial expression patterns of the Vg gene was examined by Real-time PCR using SYBR Green dye.

Result: The *An. culicifacies* vitellogenin (*AncuVg)* showed significant and strong expression in fat body tissues only as compared to other tissues at specific time interval after blood feeding. Time and tissue specific expression of the gene showed induction of the gene at 3 h PBM and peak expression was observed after 24 h of blood feeding.

Discussion: The peak expression of Vg gene observed at 24h corresponds to the peak levels observed earlier for 20-E after 24h of blood meal and also corresponds with the timing of ookinete invasion and oocyst formation.

Conclusion: The molecular events of vitellogenesis as observed in case of *An. culicifacies,* may prove beneficial to develop novel control strategies to combat parasite transmission because the timing of Vg expression coincides with ookinetes invasion in general.

Key Words: *Anopheles*, Fat body, Hematophagy, Mosquito, Vitellogenin

INTRODUCTION

Hematophagy, or blood feeding behaviour is exhibited by most of the arthropod vectors of human pathogens both for reproduction and for transmission[1]. Hematophagous mosquitoes are broadly categorized into two groups with respect to egg development. Anautogenous mosquitoes require a blood meal before they can lay their first batch of eggs, whereas autogenous mosquitoes can use their protein reserves—stored in fat body tissue during larval development—to synthesize yolk proteins and develop one batch of eggs before taking a blood meal. One important distinction is that anautogenous mosquitoes must feed on vertebrate hosts more frequently than autogenous mosquitoes, thereby increasing their overall vectorial capacity. The understanding of the mechanisms underlying anautogeny is very crucial because this reproductive strategy is the driving force behind the transmission of disease to millions of people[1-3].

These mosquitoes (*Aedes* and *Anopheles* species) also require a blood meal to synthesize vitellogenins during each gonotrophic cycle[2,3]. The developmental expression of Vgs has been documented in ananutogenous mosquitoes, *Anopheles gambiae* and *Anopheles stephensi*[1,4-6] and in autogenous *Culex tarsalis*[7]. Similarly, the mechanisms underlying the molecular regulation of one of the three vitellogenin genes

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(VgA1) in anautogenous *Aedes aegypti* has also been characterized[8-10]. However, more such studies are required to confirm the pattern and to better understand the mechanism of vitellogenesis in other mosquito species.

An. culicifacies is considered to be the principal vector of malaria in India accounting for 60–70% of malaria cases in the country and exists as a species complex of five sib-ling species named A, B, C, D and E[11-15]. In the present study, the expression of the vitellogenin gene during different stages of development following blood meal has been studied. The present investigations complement our previous study where, the complete vitellogenin gene of *An. culicifacies* was cloned and characterized including its upstream regulatory region[16].

EXPERIMENTAL PROCEDURES

Mosquitoes

The Dhera strain of *An. culicifacies* Species A was maintained in an insectary at temperature of 28 ± 2°C, 75 ± 5% relative humidity and 14/10-h light-dark cycles[17]. Larvae were reared in enamel trays at a standard density of 300 larvae/450 ml of water and were fed on yeast extract and dog biscuits in the ratio of 2:3 (w/w). It passes through 4 instars for about 12-13 days. After pupation, the pupae were transferred to fresh bowl and were kept in cloth cages for emergence to adult mosquitoes.

Adult mosquitoes were maintained on 5% glucose and fed on rabbit blood for ovarian development. Mosquitoes were starved by denying access to sugar and water for 8 h before blood feeding and those that had fed to repletion were separated from the cohort and shifted to a new cage. After blood feeding 10-15 blood fed females were taken after regular time intervals to analyse gene expression profile. During the complete procedure, a bowl filled with water was placed in the cage to enable oviposition.

RNA isolation and cDNA synthesis

Total RNA was isolated from 5-7 day old sugar-fed males and non-blood fed and blood-fed females at several time points post blood meal (PBM) using TRI reagent (Sigma) according to the manufacturer's protocol after homogenization with a motor-driven pellet pestle mixer. RNA samples also were extracted from immature stages and dissected tissues (i.e. fat body, midgut, salivary gland, ovaries). For each stage, tissues were dissected from 20 mosquitoes in a drop of DEPC treated water and were immediately processed for RNA isolation and stored at −80°C after treatment with amplification-grade RNase-free DNase I (Invitrogen) until used further for cDNA synthesis. All the plastic ware and glassware were treated with DEPC water before use.

RNA integrity was confirmed on a 1% agarose gel and was quantified using Nanodrop ND2000c spectrophotometer. For each stage 1 µg RNA was used for cDNA synthesis using the Superscript RT for PCR kit (Invitrogen) in 20µl volume with oligo$(dT)_{20}$ primers at 37°C for 1hr followed by termination of the reaction at 70°C for 20 min. To minimize possible variations in reverse transcriptase efficiency, all cDNA synthesis were proceeded siumeltaneously.

Quantiative Real time PCR (qRT-PCR)

Real-time PCR was performed using SYBR Green dye on Real-Time PCR system (Applied Biosystem). In order to quantify relative gene expression, standard curves were generated, using 10-fold serial dilutions of cDNA pools containing high concentrations of the gene of interest. Each qPCR reaction was carried out in a total volume of 20 µL, using 2 µL cDNA template with SYBR Green chemistry and gene specific primers. The primer pair was derived from two different exons with an intervening intron in order to detect amplification products from genomic DNA that may have precipitated during the RNA preparation. Primers were designed using the primer premier 5.0 to yield amplification products ranging in size between 100 and 150 bp (Vg: forward, 5'-CCTACATGCGTTGTTGATGG-3', reverse, 5'-TGACGACTATGCACTCCAGC-3'; beta-actin: forward, 5'-AGCGGGAAATCGTGCGTGAC-3', reverse, 5'-CAATGGTGATGACCTGGCCAT-3').

The amplification of the Vg gene and an endogenous control gene was performed simultaneously and the relative expression levels between Vg and endogenous control gene assessed. The relative abundance of the gene of interest in each RNA sample was estimated from the respective standard curves and the gene expression level was normalized against the β-actin expression level. Cycling conditions were kept constant for all assays. A 2-step qRT-PCR program described earlier[18] was used in the amplification process. Melting curves were visually inspected to verify a single amplification product with no primer dimers.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism3.0 software[19]. All data were presented as the mean ± standard deviation. Differences between test samples and their respective controls were evaluated by unpaired Student's t-test. The significant difference of expressions was shown at $P < 0.05$.

RESULTS

The temporal and spatial expression patterns of the Vg gene were examined using qRT-PCR. The RNA was extracted from the female fat bodies after blood feeding at different

time intervals. In addition, RNA was also extracted from midguts, ovaries and salivary glands. During the present study it was observed that the level of expression of the vitellogenin gene varied during different stages of development. For each gene (vitellogenin and beta-actin), a single peak on the melting curve was observed, indicating that target PCR products were amplified selectively and also the primer dimers and other products were not formed.

The expression was observed both in males and both sugar-fed and blood fed females excluding larvae and pupae samples (Fig. 1). The level of vitellogenin mRNA in these stages was negligible, indicating that the vitellogenin expression in male, and non-blood fed females is merely basal level transcription. It could also be ascribed to the presence of transcription initiation complex which could access the promoter region of the Vg genes. However, the *Anopheles culicif*acies vitellogenin (*AncuVg)* showed significant and strong expression in fat body tissues only as compared to other tissues viz. salivary glands, midgut and ovaries at specific time interval after blood feeding suggesting that the *AncuVg* is expressed only in fat bodies (Fig. 2).

The presence of the mRNA was detected in the fat body within 3 h of a blood meal. The expression increased several folds after blood feeding. The peak expression level was observed after 24h PBM then decreased gradually to almost negligible at 72 h PBM (Fig. 3). All the PCR products were also analysed by electrophoresis in ethidium bromide stained agarose gels (1%) (Figs. 4-6) showing expected results.

DISCUSSION

Vg is synthesized abundantly in the female fat body in tissue-, sex- and stage-specific manner, secreted into haemolymph, and subsequently sequestered in the developing oocytes through receptor-mediated endocytosis[20-22]. During the present study, the *AncuVg* expression was found in males and non-blood-fed females at very insignificant levels as compared to that in blood fed females (Fig. 1), indicating that this gene may be expressing constitutively in all stages prior to blood feeding. However, the level of Vg RNA was too low to support the process of egg development. This may be because of the presence of certain rare synonymous codons that might have initiated the process of vitellogenesis after accumulating at the 5' end in the absence of any external amino acid supply[23]. Also, as described earlier, the induction of Vg expression after a blood meal requires the Target of Rapamycin (TOR) pathway and ecdysone signaling[24,25]. Thus, once a female mosquito ingests blood, the vitellogenesis process may be triggered under the combined action of JH and 20-E along with the presence of enough amino acid residues.

The significant vitellogenin gene expression is thus restricted to adult female mosquitoes only. However, males of several species have been reported to express vitellogenin gene including *Oncopeltus fasciatus*[26],*Rhodnius prolixus*[27], and the sea urchin *Strongylocentrotuspurpuratus*[28] but their physiological significance remains to be investigated. In addition, treatment with steroid hormones (estrogen for vertebrate and 20-HE for insects) also stimulates vitellogenin gene expression in males of the chicken, *G. gallus*[29], the frog, *X. laevis*[30], the fruit fly *D. melanogaster*[31], the flesh fly *Sarcophaga bullata*[32], *An. gambie*[33], and *Cherax quadricarinatus*[34]. Thus, it is possible that an increase in tRNA levels especially those required to increase translation of rare synonymous codons has been stimulated by ecdysone along with the induction of Vg transcription after blood feeding.

Time and tissue specific expression of the gene showed induction of the gene at 3 h PBM and peak expression was observed after 24 h of blood feeding. These results of *AncuVg* gene are in accordance with the previous studies of Vg gene expression in fat bodies[35-37]. The temporal expression pattern of the Vg gene in *An. culicifacies* (Fig. 3) was found to be similar to that in *An. gambiae*[6], VgA1 in *Ae. aegypti*[9] and in *Cx. tarsalis*[7]. The only difference lies in the initiation of Vg expression by 3h after blood feeding in *An. culicifacies* and other autogenous mosquitoes as compared to at 12-24h after emergence in case of autogenous mosquitoes[7].

These results also are in accordance with the pattern shown during the hormonal regulation of anautogeny. The peak expression of Vg gene observed at 24h in the present study after blood meal corresponds to the peak levels observed earlier for 20-E after 24h of blood meal[9] and also corresponds with the timing of ookinete invasion and oocyst formation as observed earlier in case of *An. gambiae* with infection of *P. nigerensis*[6]. This invasion was found to coincide with the time at which vitellogenesis is first affected. Similar results have also been observed with infection of *P. berghei* in *An. gambiae*[38] and in *An. stephensi*[4]. A significant decrease in Vg mRNA level at 24h PBM was observed when the ookinetes were invading and transforming into oocysts[6], indicating that the infection has a negative effect on vitellogenesis in the fat body, thus eventually causing a significant reduction in fecundity[4]. Thus, the peak observed at 24 h PBM in *AncuVg* gene indicate the possibility of similar hormonal regulation pathway in *An. culicifacies* and also correlating the similar mode of parasite infection as observed earlier in other mosquitoes.

CONCLUSIONS

The expression pattern of *AncuVg* shows that the gene is expressed significantly in response of blood meal. The results of present study indicates that the fat body is the only site of Vg gene expression in *An. culicifacies* female mosquitoes after blood feeding and that too showing its peak expression

at 24h PBM coinciding with the time of ookinete invasion and oocyst formation as reported earlier. Thus, the molecular events of vitellogenesis as observed in case of *An. culicifacies,* may prove beneficial to develop novel control strategies to combat parasite transmission because of the similar timing of Vg expression and ookinetes invasion in general.

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Source of Finding

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Conflict of Interest

The authors declare they have no conflict of interest.

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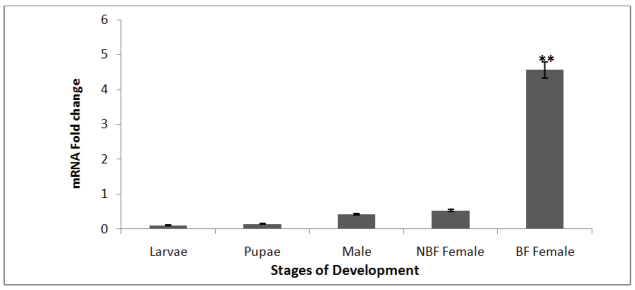

Figure 1: The developmental expression pattern of Vg gene in *An. culicifacies*.

Showing sex- specific expression of vitellogenin gene in *Anopheles culicifacies*using Real-time PCR. Higher expression of *AncuVg* gene was observed in blood fed females with basal levels of expression in males and non blood fed females. Results above are expressed as percent of maximum levels recorded. Results were normalized to β-actin mRNA levels. (BF- Blood fed, NBF- Non Blood fed).

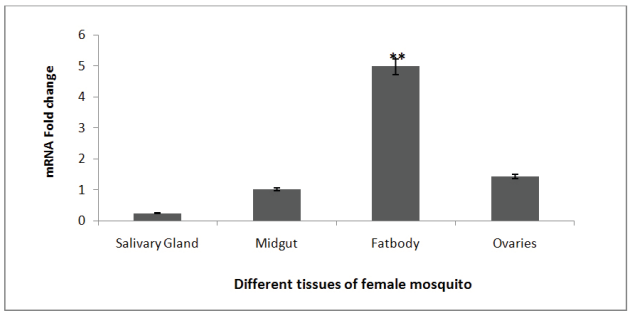

Figure 2: Tissue Specific expression of Vg gene in *An. culicifacies*.

Showing tissue- specific expression of vitellogenin gene in *Anopheles culicifacies*usingReal-time PCR. Higher expression of *AncuVg* gene was observed in fat body tisssues with basal levels of expression in midgut and ovaries. Results were normalized to β-actin mRNA levels.

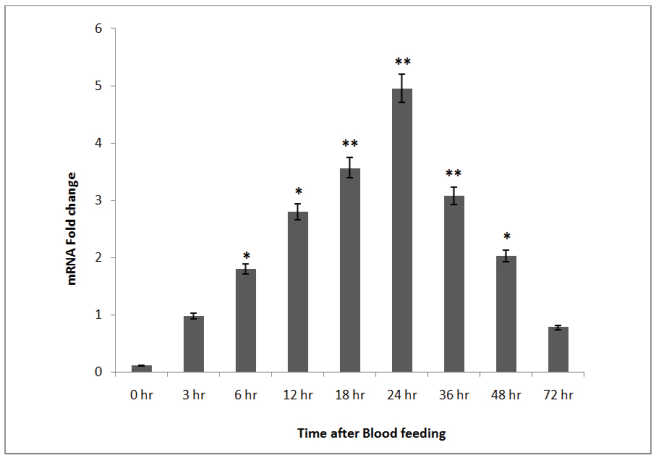

Figure 3: Stage Specific expression of Vg gene in *An. culicifacies* after blood feeding.

Showing stage- specific expression of vitellogenin gene in *Anopheles culicifacies* using Real-time PCR. *AncuVg* gene expression was found to be initiated within 3 h following a blood meal and reaching its peak at 24 h and then showing a decline afterwards. Data was normalized to the expression level of β-actin gene. Each value is expressed as mean with S.D. from n = 3 independent experiments. Statistical analyses of the data were carried out using unpaired Student's t-test. (*P < 0.05,**P < 0.01).

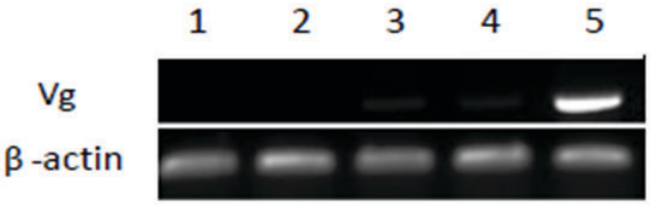

Figure 4: Sex - specific expression of the vitellogenin gene.

Agarose gel (1%) showing relative quantity of RT-PCR products. (Lane 1-Larvae, Lane 2- Pupae, Lane 3- Males, Lane 4- Non Blood fed females and Lane 5- Blood fed females).

Figure 5: Tissue specific expression of the vitellogenin gene.

Agarose gel (1%) showing relative quantity of RT-PCR products. (Lane 1-Salivary glands, Lane 2- Midgut, Lane 3- Ovaries and Lane 4- Fat bodies).

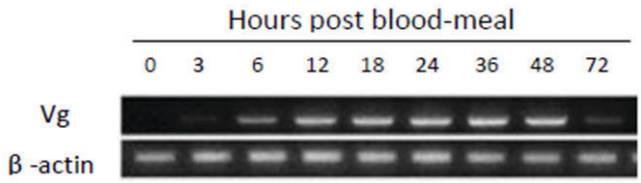

Figure 6: Stage specific expression of the vitellogenin gene following a blood meal.

Agarose gel (1%) showing relative quantity of RT-PCR products in fat body tissues at different time intervals after blood feeding.