Supplementary Information

Materials and Methods

Selection of genes based on microarray data and construction of promoter datasets:

In one of our previous studies, we established Differentially Expressed Gene (DEG) sets by comparing transcripts from the fat body of female *Aedes aegypti* at nine different time points post blood meal (PBM) with that of pre-blood-meal female mosquitoes (72 h post eclosion; PE) (1). Hierarchical clustering of the DEGs resulted in 12 different clusters. Out of these 12 clusters, 1, 11, and 6 were grouped as Early Genes (EGs), clusters 8, 7, and 4 as the Early Mid Genes (EMGs), cluster 5 carried the Late Mid Genes (LMGs) and clusters 2 and 3 were grouped as Late Genes (LGs). We have used genes within six different clusters viz 1 and 6 from EGs, 4 and 7 from EMGs, 5 from LMGs and 2 from LGs for this study. Cluster 2 which was the largest among all clusters was divided into two on the basis of gene expression profiles, making it a total of seven clusters. All genes in each of the seven clusters were sorted by their logFC value; ~200 top genes matching the average expression profile of each cluster were selected for further analysis. Regions 2kb upstream of the translation start sites of all selected genes were extracted from vectorbase ([https://www.vectorbase.org/](https://www.vectorbase.org/)), using an in-house R script. *Aedes aegypti* genome sequence (gene build 1.3, aegypti. SUPERCONTIGS-Liverpool.AaegL1.fa) was the source for the sequences in FASTA format. Information regarding the start sites within the gene feature file (aegypti.BASEFEATURES_Liverpool-AaegL1.3.gff3) was used for extraction of the required upstream regions. Similarly for checking evolutionary conservation, upstream sequences were also extracted from *Anopheles gambiae* (genebuild 3.7) and *Culex quinquefasciatus* (gene build 1.3).

Identification of over-represented motifs:

Two different motif detection programs, MEME (2) and Weeder (3) were used to identify the over-represented motifs. These were run locally in Biocluster maintained by Bioinformatics core facility of University of California, Riverside (UCR). The input options for MEME and Weeder were set to detect 6-9 nucleotide length motif patterns. The parameters used were as follows: Meme: -dna -mod anr -nmotifs 100 -minw 6 -maxw 9 -minsites 20 -maxsize 4000000 - maxiter 5 –revcomp; Weeder: -R 50 -O AA -W 6 -e 1 -V -S -M -T 100 (6-mer) -R 50 -O AA -W 8 -e 2 -V -S -M -T 100 (8-mer).
Test of motif enrichment within co-regulated gene clusters:

A two-step strategy was used to detect the enrichment of these motifs in different clusters; the equality of proportion test was used to confirm the overrepresentation of these motifs by comparing their occurrences in the selected promoter sequences to that in the promoter sequences of all protein-coding genes (background set). Then, the test for hypergeometric distribution was applied to check for the enrichment of these motifs in specific co-regulated gene clusters. The hypergeometric test calculated the statistical significance (p-value) of motif enrichment in R statistical computing environment (R Development Core Team, 2011). A heatmap was constructed using the p-values from the hypergeometric test between the number of motif occurrences in each cluster and that in all gene clusters.

Detection of positional and orientation bias:

The 2kb promoter regions were divided into ten bins of 200 bases pairs each. The frequency of motifs within each bin was calculated by scanning the bins in both forward and reverse directions. Test of equality of proportions was used to detect the region of positional bias for each motif. Comparing the occurrences of each motif in forward and reverse strands checked the orientation bias. Chi-square test was used to check the statistical significance of the difference between the observed and expected motifs in each strand.

Test for evolutionary conservation:

As the first step towards checking evolutionary conservation Reciprocal Best Blast or reciprocal best hit (RBH) was used to identify candidate orthologs of genes, the promoters of which carried the motifs. The RBH method is more stringent and effective in the selection of conserved orthologs with low false positive rate compared to single best hit (non-reciprocal BLAST search) (4). Once the orthologs were identified in An. gambiae and C. quinquefasciatus, their promoter sequences were extracted and aligned with MUSCLE (5) to check for evolutionary conservation of the motif. If a motif, which appeared in the Ae. aegypti gene promoter, also appeared within +/- 200 bps of the said position in the orthologous promoter sequence of either An. gambiae and/or C. quinquefasciatus, we considered it to be conserved. Up to five promoters were typically checked, allowing one base degeneracy while checking for the conservation.

JASPAR motif database search for identification of putative Transcription Factors (TFs):
The over-represented motifs were queried against the JASPAR Insecta database motifs by using TOMTOM motif comparison tool at the p-value cutoff of 0.01 (6). The default parameters of TOMTOM were used for the comparisons.

**Determination of tissue specificity:**

FlyAtlas 2 database (7) was used to check the tissue-specific expression of the transcription factors identified by JASPAR, in *Drosophila*. The TFs that were detected by JASPAR with a p-value of < 0.05 and were expressed in the fat body (FB) of *Drosophila*, were selected.

**Construction of regulatory networks:**

The regulatory networks of TFs were constructed using the GeneMANIA - a prediction server (www.genemania.org) for biological network integration and predicting gene function (8). Information related to *Drosophila melanogaster* TFs were used to construct networks with TFs identified from cluster-specific motifs. We used the 15 TFs predicted for the cluster 1 motifs to build a network. Of the interactions shown, 69.24% were predicted, 15.55% were based on co-expression, 13.60% were based on physical (8.04%) and genetic interactions (5.56%) and only 1.62% was based on co-localization (Figure S3A). Similarly, the network for the LMG cluster - Cluster 5, with 7 nodes (Figure S3B) was built mainly upon known Genetic (40.97%) and physical (33%) interactions. Shared protein domain (9.8%), Co-expression (9.61%) and Co-localization (6.62%) were other contributing factors.

**Experimental animals:**

*Ae. aegypti* mosquitoes were reared at 27°C and 80% humidity as described previously (1). The female *Ae. aegypti* were dissected 24h post eclosion (24PE) with 1% PBS buffer solution. White Leghorn chickens were used for blood feeding. All procedures were performed following the University of California, Riverside Animal care protocol.

**dsRNA synthesis and Microinjection:**

PCR were performed using Platinum Supermix (Invitrogen) for dsRNA synthesis. Primer sequences were designed by using Vectorbase sequence information. dsRNA was synthesized by using
Megascript T7 kit (Ambion). Samples were subjected to phenol/chloroform extraction and ethanol precipitation; dsRNA was then suspended in RNase-free water to a final concentration of 4 μg/μl. At 24h PE, about 0.3μl of 4 μg/μl dsRNA was injected into the thorax of each anesthetized female mosquito for RNAi-depletion. dsRNA synthesized for the luciferase gene was used for control injections (iLuc). The Picospritzer II (General Valve Corporation, Fairfield, NJ) was used to introduce corresponding dsRNAs.

**Total RNA extraction and cDNA synthesis:**

RNA was extracted from 7-10 FBs dissected from adult female mosquito by using TRIzol (Invitrogen) following manufacturer’s protocol. The cDNA was synthesized with ~2 μg of extracted total RNA by using SuperScript III Reverse Transcriptase kit (Invitrogen) with DNase I (Invitrogen) treatment.

**Quantitative Realtime Polymerase Chain Reaction (qRT-PCR) based gene expression analysis:**

qRT-PCR was conducted using iCycler iQ System (Bio-Rad) and IQ SYBR Green Supermix (Bio-Rad) following manufacturer’s protocol. The housekeeping genes, S7 ribosomal protein (RPS7) or Actin were used as reference genes for normalization. Experiments were done with triplicate samples and relative expression (RE) to RPS7 or Actin was checked for the analysis. The RE was calculated as RE=2^ΔΔCt in Microsoft Office Excel. Standard deviation was calculated in Microsoft Office Excel. p-values were computed with unpaired t-test using the online version of GraphPad (http://www.graphpad.com/quickcalcs/ttest1.cfm).

**In vitro fat body culture (IVFBC):**

A total of 9-12 FBs were dissected followed by incubation in complete medium as previously described (9). For hormonal treatments, the complete culture medium was supplemented with amino acids and 1X10^-6 M 20E (Sigma) (1). 20X10^-6 M cycloheximide (CHX, dissolved in DMSO) were added to the culture medium to access the effects of protein synthesis blocker. The controls were treated with DMSO only. The system was incubated for 8h followed by sample collections and processing for qRT-PCR based gene expression analysis.

**Chromatin immunoprecipitation (ChIP) assay:**

ChIP experiments were performed in *Drosophila* S2 cell culture system. 1Kb upstream promoter regions of genes *AAEL012037* and *AAEL002658*, harboring Ecdysone receptor response elements for repression (EcREr), were PCR amplified and cloned into Luciferase reporter vector PGL3basic (Promega)
followed by sequence confirmation (AAEL0120371Kb-Luc and AAEL0026581Kb-Luc). Simultaneously, Ae. aegypti Ecdysone receptor isoform A with a C-terminal Myc tag was sub-cloned into the pAc5.1 expression vector (Thermo Scientific) (EcRA-Myc). Transient transfections of S2 cell line were performed using FuGENE HD reagent (Promega) according to Saha et al., 2019 (10). Formaldehyde fixation of the chromatin, preparation of nuclear extract and fragmentation of protein bound DNA via sonication was done as previously described (10). Immunoprecipitation were carried out by incubating the chromatin with 3µl of anti-EcR antibody (DSHB, Iowa City, USA) for 2h. This was followed by overnight binding to 50µl pre-washed protein A agarose beads at 4°C with shaking. The beads were washed following protocol described in Saha et al., 2019 (10). Anti-IgG antibody (Sigma) was used as mock control. The immunoprecipitated chromatin was eluted with 0.1 M NaHCO₃ and 1% SDS, followed by reverse cross-linking via an overnight incubation at 65°C. DNA was extracted by phenol-chloroform method using ethanol precipitation. qRT-PCR based quantification for the enrichment of specific target regions were performed and the data was represented as % input DNA. A primer pair targeting the plasmid backbone was used as control region. The primers used are mentioned in Supplemental Table S3.

To access indirect DNA-protein interaction between AaMi2 and EcRεr, a dual crosslinking based modified protocol of chromatin immunoprecipitation was used. Ae. aegypti Mi2 with a C-terminal Flag tag was sub-cloned into the pAc5.1 expression vector (Thermo Scientific) (Mi2-Flag) and transfected into S2 cells along with EcRA-Myc, AAEL0120371Kb-Luc and AAEL0026581Kb-Luc, as required. Cells were first crosslinked for 30 mins with 5 mM dimethyl 3,3-dithiobispropionimidate-2HCl (DTBP) in PBS. Cells were then rinsed in 100 mM Tris-HCl [pH 8.0] with 150 mM NaCl followed by crosslinking with 1% formaldehyde in PBS for 10 min at room temperature (11). Subsequent procedure for ChIP was same as above. Commercially available anti-Mi2 antibody (Active Motif, USA) along with anti-EcR antibody was used for the purpose. Alternatively, anti-Flag antibody (Abcam) was used for testing the necessity of EcR in Mi2 and EcRεr association.

**Electrophoretic mobility shift assay (EMSA):**

CDS regions for Ae. aegypti EcR-A and USP-B were cloned into the pAc5.1-V5 vector (Invitrogen). Transfections were performed using Fugene HD (Promega) following standard protocols (3.0:1 ratio of transfect:DNA). Drosophila S2 cells were maintained at 26°C in Schnedier’s Drosophila medium (Life
Technologies) supplemented with 10% (vol/vol) FBS. Cells were plated in 100mm dish and the transfection with EcR-A and USP-B was performed at ~80% confluency (8.8 x 10^6 cell numbers). At 1h post transfection, 20E (20 μM) was added to the culture medium. Nuclear protein extracts from S2 cells were prepared with NE-Per Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Chino, CA) 24 h post transfection following manufacturer’s protocol. Electrophoretic mobility shift assays (EMSA) were performed using Lightshift Chemiluminescent EMSA Kit (ThermoFisher Scientific) following manufacturer’s protocol. DNA oligos were ordered from IDT with a 5’ biotin label and used at a concentration of 0.7 μM per reaction. Each reaction contained 3μl of prepared S2 cell nuclear extracts. The anti-EcR antibody, anti-USP antibody (Abcam) and anti-Rabbit IgG antibody (Abcam) were preincubated with nuclear extracts at 4°C for 1 h before the addition of biotin-labeled probes in the super shift assay. The DNA-protein complex was resolved on DNA Retardation Gel (Invitrogen).

**Co-immunoprecipitation (Co-IP):**

Cloned expression vectors EcRA-Myc and Mi2-Flag were transfected, either separately or together, to *Drosophila* S2 cells as described above. Cells were harvested after 48h incubation, and co-IP experiments were performed using Protein G Immunoprecipitation Kit (Roche), as previously described (12). IPs and Western blots were performed with various combinations of the following commercially available antibodies: anti-Myc, anti-Flag and anti-EcR. For input control, EcRA-Myc and Mi2-Flag were transfected separately or together in S2 cells and detected using WB with the antibodies mentioned above.

**Luciferase reporter assay:**

Culturing and maintenance of *Drosophila* S2 cells and Luciferase reporter assays were performed as previously described (13). Overexpression vectors EcRA-Myc and Mi2-Flag along with reporter plasmids AAEL0120371Kb-Luc and AAEL0026581Kb-Luc were utilized for the experiments, as and when required. Furthermore, the EcRr site was mutated by incorporating a restriction site in place of the EcREr in the reporter plasmids (AAEL0120371KbΔECRE-Luc and AAEL0026581KbΔECRE-Luc) to specifically analyze the contribution of the identified motif. Transient transfection assays on *Drosophila* S2 cell line were performed using FuGENE HD reagent (Promega) following manufacturer’s protocol. 100ng of desired Firefly luciferase reporter plasmid and 10ng of control Renilla luciferase reporter vector pCopia were co-transfected into S2 cells. 400ng of expression vectors EcRA-Myc and Mi2-Flag co-transfected into the cell
culture system, when desired. Total concentration of transfected expression plasmid in each well was normalized by adding empty expression vector pAc5.1. Hormonal treatment of S2 cells were performed by adding $10^{-6}$ M 20E to the culture medium. In order to negate the effect of endogenous factors in our experiments, cells were treated with dsRNA targeting DmEcR or DmMi2 in desired concentration, when required. Luciferase assay were performed using Dual Luciferase Assay Kit (Promega), as described previously (13).

**Western blotting:**

Tissue protein extractions, SDS-PAGE and immunoblotting were performed as described previously (14). Commercially available anti-Myc tag (Sigma) and anti-Flag tag (Abcam) mouse monoclonal antibodies were utilized for western blot analysis. For confirmation of knockdown of endogenous factors DmEcR and DmMi2 in the cell culture assays, anti-EcR (DSHB, Iowa City, USA) and anti-Mi2 (Active Motif, USA) antibodies were utilized. Mouse monoclonal antibody against GAPDH (Invitrogen) was used as loading control.

**Micrococcal nuclease digestion assay:**

Micrococcal nuclease (MNase) protection assays were performed as described in Chereji et al., 2015 (15), with few modifications. In brief, 100 adult female mosquitos were injected with desired dsRNA followed by blood feeding and tissue collection at 24h PBM. Dissected FBs were collected in 5 ml of homogenization buffer (60mM KCl, 15 mM NaCl, 4mM MgCl₂, 15 mM HEPES pH 7.6, 0.5 mM DTT, 0.5% Triton X-100, protease inhibitors). Homogenized tissue were supplanted with 2% of formaldehyde and incubated for 15 min at room temperature for chromatin cross-linking, followed by 1M glycine (to a final concentration of mM) treatment to stop the reaction. Nuclei and subsequent chromatin collections were done as described in Petesch et al. (2008) (16). The pelleted chromatin was washed twice (Wash buffer: 25% glycerol, 5 mM Mg Acetate, 50 mM Tris pH 8.0, 0.1 mM EDTA, 5 mM DTT) followed by re-suspension in MNase digestion buffer (Buffer MN: 60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 0.25 M sucrose, 1.0 mM CaCl₂). The extract was divided in two equal fraction; while one half was used as undigested control and other half was subjected to MNase digestion using 100U of the enzyme (NEB) dissolved in MN buffer (17). After a 30 mins room temperature incubation the reaction was stopped by adding EDTA and SDS to final concentrations of 12.5 mM and 0.5%, respectively. Digested chromatin were
then collected by centrifuging at 14,000 rpm (16,000 g) for 10 min at 4°C in a microcentrifuge dissolved in 250 µl elution buffer (0.1 M NaHCO3 and 1% SDS). After de-crosslinking, DNA was extracted by phenol-chloroform method and subjected to qRT-PCR based enrichment analysis. Fold difference was calculated by comparing MNase treated samples in comparison to undigested DNA.

References for materials and methods

1. S. Roy, et al., Regulation of Gene Expression Patterns in Mosquito Reproduction. *PLoS Genetics* **11**, 1–23 (2015).
2. T. L. Bailey, et al., MEME Suite: Tools for motif discovery and searching. *Nucleic Acids Research* **37**, 202–208 (2009).
3. G. Pavesi, P. Mereghetti, G. Mauri, G. Pesole, Weeder web: Discovery of transcription factor binding sites in a set of sequences from co-regulated genes. *Nucleic Acids Research* **32**, 199–203 (2004).
4. Y. Xu, JP Gogarten, *Computational Methods for Understanding Bacterial and Archaeal Genomes*, Y. Xu, JP Gogarten, Ed. (Imperial College Press, 2008).
5. R. C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797 (2004).
6. S. Gupta, J. A. Stamatoyannopoulos, T. L. Bailey, W. S. Noble, Quantifying similarity between motifs. *Genome Biology* **8** (2007).
7. S. W. Robinson, P. Herzyk, J. A. T. Dow, D. P. Leader, FlyAtlas: Database of gene expression in the tissues of Drosophila melanogaster. *Nucleic Acids Research* **41**, 744–750 (2013).
8. D. Warde-Farley, et al., The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Research* **38**, 214–220 (2010).
9. Z. Zou, et al., Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **110** (2013).
10. T. T. Saha, et al., Synergistic action of the transcription factors Krüppel homolog 1 and Hairy in juvenile hormone/Methoprene-tolerant-mediated gene-repression in the mosquito Aedes aegypti.
11. N. Fujita, P. A. Wade, Use of bifunctional cross-linking reagents in mapping genomic distribution of chromatin remodeling complexes. *Methods* **33**, 81–85 (2004).

12. S. W. Shin, Z. Zou, T. T. Saha, A. S. Raikhel, bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 16576–16581 (2012).

13. T. T. Saha, *et al.*, Hairy and Groucho mediate the action of juvenile hormone receptor Methoprene-tolerant in gene repression. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E735–E743 (2016).

14. X. Wang, *et al.*, Hormone and receptor interplay in the regulation of mosquito lipid metabolism. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E2709–E2718 (2017).

15. Ř. V. Chereji, *et al.*, Genome-wide profiling of nucleosome sensitivity and chromatin accessibility in Drosophila melanogaster. *Nucleic Acids Research* **44**, 1036–1051 (2016).

16. S. J. Petesch, J. T. Lis, Rapid, Transcription-Independent Loss of Nucleosomes over a Large Chromatin Domain at Hsp70 Loci. *Cell* **134**, 74–84 (2008).

17. L. M. Li, D. N. Arnosti, Fine mapping of chromatin structure in Drosophila melanogaster embryos using micrococcal nuclease. *Fly* **4**, 213–215 (2010).
Fig S1A. Expression profiles of Early, Early-mid, and Late-mid gene clusters (modified from Roy et al, 2015 which is licensed under CC BY [URL: https://creativecommons.org/licenses/by/4.0/]): 20E titer (ngs/gm) shown as dashed black line. Average expression (Log2 fold change) of the Early genes (EGs) Early-mid gene (EMGs) and Late-mid gene (LMGs) are shown as solid lines. While Early-mid genes are upregulated at the high titer of 20E, Early and Late-mid genes are downregulated during the same period.

Fig S1B & S1C: Genome-wide analysis of lengths of intergenic regions and 5' UTRs in the Ae. aegypti: (A) The length of the intergenic region between Ae. aegypti genes plotted in 2Kb bins from less than 2Kb up to 20Kb shows that the intergenic regions of most genes are less than 2Kb. (B) The length of the 5' UTRs of Ae. aegypti genes plotted in 50b bins from less than 50b up to 1Kb shows that in most cases the 5' UTRs are less than 200b.
Fig S2. Flowchart for motif finding pipeline: 2Kb upstream regions of ~200 co-expressed genes in each cluster were extracted and used as an input for the motif finding programs MEME and Weeder. The motifs that were commonly identified by both programs were subjected to the tests for positional bias, orientation bias and evolutionary conservation. These motifs were then used as inputs for the JASPAR database to identify the putative TFs that can bind to these motifs. The TFs corresponding to those motifs that showed positive results in two out of the three bioinformatics tests were used to build cluster specific networks using GeneMANIA.
**Fig S3 A-B. Putative regulatory network diagrams of TFs identified within EG and LMG clusters:** (A) Network of TFs related to TFBSs detected within the CRRs of Early (Clusters 1) and (B) Late-mid (Cluster 5) and Late (Clusters 2A and 2B) respectively, built with GeneMANIA. GeneMANIA color code - blue: Genetic interactions; green: Predicted; red: Physical interactions; yellow: Co-expression; purple: Shared protein domains; pink: Co-localization.

**Fig S3 C-D. Gene expression profiles compared to the average expression of the group:** (C) Expression profiles of EGs AAEL008222 and AAEL014526 compared to the average expression profiles of all EGs. 0 hrs post blood meal represent 72 hr post eclosion (PE). (D) Expression profiles of LMGs AAEL012307 and AAEL002658 compared to the average expression profiles of all LMGs. 0 hrs post blood meal represent 72 hr post eclosion (PE).
Fig. S4: Indirect repression of the EG pancreatic lipase-related protein 2 (AAEL008222) by 20E through EcR, Br and E74: (A-C) RNAi depletion of E74, Br, and EcR results in the upregulation of the AAEL008222 transcript indicating a downregulation of the transcript by 20E through EcR, Br, and E74. All relative expressions calculated against housekeeping gene RPS7. Injecting double stranded RNA for the Luciferase gene (iLuc) served as the control for all RNAi experiments. (D) Addition of CHX compromised the 20E mediated repression AAEL008222, indicating that protein synthesis is required in 20E mediated repression. All relative expressions calculated against housekeeping gene Actin. (E) Relative expression of AAEL008222 in tissues subjected to IVFBC in the culture media supplemented with amino acids without (iLuc) and with 20E (iLuc+20E) showed a downregulation of the AAEL008222 transcript with the addition of 20E. RNAi Depletion of the E74 results in a rescue of the repressive effects of 20E on the AAEL008222 transcript. (F) RNAi depletion of E74A isoform had no significant effect on the AAEL008222 transcript indicating that E74A had no role in the downregulation of this transcript. (G) Relative expression of AAEL008222 in tissues subjected to IVFBC in the culture media supplemented with amino acids, without (iLuc) and with 20E (iLuc+20E) showed a downregulation of the AAEL008222 transcript with the addition of 20E. RNAi Depletion of the E74B isoform resulted in a rescue of the repressive effects of 20E on the AAEL008222 transcript. (A-G) All data is representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, * p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001. Injecting ds RNA for the Luciferase gene (iLuc) served as the control.
Fig S5. Direct repression of EcR target gene *amp dependent ligase (AAEL002658)* by 20E: (A) RNAi depletion of EcR results in upregulation of the AAEL002658 transcript, indicating a downregulation of the transcript by 20E through EcR. Relative expression detected by qRT-PCR, in FB tissue collected from female mosquitoes after knock-down of the EcR (iEcR). Injecting double stranded RNA for the Luciferase gene (iLuc) served as the control in the RNAi experiments. Expressions calculated against housekeeping gene RPS7. (B) Addition of Cycloheximide (CHX) does not compromise the 20E mediated repression of target gene *AAEL002658*, indicating that protein synthesis is not required in 20E mediated repression. Expressions calculated against housekeeping gene Actin. (A-B) Data representative of three biological replicates, with three technical replicates and are illustrated as average ± SD, **p-value < 0.01; ***p-value<0.001.
Fig S6. EcR interacts with a unique EcREr (TTGATTGA motif): (A-B) ChIP assays demonstrating the binding of EcR protein to specific EcREr DNA elements (A) in the promoter of target gene (B) only in the presence of 20E. The promoter of target gene AAEL002658 was cloned (AAEL0026581kb-Luc) and transfected into Drosophila S2 cells along with plasmids overexpressing EcR-A (EcRA−Myc). The endogenous DmEcR was knocked down by RNAi. ChIPs were performed with anti-EcR antibody (DSHB, Iowa City, USA). anti-IgG antibody was used as mock control. Quantifications were performed by qRT-PCR using primer pairs targeting EcREr regions in the target gene promoters. Primers targeting the plasmid backbone were utilized as controls. Data was represented as % of input DNA. Error bars represent ± SD. (C) Electrophoretic mobility shift assay showing specific binding of the biotin labeled probe (synthesized DNA oligo carrying the unique EcR binding TTGATTGA motif and its flanking regions from the promoter of the EcR target gene AAEL002658) to the nuclear extract (prepared after overexpressing EcR-A) from Drosophila S2 cells. Column 1 – Probe Only; Column 2 – Labelled Probe; Column 3 – 30x Unlabeled specific Probe; Column 4 – 100x unlabeled specific probe; Column 5 – Drosophila EcR Antibody; Column 6 – Drosophila USP Antibody; Column 7 – Both AB; Column 8 – IgG (non-specific) Rabbit Antibody. NSB stands for non-specific binding; FP signifies free probe. Binding was detected (Column 2) with labelled probe and nuclear protein extracts from S2 cells. Competition with increasing amounts (30x - Column 3 and 100X - Column 4) of the unlabeled specific probe confirmed the binding specificity. The specific band disappeared with the addition of anti-EcR antibody without (Column 5) or with the addition of anti-USP antibody (Column 7). Addition of anti-USP antibody by itself had no effect on the specific binding (Column 6). Similarly, addition of IgG (non-specific; Column 8) Rabbit antibody had no effect on the binding. (D) EMSA with mutated TTGATTGA motif (EcREr), but intact flanking regions from the CRR of the EcR target gene AAEL012037 and nuclear extract (prepared after overexpressing EcR) from Drosophila S2 cells confirming the specificity of the EcREr; Column 1 – Labelled probe only (without nuclear extract); Column 2 – Labelled Probe; Column 3 – 100x Unlabeled specific Probe; Column 4 – Labelled mutated probe 1; Column 5 – Labelled mutated probe 2.
Fig. S7. USP is not involved in 20E-EcR mediated gene repression: dsRNA targeting USP-A, USP-B or USP core region (USP-core) did not alter the expression of target genes, AAEL002658 and AAEL012037, demonstrated to be repressed via 20E-EcR action. iLuc was used as a control. Gene expression analysis was performed by qRT-PCR. All experiments were performed in triplicates with similar results. Error bars represent ± SD.
Fig. S8. AaMi2 is a potential co-repressor of EcR in 20E mediated gene repression: (A) Repression of the 20E-EcR target gene AAEL002658 is compromised by the dsRNA mediated knockdown of AaMi2, indicating its possible role as a EcR co-repressor in the 20E gene repression pathway. **p-value<0.01, ***p-value<0.001. (B) RNAi and IVFBC tandem experiments showing a loss of the repressive function of the hormone 20E on tissue samples obtained from iEcR and iMi2 mosquitoes. A clear repression in the target gene AAEL002658 was observed in control iLuc samples when treated with 20E. (A-B) Gene expression analysis were performed qRT-PCR. Error bars represent ± SD. All experiments were performed in triplicates with similar results.
Fig. S9. ChIP assays demonstrated the indirect association of AaMi2 with EcREr in the promoter region of 20E-EcR target gene AAEL002658: (A) The constructed plasmid AAEL002658_{1kb}-Luc, harboring promoter EcREr binding site was transected into Drosophila S2 cells along with EcRA-Myc and Mi2-Flag. Immunoprecipitations were performed with Drosophila anti-EcR and anti-Mi2 antibodies. anti-IgG antibody was used as mock control. ChIP with anti-Mi2 antibody shows a significant enrichment for EcREr region. (B) ChIP assays performed with constructed plasmid AAEL002658_{1kb}-Luc and either both EcRA-Myc and Mi2-Flag (EcRA-Myc+/Mi2-Flag+) or Mi2-Flag only (EcRA-Myc-/Mi2-Flag+), demonstrating the need for functional EcR protein for the association of Mi2 with EcREr elements. Cells devoid of both EcRA-Myc and Mi2-Flag (EcRA-Myc-/Mi2-Flag-) were used as controls. Immunoprecipitations were performed with commercially available anti-Flag antibodies. Endogenous DmEcR and DmMi2 were knocked down using corresponding dsRNAs. (A-B) Quantifications were performed by qRT-PCR using primer pairs targeting the EcREr regions in the target gene promoter. Primers targeting the plasmid backbone were utilized as controls. Data was represented as % of input DNA. Error bars represent ± SD.
Fig. S10. 20E represses the reporter activity of the target gene **AAEL012037** promoter: (A) Reporter plasmid **AAEL012037**<sub>1kb</sub>-Luc was transected into *Drosophila* S2 cells. A titration for the hormone was performed by supplementing the culture medium with 10⁻⁶ M, 10⁻⁷ M, 10⁻⁸ M and 10⁻⁹ M 20E. (B) The effect of overexpression of ecdysone receptor on the repression of luciferase activity under the control of target gene promoter AAEL012037<sub>1kb</sub>-Luc. The endogenous ecdysone receptor (*DmEcR*) was silenced by RNAi. Cloned overexpression vector EcRA-Myc was transected to *Drosophila* S2 cells at 50, 100, 200 and 400 ng. All cells were treated with 10⁻⁶ M 20E. (C) Western blots showing the expression of tagged proteins EcRA-Myc and Mi2-Flag. Commercially available anti-Myc (Sigma) and anti-Flag (Abcam) antibodies were used for the purpose. Anti-GAPDH antibody (Invitrogen) was used as an internal control. (D) Western blots showing the efficacy of dsRNA mediated knockdown of endogenous *DmEcR* and *DmMi2* proteins in S2 cells. Anti-GAPDH antibody (Invitrogen) was used as an internal control.
**Fig. S11. Functional analysis of 20E/EcR/Mi2 repressor complex:** (A) Luciferase reporter assays demonstrating direct repression by 20E/EcR through target gene promoter harboring EcREr. *Drosophila* S2 cells were co-transfected with expression vector EcRA–Myc and reporter plasmid AAEL002658<sub>1Kb</sub>-Luc, along with 20E supplementation of culture medium when required. Reporter plasmid with a mutated EcREr (AAEL002658<sub>1Kb</sub>ΔEcREr-Luc) was also used to demonstrate the necessity of the DNA element in trans-repression of reporter gene. (B) The putative EcR binding site along with flanking regions harbored within 1Kb region of the AAEL002658 gene promoter and the mutated sequences utilized in this experiment are indicated. (C) Luciferase reporter assay demonstrating the necessity of the functional AaMi2 protein for 20E/EcR mediated repression of target gene AAEL002658. Expression vector EcRA-Myc was co-transfected with the reporter plasmid AAEL002658<sub>1Kb</sub>-Luc into *Drosophila* S2 cells. Hormonal treatments with 10<sup>-6</sup> M 20E resulted in clear repression of luciferase activity. dsRNA-mediated
Fig. S12. (A-B) Cell culture based ChIP assays demonstrating EcR-B does not interact with the identified EcRer DNA elements in the CRRs of target genes AAEL012037 and AAEL002658. EcRB-V5 and either AAEL012037_{1Kb}-Luc or AAEL002658_{1Kb}-Luc was transfected into S2 cells. ChIPs were performed with anti-EcR antibody. anti-IgG antibody was used as mock control. Endogenous DmEcR was knocked down using dsRNA. Data was represented as % of input DNA. Error bars represent ± SD. (C-D) Luciferase reporter assays demonstrating that EcR-B does not alter the reporter activity in the presence of 20E. S2 cells were co-transfected with expression vector EcRB–V5 and reporter plasmids AAEL012037_{1Kb}-Luc or AAEL002658_{1Kb}-Luc, along with 20E supplementation of culture medium when required. DmEcR was knocked down via RNAi in all samples. Error bars represent ± SD.
Table S1: List of overrepresented motifs

| Early gene (EG) clusters | Early-mid gene (EMG) clusters | Late-mid gene (EMG) cluster | Late gene (LG) clusters |
|--------------------------|-------------------------------|-----------------------------|------------------------|
| Cluster 1 motifs         | Cluster 4 motifs              | Cluster 5 motifs             | Cluster 2A motifs      |
| CATA[CG][AT]AAA          | AGC[AGT][A][AG][AC]           | ATC[GT]CTCGC                | CA[AC][G][AG][AA]     |
| G[CG]GTTATGA             | CTG[T][CG][AA]               | [AT]CTGACTG                 | G[GC][G][GC][CTAAC]   |
| CAC[ACG][C][AG][CCC]     | AAA[AT]ATGGGG                | TTGATTGA                    | AAAAG[CG][GCC]        |
| GAA[AC][T][CT]GTCA       | GAC[TA][TG][CCC]             | G[CT]AACGGGAA               | GCCG[GT][A][AC][GCC]  |
| [AG][GTC][A][AT]GGA      | CTCT[G][GT][AT][GC]          | AA[CA][TG][GT][TG]         | ACCCTCA[ACGT][A][CT]  |
| GCC[AG][CG][G][AC][C]    | CAG[AT][AC][G][CTG]          | AC[GC][AT][G]AGCA          | AAAA[AG][AC][TCA]     |
| CA[AC][AT]GCGG           | GG[AT][AC][G][CAC]           | ACTCA[CG][TC]               | AT[AGT][CCGA][AC]     |
| C[AGT][C][AG][GGAAG]     | CAAGA[GC][C][AC]             |                             | GCGA[TA][TCC]         |
| GCAG[AC][G][CA][A]       | [CT]AA[T][AG][AAA]           |                             | CGT[GT][ATC][GGCA]    |
| T[CT][AC][CG][GC]        | TGCC[AC][AC][AAA]            |                             | CG[CT][GT][TG][GGCA]  |
| GAAA[CT][A][AC][CA]      | GAGGA[AC][T][AA]             |                             | CG[CC][GC][GC][CT][G] |
| [ACT][ACGT][C][A][T][A]  | AT[AG][AC][T][G][CT][CA]     | AA[AAC][AG][CATG]           |                        |
| AA[AC][CAA][AAC]         | ATC[TA][G][CG][TC]           |                             |                        |
| ATCCAGT[AG]              | AAAG[AG][AT][G][AAA]         |                             |                        |
| [AC][TG][AG][GCC]        | AAAG[AG][AT][G][AAA]         |                             |                        |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |
| Cluster 6 motifs         | Cluster 7 motifs              | Cluster 2B motifs           |
| CA[AG][AGC][T][CA]       | GCC[CG][T][AC][AG][CA]       | CA[AG][A][AC][TT][G]       |
| CG[AT][AT][CC][CA]       | T[CA][CT][TG][CG]            | GA[AC][T][CA][AG][T]       |
| CAAAAT[AGT][A][G]        | AAAAG[AG][A][AG][A]          | AA[AAC][AG][CA][AG][T]     |
| GCCA[AT][A][AA][CT]      | TCAC[AC][G][T][GAA]          | AAA[AG][T][G][C][G][A]     |
| CGAC[GA][C][G][CT][C]    | AT[CA][C][GA][G][AC]         | AAA[AC][AG][G][AC][GGG]    |

---

**Early gene (EG) clusters**: Early gene clusters

**Early-mid gene (EMG) clusters**: Early-mid gene clusters

**Late-mid gene (EMG) cluster**: Late-mid gene cluster

**Late gene (LG) clusters**: Late gene clusters

**Cluster 1 motifs**: Cluster 1 motifs

**Cluster 4 motifs**: Cluster 4 motifs

**Cluster 5 motifs**: Cluster 5 motifs

**Cluster 2A motifs**: Cluster 2A motifs

**Cluster 6 motifs**: Cluster 6 motifs

**Cluster 7 motifs**: Cluster 7 motifs

**Cluster 2B motifs**: Cluster 2B motifs
Table S2: Data for construction of gene regulatory networks

| Cluster | Co-expression | Physical interactions | Genetic interactions | Shared protein domains | Co-localization | predicted interaction |
|---------|--------------|-----------------------|----------------------|------------------------|-----------------|-----------------------|
| EGs     | 1            | 13.60%                | 8.04%                | 5.56%                  | 0.00%           | 1.62%                 | 69.24%               |
|         | 6            | 28.57%                | 19.66%               | 30.58%                 | 1.93%           | 0.00%                 | 27.11%               |
| LMGs    | 5            | 9.61%                 | 33%                  | 40.97%                 | 9.80%           | 6.62%                 | 0.00%                |
## Table S3: List of Primers Used

### Primers for RNAi

| Organism                  | Target_ID          | Direction | Primer Sequence                                      | Descriptors |
|---------------------------|--------------------|-----------|------------------------------------------------------|-------------|
| **Aedes aegypti**         | XM_021851078.1     | Forward   | TAATACGACTCACTATAGGGAGGACAGCATCGTGTTG               | Mi-2        |
|                           |                    | Reverse   | TAATACGACTCACTATAGGGAGGACAGCATCGTGTTG               |             |
|                           | AAEL019431         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | EcR         |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL022402         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | HR38        |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL005730         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Alien       |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | XM_021853263.1     | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | SMRTER      |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000395-RA      | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Usp-A       |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000395-RB      | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Usp-B       |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000395         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Usp_Core    |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL008426         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Br          |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000741         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | E74         |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000741         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | E74A        |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | AAEL000741         | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | E74B        |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
| **Drosophila melanogaster** | FBgn0262519       | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | DMI-2       |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |
|                           | FBgn00000546       | Forward   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              | Dmel/EcR    |
|                           |                    | Reverse   | TAATACGACTCAGTATAGGGAGGACAGCATCGTGTTG              |             |

### Primers for Q-PCR

| Organism                  | Target_ID  | Direction | Primer Sequence                                      | Descriptors                  |
|---------------------------|------------|-----------|------------------------------------------------------|------------------------------|
| **Aedes aegypti**         | AAEL014526 | Forward   | ATCCTGGTTCTGCGGTCTTCG                                 | Sideroflexin-1               |
|                           |            | Reverse   | GTAGACGACCTCCGCTGC                                   |                             |
|                           | AAEL008222 | Forward   | GGGTGGCTGCTAGTTAGTC                                   | Pancreatic lipase-related    |
|                           |            | Reverse   | GAGGCATCGGTAATCGCAAG                                  | protein 2                    |
|                           | AAEL002658 | Forward   | TGGTACCCTCTGCTACATAGGC                                | AMP dependent ligase         |
|                           |            | Reverse   | GCCGTCCAGAGACAAACACTTT                                |                             |
|                           | AAEL012037-RA | Forward | GGAATCACCTCGCCGAGCAAA                                 | sulphate transporter         |
|                           |            | Reverse   | GCCGTAATCCGCTCCGCTG                                  |                             |
### Primers for Luciferase assay

| Organism       | Target_ID | Direction | Primer Sequence                           | Descriptors  |
|----------------|-----------|-----------|------------------------------------------|--------------|
| Aedes aegypti  | AAEL002658-RA | Forward  | ATGCCTAGCTGAATGGCTTTTTCTTGTGTCA          | AAEL0026581KB-Luc |
|                |           | Reverse  | ATGCAAGCTTTTCATCGCAACTCGACAATGTG        |              |
|                | AAEL002658-RA | Forward  | ATATCTCGAGTCCAGTGCTAGTAAATTAG          | AAEL0026581KB∆EcRE-Luc |
|                |           | Reverse  | ATATCATATGTAGCGAAGACTGCCTCACTA         |              |
|                | AAEL012037-RA | Forward  | ATATCTCGAGTCCAGCAAAGACGACAGGAAGAGG    | AAEL0120371KB-Luc |
|                |           | Reverse  | ATATCATATGCGACCATGAAGCTGAGAGTG         |              |
|                | XM_021851078.1 | Forward | ATATACGCGCTACTGTGCTTTTCTTGTGTCA       | Mi-2-Flag    |
|                |           | Reverse  | ATATACGCGCTACTGTGCTTTTCTTGTGTCA       |              |
|                | AAEL019431-RA | Forward  | GCGCGGCAATTCCGCTTGGAGCAGGAGAAG       | EcR-Myc     |
|                |           | Reverse  | GCGCGGCAATTCCGCTTGGAGCAGGAGAAG       |              |
|                | AAEL019431-RB | Forward | GCGCGGCAATTCCGCTTGGAGCAGGAGAAG       | EcR-V5      |
|                |           | Reverse  | GCGCGGCAATTCCGCTTGGAGCAGGAGAAG       |              |
### Primers for Chip

| Organism       | Target_ID   | Direction | Primer Sequence                     | Descriptors                      |
|----------------|-------------|-----------|-------------------------------------|----------------------------------|
| *Aedes aegypti*| AAEL002658-RA| Forward   | TGGCTGTTTTCTTGTGTCATTT              | AMP dependent ligase             |
|                |             | Reverse   | TGTATCGAAACACATTCAATGCAC             |                                   |
|                | AAEL012037-RA| Forward   | CCTATGAACCGCTATTGCTCG               | sulphate transporter             |
|                |             | Reverse   | AGTCATCGATGAGGCCAG                  |                                   |
| pGL3 Basic     |             | Forward   | TGGGCTGAATACAAATCAC                 | Primers targeting the plasmid backbone (control) |
|                |             | Reverse   | CAACTCCGATAAAATAACGCG              |                                   |

### Primers for EMSA

| Organism       | Target_ID   | Direction | Primer Sequence                     | Descriptors          |
|----------------|-------------|-----------|-------------------------------------|----------------------|
| *Aedes aegypti*| AAEL002658-RA| Forward   | GTTTTAAATATTGATGATCCAGTAGCTT        | AMP dependent ligase |
|                |             | Reverse   | AAGCAGTGGATCAATCAATATTGGAC          |                      |
|                |             | Forward   | /5BiosG/GTTCAAAATATTGATGATCCAGTAGCTT| Biotin labelled      |
|                | AAEL012037-RA| Forward   | TTCATGCTGTTGATGATAGCGAAGAC          | sulphate transporter  |
|                |             | Reverse   | GTCTTCGCTATCAATCAACGACCATTG         |                      |
|                |             | Forward   | /5BiosG/TTTCAATGCTGTTGATGATCCAGAAC | Biotin labelled      |
|                |             | Mutated 1 | /5BiosG/TTTCAATGCTGTTCAATCACAGAAC  | Biotin labelled      |
|                |             | Reverse   | TCTTCGCTATGGAATACGACCAGTCAGAAGAC   |                      |
|                |             | Mutated 2 | /5BiosG/TTTCAATGCTGCTGAGCGAGAGAC   | Biotin labelled      |
|                |             | Reverse   | GTCTTCGCTATGCGTGCGGACCATGAA        |                      |

### Primers for Mnase Protection

| Organism       | Target_ID   | Direction | Primer Sequence                     | Descriptors          |
|----------------|-------------|-----------|-------------------------------------|----------------------|
| *Aedes aegypti*| AAEL012037-RA| Forward   | TTTTTGCGCTGCTCAATTATGTCT             | sulphate transporter (A) |
|                |             | Reverse   | ACGTCGTTACTGCTGTGGTTCTA             |                      |
|                | AAEL012037-RA| Forward   | AGTAGGGGAAAGGCACTATCCA              | sulphate transporter (B) |
|                |             | Reverse   | CACCCTTCTACTGCGTCATGACTG            |                      |
|                | AAEL012037-RA| Forward   | CCCCTATGAACGCTATTTGCTC              | sulphate transporter (C) |
|                |             | Reverse   | GCTGATATGAGCCTGCTGCG               |                      |
|                | AAEL012037-RA| Forward   | CGGAACGTGCTACTCGCCCT               | sulphate transporter (D) |
|                |             | Reverse   | CGCCGATATGCTCGAGAGGT               |                      |
|                | AAEL012037-RA| Forward   | CCTCTGAATGGAAACGAGCC                | sulphate transporter (E) |
|                |             | Reverse   | CGTAGTAGGCTCGGCGGCTGCT             |                      |