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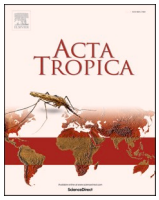

Sequence analysis and developmental expression dynamics of cAMP response element-binding protein in *Aedes albopictus*

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ABSTRACT

Mosquito-borne diseases remain a major global health challenge, and understanding the molecular mechanisms underlying mosquito physiology and adaptation is essential for developing new vector control strategies. cAMP response element-binding protein (CREB) plays a pivotal role in regulating specific gene expression programs governing cell survival, differentiation, plasticity, and the maintenance of physiological homeostasis. In this study, we aimed to investigate the evolutionary and developmental characteristics, as well as sex-specific expression patterns of CREB transcription factors in mosquitoes, especially *Aedes albopictus*. A combination of bioinformatics and molecular approaches, including multi-species phylogenetic analysis, three-dimensional structure prediction, and developmental expression profiling via RT-qPCR and Western blotting, were used to characterize the evolutionary and expression features of CREB in mosquitoes. As a result, it revealed a divergence of mosquito CREBs into two major clades, CREB-A and CREB-B. These mosquito CREBs contained the basic leucine zipper (bZIP) domain and/or kinase-inducible domain (KID), showing strong evolutionary conservation across species. Furthermore, dynamic, stage-dependent, and sex-specific transcriptional and protein expression patterns for both CREB-A and CREB-B were found in *Aedes albopictus*. The findings indicated that mosquito CREB proteins may have experienced evolutionary and functional divergence while retaining conserved KID and bZIP domains in CREB-B and bZIP domain in CREB-A, suggesting possible complementary roles in development and physiology.

1. Background

Mosquito-borne diseases, transmitted through the bites of blood-feeding mosquitoes carrying viruses, protozoa, or other pathogens, represent one of the heaviest global infectious disease burdens. To date, three genera of mosquitoes-*Anopheles, Aedes*, and *Culex,* are known to transmit diseases in humans. *Anopheles* mosquitoes are main vectors for malaria, which is responsible for approximately 220 million cases and >400, 000 deaths annually (WHO, 2025). *Aedes* mosquitoes, especially *Ae. aegypti* and *Ae. albopictus*, transmit devastating arboviral diseases, including dengue fever, yellow fever, chikungunya fever, and Zika fever (Ryan et al., 2019). *Culex*, the most widespread genus of mosquitoes in the world, are primarily for the transmission of West Nile virus and St. Louis encephalitis (Gorris et al., 2021).

Recently, driven by climate change, rapid urbanization, and the global surge in trade and travel, mosquito-borne diseases have breached their previously tropical confines and are now embedding locally in temperate regions, due to the introduction of species beyond their native range (Zhang et al., 2024). For instance, following a period of unprecedented spring heat in 2025, three mosquitoes of *Culiseta annulata* were sighted in the mountains of Iceland for the first time (Chapman, 2025). The expansion of mosquito habitats further exacerbates the risk of infections. To suppress these mosquito-borne diseases by preventing mosquitoes from feeding on humans, mosquito repellent, insecticide-treated nets, and indoor residual spraying are considered as the key global health strategies (Zhou et al., 2022). However, extensive and long-term use of insecticides in both public health and agriculture has consequently led to the emergence and spread of insecticide

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resistance, which is regarded as one of the major obstacles to the effective control of mosquito-borne disease (Hazarika et al., 2025; Wang et al., 2023). Among various insecticide resistance mechanisms, including target-site resistance, metabolic resistance, cuticular resistance and behavioral resistance (Liu, 2015), cytochrome P450 monooxygenases, which metabolizes widely used insecticides such as pyrethroids and organophosphates, play a crucial role in metabolic resistance (Feyereisen, 2015; David et al., 2013). Moreover, the up-regulation of specific cytochrome P450 genes, particularly members in CYP6 and CYP9 families of insecticide-resistant mosquitoes (David et al., 2013; Tchouakui et al., 2020), is regulated through multiple signaling pathways, such as GPCR/cAMP/PKA, MAPK/CREB, CncC/Keap (Hazarika et al., 2025; Wilding, 2018; Li et al., 2015, 2021). Among these, cAMP response element-binding protein (CREB) acts as a transcription factor that regulates the expression of cytochrome P450 genes in various organisms (Li et al., 2021). Notably, a recent study has shown that CREB could directly bind to promoter region of CYP6CM1, transcriptionally activate detoxification-related cytochrome P450 genes to confer imidacloprid resistance (Yang et al., 2020).

Normally, mammalian CREB1 has a domain organization of Q1 (glutamine rich 1)-KID (kinase-inducible domain)-Q2 (glutamine rich 2)-bZIP (basic leucine zipper) with consistent exon usage (Wang et al., 2018), although CREB isoforms may differ in domain composition across species. In general, the bZIP domain is responsible for binding to the consensus cAMP response element (CRE) region on target DNA and enables dimerization. The KID domain adopts an α-helical structure that specifically binds to the KIX domain of CREB-binding protein (CBP), and serves as the central regulatory switch for CREB transcriptional activity upon its phosphorylation (Sakamoto et al., 2011; Lonze and Ginty, 2002). The Q1 and Q2 domains are considered to be responsible for recognizing and binding to the canonical CRE (Johannessen et al., 2004; Martinez-Yamout et al., 2023). Mammalian CREB3 has a transactivation domain (TAD), a bZIP domain and a transmembrane region (TM), while CREB5 has a TAD and a bZIP domain. These CREBs have been firmly established as central regulators of complex physiological processes. For instance, it functions as a molecular switch for memory formation, where it governs synaptic plasticity and orchestrates the transcriptional programs required for both the establishment and the long-term consolidation of memory (Kida, 2012). In mammals, many studies have found that CREBs also contributed critically to whole-organism metabolic homeostasis under diverse physiological conditions, such as modulating hepatic gluconeogenesis (Herzig et al., 2001; Zhao et al., 2023), coordinating hormonal and nutritional signals (Altarejos and Montminy, 2011; Wade et al., 2021), and regulating adipocyte differentiation and lipid metabolism (Herzig et al., 2003). In *Drosophila*, dCREB-A regulated secretory pathway genes (endoplasmic reticulum/Golgi apparatus/secretory vesicles) in salivary glands/neurons (Fox et al., 2010; Johnson et al., 2020), mitigated TDP-43 neurodegeneration (Ho et al., 2024), and had mammalian orthologs Creb3L1/L2 (Fox et al., 2010). dCREB-B controlled long-term memory consolidation via activator/repressor isoforms in mushroom body/DAL neurons (Lin et al., 2021, 2022), enhanced presynaptic release at neuromuscular junction for plasticity (Davis et al., 1996), regulated circadian rhythms (Tanenhaus et al., 2012), maintained fat body metabolic homeostasis (Iijima et al., 2009), and provided neuroprotection against polyglutamine toxicity (Soares et al., 2024; Iijima-Ando et al., 2005). In addition, CREB-dependent transcription contributes to developmental transitions across multiple biological systems by regulating genes associated with cell cycle progression, differentiation programs, and lineage specification (Chowdhury et al., 2024; Ichiki, 2006).

Although these roles of CREBs have been revealed across many organisms, their functional significance in mosquito species remains largely unexplored. A systematic characterization of CREB molecular properties, regulatory mechanisms, and expression dynamics are therefore essential for elucidating how intracellular signaling pathways shape gene regulation in these medically important disease vectors. Given that the *Ae. albopictus* is a major vector for dengue virus, yellow fever virus, chikungunya virus, and Zika virus, as well as its rapid global expansion and adaptability to diverse environments (Abbasi, 2025; Laporta et al., 2023), we employed *Ae. albopictus* in the present study to delineate transcriptional and protein expression dynamics of CREBs across developmental stages and between sexes, and identify mosquito CREB through phylogenetic analysis and structural prediction, aiming to provide fundamental insights into their potential regulatory roles in mosquito physiology and adaptation.

2. Materials and methods

2.1. Homologous sequences identification

Amino acid sequences of CREB proteins across seven organisms, including *Homo sapiens, Xenopus laevis, Rattus norvegicus, Mus musculus, Arabidopsis thaliana, Danio rerio*, and *Drosophila melanogaster*, were retrieved from the UniProt database (Table S1). To ensure that only bona fide CREB family members were included, each candidate sequence was subjected to domain validation using the NCBI Conserved Domain Database (CDD) and the SMART (Simple Modular Architecture Research Tool) (Lu et al., 2020; Letunic et al., 2021). Those sequences containing bZIP domain were retained as the inputs to confirm the presence of CREBs in selected 7 taxa and further explore their homologs in 25 mosquito species (Table S2), using BLASTp embedded in TBtools-II (v.2.341) (Camacho et al., 2009; Chen et al., 2023). Sequences with significant similarity of the E-value threshold at 1e-5 or lower were identified, and then the sequences lacking the complete bZIP domain were excluded. Finally, the mosquito CREB sequences of 21 *Anopheles* species (*An. gambiae, An. arabiensis, An. coluzzii, An. quadriannulatus, An. merus, An. melas, An. christyi, An. epiroticus, An. maculipalpis, An. stephensi, An. maculatus, An. minimus, An. culicifacies, An. funestus, An. dirus, An. farauti, An. atroparvus, An. sinensis, An. aquasalis, An. albimanus, An. darlingi*), *Cx. quinquefascitus, Cx. pipiens pallens, Ae. aegypti*, and *Ae. albopictus*, together with CREBs of *H. sapiens* and *D. melanogaster*, were subsequently aligned using the MUSCLE algorithm implemented in MEGA (v.11) (Tamura et al., 2021), and low-quality or gap-rich regions were removed using the TrimAl with a site coverage cutoff of $\geq$ 40 % (Capella-Gutiérrez et al., 2009).

2.2. Phylogenetic tree construction

Phylogenetic relationships among CREB proteins of selected organisms and 25 mosquito species were inferred separately using IQ-TREE (v.3.0.1) (Nguyen et al., 2015). Auto mode was selected for automatic sequence type identification and substitution model selection. Standard bootstrap analysis (1000 replicates) was performed to assess node confidence, with maximum iteration set to 1000 and minimum correlation coefficient set to 0.99. Both SH-aLRT and approximate Bayes tests were enabled to provide additional branch support. Perturbation strength was set to 0.5, and the IQ-TREE stopping rule was defined as 100.

2.3. Protein domain architecture, sequence analysis, and structural visualization

To identify conserved structural and functional motifs of CREB proteins, domain analysis was performed using MEME (Bailey et al., 2015), the NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?tdsourcetag) and the SMART (https://smart.embl.de/help/smart_about.shtml). Identified domains were annotated, and a schematic diagram illustrating their relative positions and structural organization was manually generated using TBtools-II (v2.341) (Chen et al., 2023). In addition, protein sequences were analyzed with TMHMM 2.0 to predict potential transmembrane helice (Krogh et al., 2001). NLStradamus was employed under default parameters to identify

putative nuclear localization signal (Nguyen Ba et al., 2009).

Conserved residues were identified based on sequence identity and similarity metrics using Jalview (v.2.11.5.0) (Waterhouse et al., 2009), and functionally critical amino acid residues were highlighted. The three-dimensional (3D) structures of CREBs were predicted via AlphaFold (Jumper et al., 2021) or SWISS-MODEL (Waterhouse et al., 2018). Upon individual submission of each protein sequence, the optimal template was automatically selected with the highest sequence identity and GMQE score. The solved *H. sapiens* CREB1 structure (PDB ID: 5ZK1) was used to show the binding pose of bZIP domain to target DNA, and interaction of CREB1 homo-dimer. *H. sapiens* CREB1 structure (PDB ID: 2LXT) was used to show the binding pose of KID domain to CBP. The predicted structures were visualized using Open-Source PyMoL (DeLano, 2002).

2.4. Mosquito rearing and sampling procedures

Ae. albopictus were reared at 26 ± 1 °C with 80 % relative humidity and a 12-h light/dark photoperiod in the insectary laboratory at the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. Samples were collected from multiple developmental stages, including eggs (500 mg per sample), larvae (10 individuals of 4th instar per sample), pupae (10 individuals per sample), adult females (three per sample), and adult males (three per sample). Male and female adults were collected at day 1, 3, 5 and 7 post-eclosion, and identified morphologically. And all individuals were normally fed prior to sample collection. All samples (3 replicates) were flash-frozen in liquid nitrogen immediately following collection and then stored at −80 °C until RNA or protein extraction. This study was approved by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (license number IPD-2024–007).

2.5. RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from frozen samples using the TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Briefly, 1 mL of Trizol reagent was added to each sample, followed by adding 200 μL of chloroform. After mixing and centrifugation, the aqueous phase was collected and mixed with 400 μL of isopropanol to precipitate RNA. The RNA pellet was washed with 75 % ethanol, air-dried and dissolved in RNase-free water. After determining the concentration and purity of RNA using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), these RNA samples were incubated with DNase I (RNase-free, Thermo Fisher, USA) at 37 °C for 30 min. The reaction was stopped by adding 50 mM EDTA and heating at 65 °C for 10 min. Reverse transcription was performed using the PrimeScript™ RT Master Mix (Takara, Japan) with oligo (dT) primers, incubated at 37 °C for 15 min, followed by enzyme inactivation at 85 °C for 5 min. The cDNA products were stored at −20 °C until downstream analysis.

Each PCR reaction (25 μL) contained 12.5 μL of TB Green Premix Ex Taq (Takara, Japan), 0.5 μL each of forward and reverse primers (10 μM), 2 μL of cDNA, and 9.5 μL of nuclease-free water. Cycling conditions were initially denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 30 s. A melting curve was generated by cooling the products to 65 °C then heating to 95 °C at a rate of 0.1 °C/s while simultaneously measuring fluorescence. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method analyzed with two *Ae. albopictus* housekeeping genes of RPS17 and Actin as references using the approach previously reported (Dzaki and Azzam, 2018; Vandesompele et al., 2002; Hellemans et al., 2007). Primers for CREB-A and CREB-B were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) based on the reference sequences obtained from NCBI. The primers were listed in Table S3.

2.6. Protein extraction and western blotting

Total protein was extracted from samples (3 replicates) using Radio Immunoprecipitation Assay Lysis buffer (Beyotime, China) adding protease inhibitor cocktail (Epizyme Biotech, China). Protein concentrations were determined using a bicinchoninic acid assay (Solarbio, China). Equal amounts of protein (40 μg) were separated by SDS-PAGE (4–20 % gels) (GenScript, USA) and transferred to 0.45 μm polyvinylidene fluoride membranes (Cytiva, China). Membranes were blocked for 1 h at room temperature (RT), and then incubated overnight at 4 °C with Anti-CREB antibody (Abcam, ab32515, 1:1000) or Anti-CREB3 antibody (Abcam, ab180119, 1:5000) antibodies. After washing three times with Tris buffered saline with Tween-20, membranes were probed with Horseradish peroxidase-conjugated mouse anti-heavy chain of Rabbit IgG (Proteintech, SA00001–7H, 1:5000) for 1 h at RT. Protein bands were visualized using enhanced chemiluminescence on a ChemiDOC™MP imaging System (Bio-rad, USA) and quantified with ImageJ software (v1.5) (Schneider et al., 2012). Protein levels were normalized to total protein (Ponceau S staining) (Sander et al., 2019), according to the manufacturer's protocol (Bio-Rad Laboratories, USA).

2.7. Statistical analysis

Comparisons of relative expression levels of CREB-A and CREB-B as well as the protein levels respectively at different developmental stages were statistically analyzed using a one-way ANOVA followed by Tukey test based on the homogeneity of variances by brown-forsythe test. Statistical analyses were performed using SPSS 26.0 (IBM, Armonk, NY, USA), and P value < 0.05 was considered statistically significant. Data are presented as mean ± standard deviation (SD) using GraphPad Prism (v.10.4.2) (Boston, Massachusetts USA) unless otherwise specified.

3. Results

3.1. Phylogenetic evolution of CREB proteins

A phylogenetic tree comprising 88 CREB protein sequences from *H. sapiens* and six model organisms was constructed in Fig. 1a, which demonstrated a clear segregation of the CREB family into three major clades, designated as CREB1, CREB5, and CREB3. Five species (*H. sapiens, M. musculus, R. norvegicus, X. laevis* and *D. rerio*) encompassed all three subfamily members, with each member presenting multiple isoforms (Fig. 1a and c). In *D. melanogaster*, three CREB-B (also known as CREB1 in *H. sapiens*) isoforms and one CREB-A (also known as CREB3 in *H. sapiens*) were identified. In addition, all the CREB proteins from *A. thaliana* were clustered with orthologs of CREB1 from other taxa. Notably, gene expansion was observed within CREB3, with four paralogs, CR3L1, CR3L2, CR3L3, and CR3L4 diverging into individual branches (Fig. 1a).

Based on these CREB proteins from the aforementioned organisms, their homologs in 25 mosquito species were identified (Fig. 1b). These CREB genes were clearly segregated into two subfamilies, named CREB-A and CREB-B, corresponding to that observed in *D. melanogaster*. Both of these two CREB members were identified in most mosquito species, and some of them harbored multiple isoforms (Fig. 1c). For instance, *An. gambiae* complex except *An. merus* contained multiple CREB-B isoforms, and a similar pattern was observed in *Aedes*. Furthermore, two types of CREB-A paralogs (named CREBA-a and CREBA-b) were identified in some *Anopheles* species as showed in Fig. 1b

3.2. Sequences and structures of CREBs in mosquito species

Mosquito CREB-A encoded a protein of ~584 amino acids (aa), whereas the CREB-B consisted of ~340 amino acids and shorter than CREB-A (Fig. 2). Comparative inspection of motif and domain

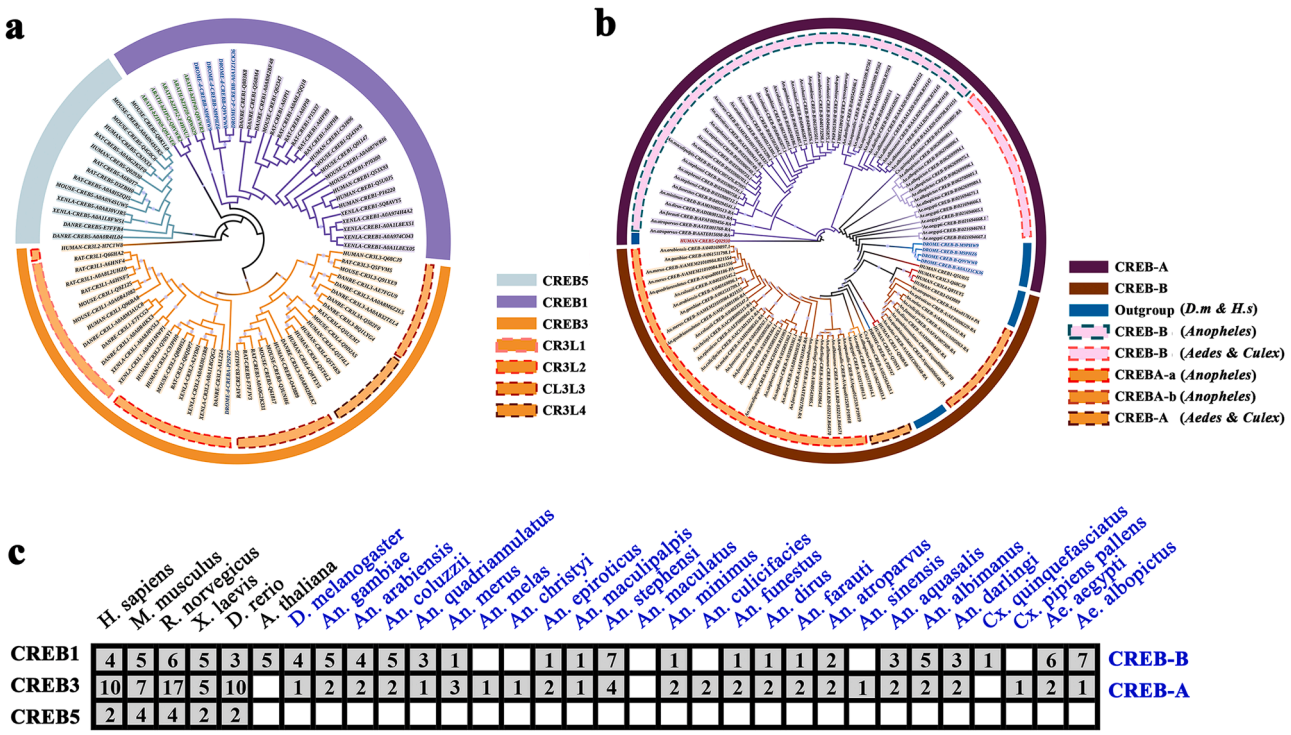

Fig. 1. Phylogenetic analysis of CREB. (a) Phylogenetic tree of CREB from *H. sapiens* and six model organisms. A total of 88 CREB protein sequences were used to construct the phylogenetic tree based on bootstrap analysis. CREB sequences were grouped into three major subfamilies: CREB1, CREB3, and CREB5. (b) Phylogenetic tree of mosquito CREB proteins. 100 CREB sequences from 25 mosquito species were grouped into two major subfamilies: CREB-A and CREB-B, using *D. melanogaster* and *H. sapiens* as the outgroup. (c) The number of CREB proteins across 32 taxa. White background indicated gene absence, numbers on gray background represented the protein hits in the genome.

organization further revealed their lineage-specific patterns. For instance, three (Motif 2–4) of four motifs (Motif 1–4) in CREB-B were present in both Culicinae and Nyssorhynchus with identical arrangement (Fig. 2a). During subsequent mosquito expansion, novel arrangements emerged, such as a duplicated Motif 1 in *An. gambiae* complex, and two Motif 2 in *An. stephensi*, indicating the recent gene gains. In addition, mosquito CREB-B harbored canonical domains (pKID and bZIP) whose exon-intron architecture was highly conserved across lineages (Fig. 2a). In contrast, five characteristic motifs (Motif 1–5) were identified in mosquito CREB-A, and some lineage-specific evolutionary trends were evident (Fig. 2a). For instance, four motifs (Motif 1, Motif 3–5) were found in CREBA-a of *Anopheles* and CREB-A of Culicinae, representing the ancestral motif arrangement pattern of CREB-A. In contrast, CREBA-b in some *Anopheles* lost Motif 5. In addition, a Motif 2 was inserted in *An. aquasalis* CREBA-b at the C-terminus, and both *An. culicifacies* and *An. minimus* gained a Motif 2 at the N-terminus of their CREBA-b. Futhermore, *An. culicifacies, An. funestus* and *An. minimus* carried either a second Motif 1 or Motif 5 in their CREBA-a. In addition, domain analysis showed that all the CREBA-a proteins contained a single conserved domain (bZIP), whereas CREBA-b in some mosquito species had recruited additional domains (e.g., Herpes_BLLF1, SP1–4_N superfamily and PHA03255) (Fig. 2a). Collectively, the exon-intron organization of CREB-A differed markedly from that of CREB-B, the most notably in its substantially longer introns as showed in Fig. 2a. It is noteworthy that transmembrane regions (TMRs) exist only in CREBA-b. Although all mosquito CREB sequences harbored NLS (nuclear localization signal), they exhibited distinct sequence features in three types of CREB in mosquito species (Fig. 2b). In all, mosquito CREB-B contained domains of Q1, KID, and a classical bZIP, while CREB-A contained a TAD and a bZIP domain (Fig. 2c).

CREB1 orthologs across mosquito species revealed universally conserved bZIP and pKID modules (Fig. 3a). In solved *H. sapiens* CREB1 (PDB ID: 5ZK1) containing bZIP domain, a single α-helix with an N-terminal basic segment rich in Arg/Lys docks in the major groove to recognize the consensus CRE half-site TGACGTCA of target gene, and a C-terminal leucine-zipper in which a Leu at every seventh position drives parallel coiled-coil dimerization. The homo-dimer adopted a V-shaped 'clamp' that engaged DNA with minimal conformational rearrangement (Fig. 3a). In mosquito species, CREB-B carried a canonical bZIP that was almost invariant in the DNA-contacting face, displaying the characteristic motif containing L-NR-R-R-K that inserted directly into the DNA groove (Fig. 3a). The KID of CREB is a critical regulatory region that orchestrates the transcriptional activity of CREB through phosphorylation by multiple kinases. Consistent with that in humans, this domain was only found in the CREB-B of fruit fly and mosquito species. The KID domain (*H. sapiens* CREB1, PDB ID: 2LXT) contains conserved Ser-133 phosphorylation sites, which is phosphorylated by PKA to enhance CREB's transcriptional activity by facilitating its interaction with the KIX domain of CBP/p300 (Fig. 3a). In mosquitoes, apart from a highly conserved serine, the key amino acids of CREB-B that bind to KIX of CBP (L-Y-IL-DL) were also highly conserved (Fig. 3a). Outside these core domains, the remaining sequences were highly divergent among lineages. Furthermore, a highly conserved Q1 sequence consisting of multiple glutamine was also identified in CREB-B of mosquito species. Mosquito CREB-A (CREB3 orthologs) had a distinct but equally conserved motif containing K-NK-Q-R-K for DNA binding in bZIP domain and highly conserved TAD sequences (Fig. 3b). No orthologs of CREB5 were identified in mosquito species, which retained the signature motif containing K-VW-L-A-L (Fig. 3c).

3.3. *CREB expression dynamics across Ae. albopictus developmental stages*

Both CREB-A ($P < 0.001$) and CREB-B ($P < 0.001$) exhibited stage-specific, dynamic transcriptional profiles (Fig. 4a). For CREB-A, transcript abundance dropped markedly from egg to larval stage ($P <$

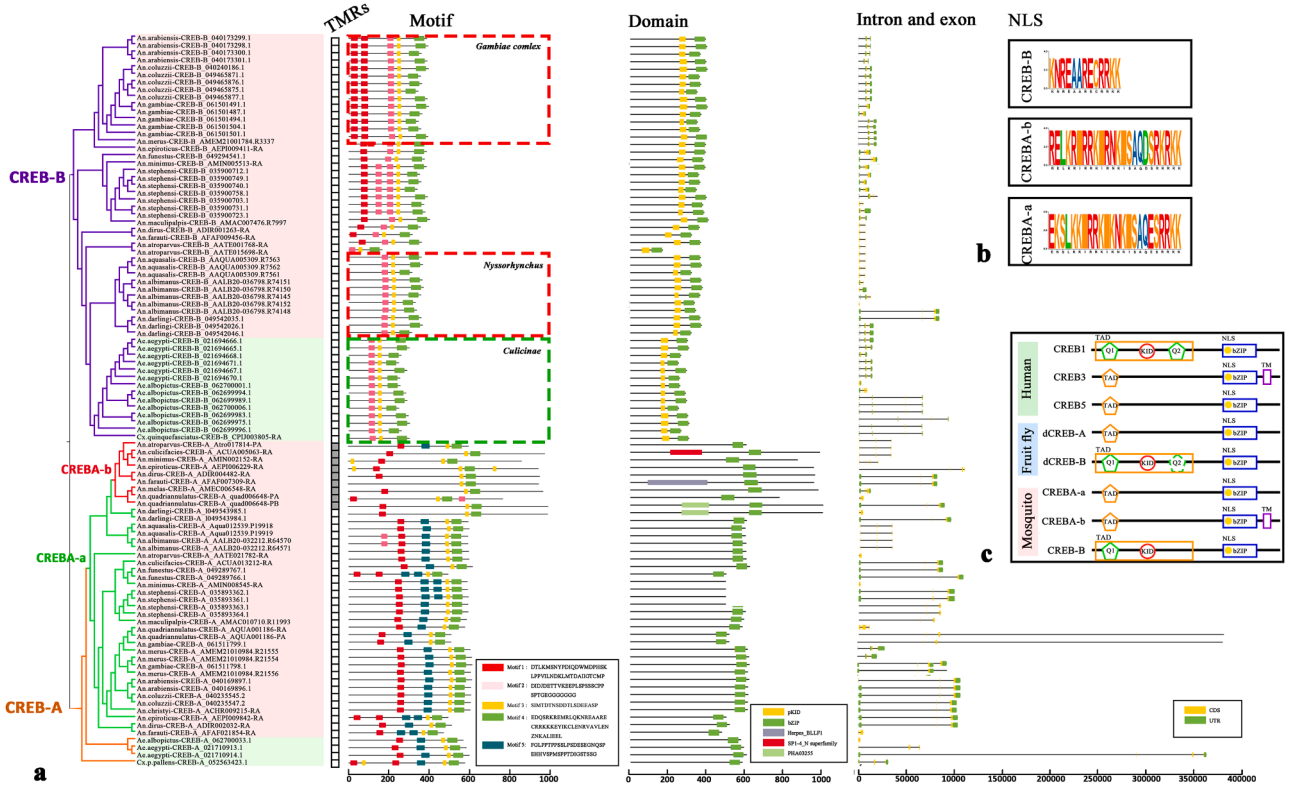

Fig. 2. Comparative analysis of CREB in mosquito species. (a) Maximum-likelihood phylogenetic tree: branches representing CREB-B clade were shown in purple, and those representing CREB-A clade were shown in orange (CREBA-a in green and CREBA-b in red); *Anopheles* species were highlighted with light red, while *Aedes* and *Culex* species (Culicinae) with light green. Transmembrane regions (TMRs) were identified in mosquito CREBs, white background indicated TMRs absence, gray background represented the hits in sequences. Distribution of conserved protein motifs and conserved domains were depicted based on their sequences. Schematic representation of exon-intron structures were also showed. (b) The characteristics of nuclear localization signal (NLS). (c) Schematic representation of the sequence features of CREB in *H. sapiens, D. melanogaster*, and mosquito species. Q1: glutamine rich 1; KID: kinase-inducible domain; Q2: glutamine rich 2; bZIP: basic leucine zipper; TAD:transactivation domain; TM: transmembrane region.

0.001), rebounded significantly in pupal stage ($P < 0.001$), and thereafter diverged between female and male adults (Fig. 4a). Particularly, expression levels of CREB-A in females increased progressively from day 1 to day 7 post-eclosion compared to pupal stage ($P < 0.001$). In males, CREB-A transcript levels also increased significantly relative to pupal stage ($P < 0.001$), further surged on day 3 ($P < 0.001$), but returned on day 5 ($P < 0.001$), and increased again on day 7 ($P < 0.001$). In contrast, CREB-B transcripts were significantly down-regulated in both larval ($P < 0.001$) and pupal ($P < 0.001$) stages compared to eggs respectively (Fig. 4a). Thereafter, CREB-B expression in female adults increased on day 1 ($P = 0.005$) and was maintained through day 3 post-eclosion ($P = 0.120$), then decreased steadily on day 5 ($P = 0.284$) and comparatively stable to day 7 ($P = 0.641$). In male adults, CREB-B transcripts increased significantly on day 3 post-eclosion relative to day 1 ($P = 0.035$) and remained stable thereafter through day 5 ($P = 0.972$) and day 7 post-eclosion ($P = 0.980$).

From the perspective of protein expression, CREB-A increased from egg to pupal stage ($P < 0.001$), while CREB-B decreased ($P < 0.001$) (Fig. 4b). For CREB-A, it increased on day 1 post-eclosion compared to pupal stage ($P = 0.003$), and remained stable one day 3 ($P = 1.000$), and increased again on day 5 ($P < 0.001$) and day 7 ($P < 0.001$) in female adults (Fig. 4b). In male adults, it decreased on day 1 compared to pupal stage ($P < 0.001$), then increased on day 3 ($P < 0.001$), but decreased again on day 5 ($P < 0.001$) and day 7 ($P = 0.003$) (Fig. 4b). For CREB-B, it decreased further on day 1 compared to pupal stage ($P < 0.001$), but increased on day 3 ($P < 0.001$) and day 5 ($P = 0.001$), but decreased again one day 7 ($P < 0.001$) in female adults (Fig. 4b). In male adults, it also decreased on day 1 compared to pupal stage ($P < 0.001$), then increased on day 3 ($P < 0.001$), and remained the level on day 5 ($P = 1.000$) and day 7 ($P = 0.533$) (Fig. 4b).

4. Discussion

Phylogenetic analysis of CREB proteins across diverse taxa revealed a complex evolutionary history potentially driving functional diversification of the CREB family (Chowdhury et al., 2024). Expansion of this gene family appeared to have originated from ancient gene duplication which subsequently led to the emergence of three major subfamilies-CREB1, CREB3, and CREB5 (Wang et al., 2019; Chowdhury et al., 2023). Specifically, human CREB1 and CREB3 correspond to CREB-B and CREB-A in mosquito species and *D. melanogaster*. Moreover, members of CREB family exhibited remarkable diversity, which was reflected in the presence of multiple alternatively spliced isoforms and paralogs (Ruppert et al., 1992; Chan et al., 2011). In addition, although the CREB family in mosquitoes is similar to that in fruit flies, containing only two types of members, their diversity exhibits lineage-specific patterns. In *Drosophila*, the CREB repertoire consisted of a single CREB-A protein alongside multiple isoforms of CREB-B, such as the activator isoform dCREB2-a and the repressor isoform dCREB2-b, which regulates long-term memory, circadian rhythms, and energy metabolism (Tubon et al., 2013; Perazzona et al., 2004; Iijima et al., 2009). However, mosquitoes displayed a more extensive repertoire of CREB isoforms, with CREB-A itself containing two paralogs likely derived from gene duplication. This expanded isoform diversity suggests a more complex CREB family in some mosquito species than in fruit flies. In *Aedes* species particularly, multiple CREB-B isoforms may reflect adaptive evolution underlying their broad ecological versatility.

Extensive lineage divergences in the motifs and domains of mosquito

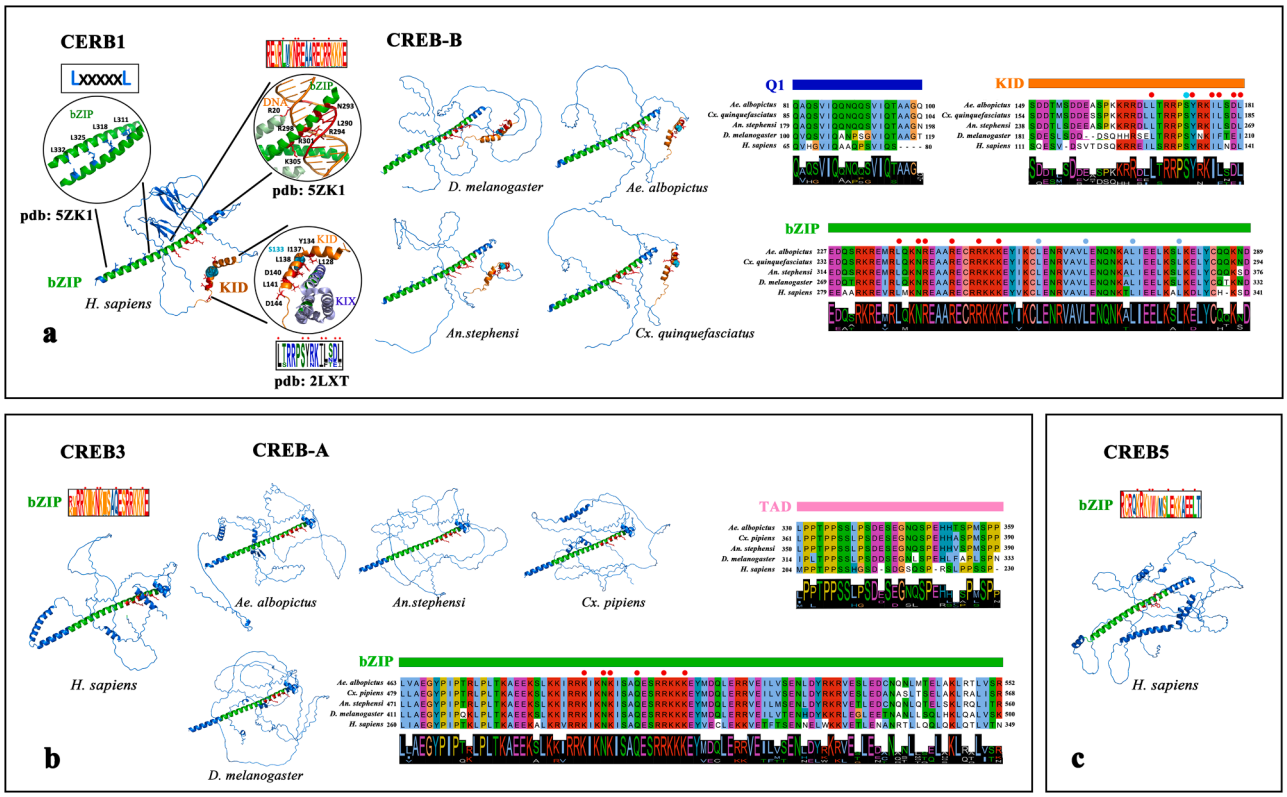

Fig. 3. Structural and sequence characteristics of the mosquito CREB-A and CREB-B. (a) Structural and sequence characteristics of *H. sapiens* CREB1, *D. melanogaster* and mosquito CREB-B. The solved *H. sapiens* CREB1 structure (PDB ID: 5ZK1) showed the binding of bZIP domain to target DNA, and forming of homo-dimer of CREB1, and the solved *H. sapiens* CREB1 structure (PDB ID: 2LXT) showed the binding of KID domain to CBP. (b) Structural and sequence characteristics of *H. sapiens* CREB3, *D. melanogaster* and mosquito CREB-A. (c) Structural and sequence characteristics of *H. sapiens* CREB5. The conserved bZIP domain was denoted with orange and pKID domain with green. Red sticks indicated the residues contacting target DNA, and blue sticks indicated the residues driving parallel coiled-coil dimerization. Cyan ball in pKID indicated the classical Ser133.

CREB-A and CREB-B proteins were found, and new domains were also been introduced, such as *An. culicifacies* CREB-A contains an additional SP1–4_N superfamily domain, which is a conserved N-terminal transactivation module shared by SP1-SP4 transcription factors and essential for transcriptional regulation (Bouwman and Philipsen, 2002). These acquisitions of motifs could potentially enhance or diversify the functions of mosquito CREB proteins. However, their overall structural organization displayed highly characteristic of the CREB family, indicating strong evolutionary constraint on core structural elements. In particular, the similarity of core elements arrangement between mosquito CREB-B and human CREB1 (both containing Q1, KID, bZIP domain), and that between *Anopheles* CREBA-b and human CREB3 (both possessing TAD, bZIP domain and TM region), as well as that between *Anopheles* CREBA-a and dCREB-A (containing only TAD and bZIP domain but lacking TM region), suggests that mosquito may also share similar regulatory mechanisms with humans (Kandel, 2012; Yuxiong et al., 2023; Gao et al., 2024) and *Drosophila* (Fox et al., 2010; Johnson et al., 2020; Ho et al., 2024). Furthermore, comparative sequence analyses revealed that mosquito bZIP region closely resembled corresponding orthologous CREB proteins from *H. sapiens* and *D. melanogaster*. Notably, DNA-contacting amino acid residues were conserved across these taxa, underscoring the evolutionary conservation and potentially essential function of this domain. Meanwhile, the divergent contact residues in bZIP domains observed in mosquito CREB-A and CREB-B suggested that they may bind to different CRE sequences, thereby fine-tuning downstream transcriptional outputs such as CYP450-mediated insecticide resistance (Steven et al., 2020; Yang et al., 2020; Dong et al., 2025). Additionally, the conserved phosphorylation site such as *Ae. albopictus* CREB-B Ser172 and *An. stephensi* CREB-B Ser261, which were the analogs to human CREB1 Ser133, may serve as the central regulatory switch for CREB transcriptional activity (Chowdhury et al., 2024).

It has been demonstrated that CREB was differentially expressed across developmental stages in multiple species. In mammals, CREB expression is detected at all developmental stages, appearing in preimplantation embryos, rising during fetal development, readily measurable in pubertal tissues, and maintained throughout adulthood (Akerele and Cheema, 2020; Liu et al., 2024; Montagud-Romero et al., 2021; Wu et al., 2020). In parallel, its phosphorylation-dependent activation varies, being negligible at the embryonic stage, significantly elevated during fetal life, down-regulated during puberty, and persistently detectable in adult tissues (Akerele and Cheema, 2020; Liu et al., 2024; Montagud-Romero et al., 2021). In *Drosophila*, CREB orthologue dCREBA is expressed during embryogenesis, becoming active at zygotic genome activation to recruit CBP (Galouzis et al., 2025). Its nuclear import via the shuttle protein JIG is essential, the loss of JIG prevents dCREBA nuclear entry and causes arrest at the early 3rd-instar larval stage (Bhuiyan et al., 2023). In *Ae. aegypti*, the transcriptional repressor AaCREB and the epigenetic regulator CBP coordinately control hormonal signaling pathways essential for reproduction and development. AaCREB attenuates yolk protein precursor gene expression via the cAMP pathway and terminates vitellogenesis (Dittmer et al., 2003). Conversely, CBP functions as a juvenile hormone-dependent coactivator that upregulates Krüppel-homolog 1 through histone acetylation at its promoter, thereby suppressing ecdysone response genes and preventing premature metamorphosis (Gaddelapati et al., 2020). Our study revealed that both CREB-A and CREB-B from *Ae. albopictus* exhibited stage-specific differential expression throughout development, with their functional roles and regulatory mechanisms remaining to be characterized. Notably, the observed discrepancy between mRNA and protein expression possibly due to specific mechanism, such as

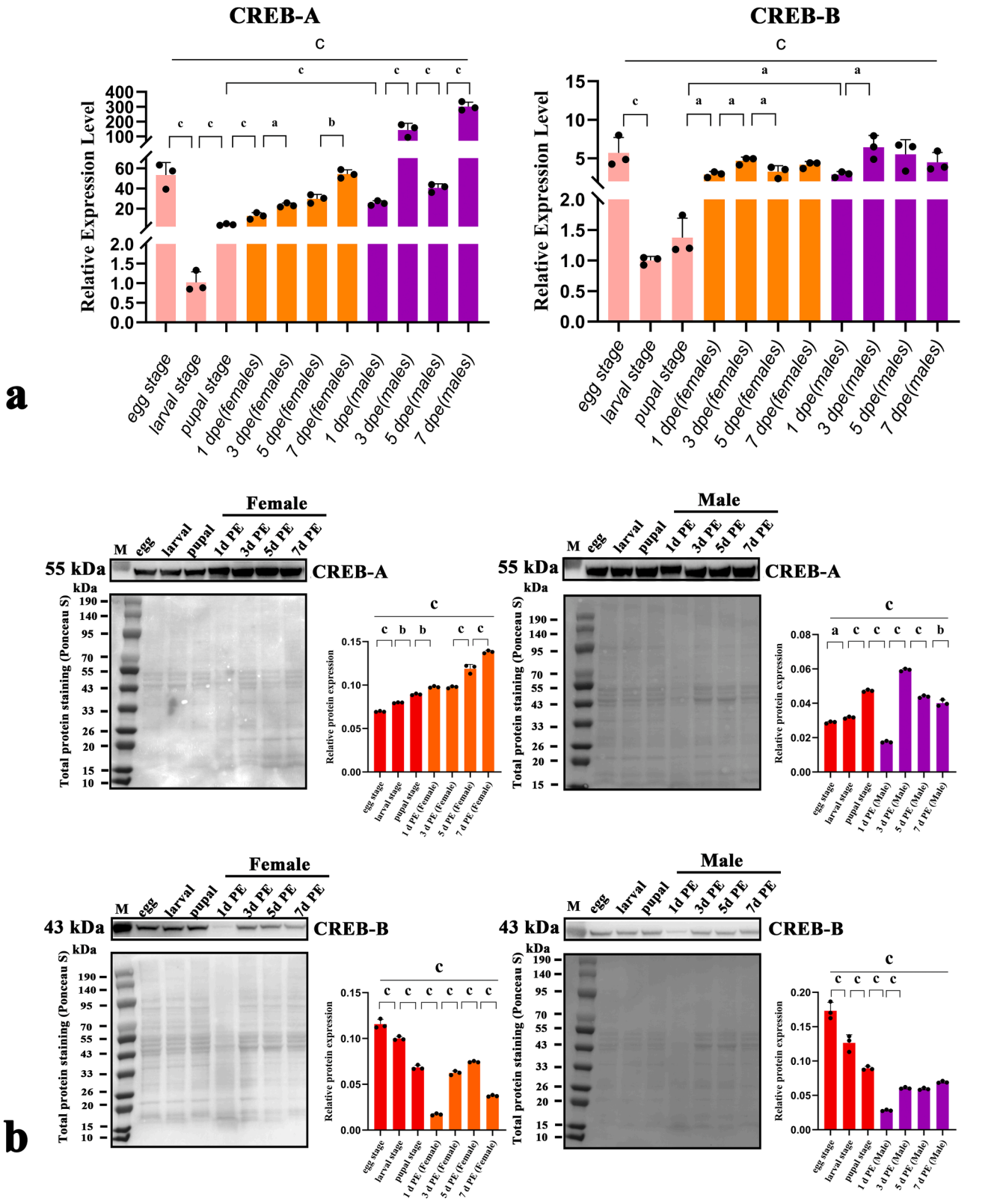

Fig. 4. Expression patterns of CREB-A and CREB-B across *Ae.albopictus* developmental stages. (a) Relative gene expression levels at egg, larval, pupal, and adult stages (1, 3, 5, and 7 days post-eclosion) of *Ae. albopictus* were quantified by RT-qPCR. Actin and RpS17 genes were used as internal reference. Expression levels were normalized to that of larvae. (b) Protein expression was analyzed by Western blotting. Samples were normalized to total protein (Ponceau S staining). Statistical differences were analyzed using a one-way ANOVA followed by Tukey test (a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$).

post-transcriptional regulation via altered mRNA stability or translational efficiency (Weerakoon et al., 2024).

This study also has some limitations. First, the CREB evolutionary analysis was mainly based on published data, and the quality of sequencing data may vary between different studies. Second, the study primarily identified and predicted CREB from *Ae. albopictus* and across different developmental stages, without conducting related functional validation studies.

In conclusion, we found that mosquitoes had CREB-A and CREB-B, which were respectively analogous to human CREB3 and CREB1. Their structural organization with core elements showed the characteristics of the CREB family, although their motifs and domains exhibited extensive lineage diversification. In particular, the observed different contact residues of the bZIP domain suggested that they may bind to different CRE sequences, thereby fine-tuning downstream transcriptional outputs. In addition, both *Ae. albopictus* CREB-A and CREB-B showed developmental stage-specific and sex-specific differential expression, which also suggested that mosquitoes may regulate their physiological activities by controlling the expression of these factors. All the findings provided a foundation for the subsequent elucidation of possible complementary roles of CREB in mosquito development and physiology.

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CRediT authorship contribution statement

Zhenyu Yue: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Xiaokai Jia:** Resources, Methodology, Investigation. **Yi Xin:** Resources, Investigation. **Ying Wang:** Resources, Methodology. **Mei Zhang:** Methodology, Investigation. **Congshan Liu:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Hua Liu:** Supervision, Project administration, Conceptualization. **Jianhai Yin:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest associated with this work.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2026.108084.

Appendix A. Supplementary data

Table S1. Amino acid sequences of CREB proteins across seven organisms

Table S2. Amino acid sequences of CREB proteins across 25 mosquito species

Table S3. Primers for RT-qPCR

Data availability

The data that supports the findings of this study are available in the supplementary material of this article.

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