

Research Paper

Functional characterization of the dual allatostatin-A receptors in mosquitoes

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ABSTRACT

The neuropeptide allatostatin-A (AstA) and its cognate receptors (AstARs) are involved in the modulation of feeding behavior, which in hematophagous insects includes the regulation of the disease vector-related behaviors, host seeking and blood feeding. In mosquitoes and other dipterans, there are two copies of AstAR, contrasting with the single copy found in other insects. In this study, we identified and cloned the dual AstAR system of two important disease vectors *Aedes aegypti* and *Culex quinquefasciatus*, and compared them with those previously described, including those in *Anopheles coluzzii* and *Drosophila melanogaster*. Phylogenetic analysis of the AstARs revealed that the mosquito AstAR1s has retained a similar amino acid sequence as the AstARs from non-dipteran insect species. Intron analysis revealed that the number of introns accumulated in the AstAR2s is similar to that in other insects, and that introns are conserved within the receptor types, but that only the final two introns are conserved across AstAR1s and 2s. We functionally characterized the dual AstARs in *An. coluzzii*, *Ae. aegypti* and *Cx. quinquefasciatus* by stably expressing the receptors in a Chinese hamster oocyte cell line (CHO) also stably expressing a promiscuous G-protein (G16), and challenged them with the endogenous isoforms of AstA from the three mosquito species. In the culicine mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*, the AstARs demonstrated differential sensitivity to AstA, with the AstAR2s displaying a higher sensitivity than the AstAR1s, suggesting a divergence of functional roles for these AstARs. In contrast, both *An. coluzzii* AstARs demonstrated a similar sensitivity to the AstA ligands. We discuss our findings in the light of AstA acting as a regulator of blood feeding in mosquitoes. A better understanding of the regulation of host seeking and blood feeding in vector mosquitoes will lead to the rational development of novel approaches for vector control.

1. Introduction

Allatostatin-A (AstA) or FGLamide neuropeptides are involved in various physiological and behavioral processes, including the neural control and modulation of feeding in insects [1,2]. In *Drosophila melanogaster*, activation of AstA-expressing neurons increases the preference for a protein-rich diet and decreases the responsiveness to sugar [3,4], and in sugar starved flies, AstA neuron activation decreases sleep-like behavior [5]. In addition, inactivation of AstA increases food intake [3–5], whereas RNAi knockdown of its receptor reduces foraging behavior [6]. Moreover, AstA has significant effects on ion transport and gut motility [7], which is attributed, at least in part, to its expression in gut endocrine cells [8,9]. Taken together, this suggests that AstA

signaling is directly involved in regulating a digestive energy-saving state in flies.

The regulation of feeding through effects on foraging, food intake, gut motility and digestive enzyme release appears to be conserved in a broad range of insects, including mosquitoes (reviewed e.g. by [10–15]). Recent semi-quantitative mass spectrometric analysis in the yellow fever mosquito, *Aedes aegypti* [16] and transcriptome together with physiological analyses in the kissing bug, *Rhodnius prolixus* [17,18] implies that the function of AstA may also be conserved in blood feeding insects. Feeding to completion on protein-rich blood in mosquitoes and *R. prolixus* induces a transient inhibition of the odor-mediated host seeking behavior [19–21]. Coinciding with the peak of this behavioral inhibition, the AstA neuropeptide level in the primary

Abbreviations: AstA, allatostatin-A; N-terminus, amino-terminus; AstAR, AstA receptor; C-terminus, carboxy-terminus; cDNA, complementary deoxyribonucleic acid; CHO, Chinese hamster oocyte; ECL, extracellular loop; EC₅₀, Half maximal effective concentration; ICL, intracellular loop; PCR, Polymerase chain reaction; TM, transmembrane

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olfactory center, the antennal lobes, of *Ae. aegypti* changes and systemic injection of AstA reduces host-seeking [16]. Similarly, there is a reduction in the abundance of AstA transcripts in the central nervous system of *R. prolixus*, shortly after blood feeding [17], correlating with the reduction in foraging for a protein-rich food source. Blood feeding, however, does not seem to affect the AstA receptor transcript levels in the heads of neither *Ae. aegypti* [22] nor the malaria mosquito, *Anopheles coluzzii* [23]. To gain further insight into the putatively retained regulatory function of AstA, we here analyze the evolution of AstA receptors (AstARs) and their peptide ligands in *Ae. aegypti*, *An. coluzzii* and the southern house mosquito, *Culex quinquefasciatus*, as well as determine whether evolution has modified receptor function.

The AstA peptides in insects are cleaved from a common precursor peptide encoded by a single gene [17,23–27]. The number of neuropeptide isoforms encoded by the precursor is, however, highly variable between species [24]. Of the five peptides identified in *Ae. aegypti*, *An. coluzzii* and *Cx. quinquefasciatus*, only AstA-2 shares a high amino acid identity with the AstA-2 described in *D. melanogaster* [23,24]. The remaining peptides, besides sharing the conserved C-terminal Y/FXFGI/I-amide motif, generally differ in length and show amino acid substitutions across species [24].

Allatostatin-A receptors were first identified and functionally characterized in *D. melanogaster* (Drome-AstAR1 and Drome-AstAR2; [28–31]). While not unique, a characteristic feature of flies and mosquitoes is the existence of two AstAR genes [32,33]. The sequences of these genes in *Ae. aegypti* (*Aedae-AstAR1* and 2), *An. coluzzii* (*Anoco-AstAR1* and 2), and *Cx. quinquefasciatus* (*Culqu-AstAR1* and 2), were first identified by Félix et al., who found *Aedae-AstAR1* and *Culqu-AstAR2* to be pseudogenes. To date, AstARs have also been functionally characterized in *An. coluzzii* [23], *R. prolixus* [34], the moth *Bombyx mori* [35], the honey bee *Apis mellifera* [36], as well as in the cockroaches, *Periplaneta americana* [37,38] and *Diploptera punctata* [39,40]. Together, these studies show that the conserved C-terminus Y/FXFGI/I-amide of the AstA peptide is sufficient for AstAR activation, and that the FXGLF motif generally has the highest affinity in most species [2]. Moreover, AstARs are activated by the synthetic pentapeptide YDFGL-amide, but with a lower affinity compared to the endogenous peptides, indicating that the length, and the extended amino acid sequence, of the neuropeptide C-terminus play a role in receptor binding [34,38]. Interestingly, even in receptors with very dissimilar sequences, the same affinity for AstAs can be observed. For example, both Drome-AstAR1 and 2 have a high affinity to all *Drosophila* AstAs, although these are highly divergent in protein sequence [28,31]. To date, however, the ligand binding sites of AstARs and most other neuropeptide receptors remain enigmatic.

2. Material and methods

2.1. Animals

Aedes aegypti (Rockefeller strain), *An. coluzzii* (Suakoko strain; formerly *An. gambiae* M-molecular form) and *Cx. quinquefasciatus* (Thai strain) were reared at $27 \pm 1^\circ\text{C}$, 70% relative humidity, and at a 12 h:12 h light: dark cycle, as previously described [41].

2.2. Nomenclature

The nomenclature used here was adopted from [42] with a few modifications. A five-letter code was used for interspecific neuropeptide isoforms, and for the intraspecific isoforms, the numbering was based on the order of the isoforms in the peptide precursor. For neuropeptide isoforms conserved in all three species, we omitted the five-letter code.

2.3. Phylogenetic and structural analysis of AstARs

Previous annotations of the mosquito AstA receptor and AstA

precursor genes were identified in the genomes available in Vectorbase (<https://www.vectorbase.org/>; AaeGL3.3; AgamP4.3; CpipJ2.2; Supplementary Table 1) and compared with those published by Félix et al. [23]. The AstAR genes were cloned from cDNA (see 2.4) by PCR using specific primers (Supplementary Table 2) based on the available sequences, and their identity confirmed by DNA sequencing. In the case of *Anoco-AstAR1*, sequencing yielded an isoform with a three amino acid deletion at the intron-exon boundary in the carboxy (C)-terminus compared with that previously published and currently annotated in Vectorbase [23]. The *Anoco-AstAR1* sequence was manually curated to confirm this isoform. Full-length open reading frames of *Ae. aegypti* and *Cx. quinquefasciatus* AstAR2 were predicted using BLAST search with the complete *An. gambiae/coluzzii* sequences and identification of potential splice sites using the NetGen2 server: (<http://www.cbs.dtu.dk/services/NetGen2/>). Phylogenetic analysis was performed using homologues of other species obtained by BLAST search from Vectorbase (<https://www.vectorbase.org/Blast>) and NCBI (<https://www.ncbi.nlm.nih.gov/Blast>) (Supplementary Table 1). The phylogenetic tree was constructed in MEGA7 [43], based on a CLUSTALW alignment of full length predicted AstAR protein sequences, using the neighbor-joining method and 2000 bootstrap replicates.

For structural analysis of the mosquito AstARs, sequences were aligned and conserved amino acids determined in GeneDoc [44]. Transmembrane domains were determined based on those described in Mirabeau and Joly [51], and modified according to TOPCONS [45] as well as motifs identified in PROSITE [46]. In addition, two-dimensional representations of the receptor three-dimensional structure were created in TOPO2 (Johns S.J., TOPO2, Transmembrane protein display software, <http://www.sacs.ucsf.edu/TOPO2/>). Sequence similarities between AstARs were determined using the sequence identity and similarity tool (SIAS; <http://imed.med.ucm.es/Tools/sias.html>).

2.4. Cloning of the allatostatin-A receptors

To clone the AstARs, 6–12 days post-emergence non-blood fed mosquitoes were transferred into RNAlater (Thermo Fisher Scientific, Roskilde, Denmark), and total RNA was then extracted and purified using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany). Total RNA was used as the template for first strand cDNA synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd., Copenhagen, Denmark), according to the manufacturer's standard protocol.

Primers were designed using the Primer Design tool (<http://www.clontech.com/>) to enable the use of In-Fusion® technology (Clontech, Saint-Germain-en-Laye, France; Supplementary Table 2). Receptor gene sequences were amplified using Q5® Hot Start High-Fidelity 2 × Master Mix (New England Biolabs, Herlev, Denmark), and the following cycle program: denaturation at 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 60 or 62°C for 30 s, and 72°C for 50 s, then a final elongation step at 72°C for 2 min (Supplementary Table 2). The PCR product was purified from a 1% agarose gel using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), and then cloned into the pIRES2-ZsGreen1 expression vector using the In-Fusion® HD EcoDry™ cloning kit (Clontech). Expression vectors were transformed into One Shot® TOP10 chemically competent *Escherichia coli* cells (Thermo Fisher Scientific), and grown on LB agar plates with 10 mg/ml kanamycin (Thermo Fisher Scientific). Vector inserts were sequenced using the pIRES Forward and pIRES Reverse primers (Supplementary Table 2). Colonies with inserts of the correct size were grown overnight in LB medium, and plasmids harvested using NucleoBond® Xtra Midi EF (Macherey-Nagel).

2.5. Bioluminescence assay

For the heterologous expression, AstARs were stably expressed in CHO cells, already stably expressing the human G-protein G16 (CHO/G16), and



Fig. 1. Mosquito allatostatin-A (Asta) peptide precursor sequences. Deduced sequences of Asta were obtained from AAEL015251 (*Aedes aegypti*, Aedae), AGAP003712 (*Anopheles coluzzii*, Anoco) and CPIJ008017 (*Culex quinquefasciatus*, Culqu). The signal peptide was predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and is boxed. Introns are indicated by arrowheads. Predicted mature neuropeptides are highlighted in light gray. Differences between neuropeptide isoforms are displayed in dark gray. Recognition sites for neuropeptide cleavage are underlined and glycine residues, post-translationally modified to the C-terminal amide of the mature Asta peptides, are indicated in italics.

assayed with endogenous Asta isoforms of the three species in an established bioluminescence assay [47]. In brief, CHO/G16 cells were cultured in monolayers in complete medium (Dulbecco's Modified Eagle Medium Nutrient Mixture F12-Ham) supplemented with 5 % fetal bovine serum and 1 % penicillin-streptomycin with 200 µg/ml hygromycin B and 400 µg/ml geneticin (Thermo Fisher Scientific). To generate stable cell clones, cells were transfected with the expression vectors using jetPEI® (Polyplus transfection, Illkirch, France). For the bioluminescence assays, CHO/G16 cells were transiently transfected with the mitochondrial targeted aequorin/pcDNAI (Molecular Probes, Eugene, OR, USA) and treated with the cofactor coelenterazine (Thermo Fisher Scientific). Neuropeptides were synthesized (Peptides & Elephants GmbH, Potsdam, Germany) and diluted into Dulbecco's phosphate buffered saline. The amidated synthetic neuropeptides represented the neuropeptide isoforms encoded by the neuropeptide precursors of the three species, identified from annotations in Vectorbase and alignment of the amino acid sequences (Fig. 1). For *Ae. aegypti*, these isoforms have been validated by direct mass spectrometric profiling [25]. The bioluminescent response to the presence of calcium released from internal stores was measured from the transfected cells in 96-well optical-bottom plates (Nunc, Thermo Fisher Scientific) in a Victor X2 Multilabel Plate Reader (PerkinElmer, Skovlunde, Denmark). For each concentration of neuropeptides tested, three technical replicates were measured and bioluminescence recorded at 5 s intervals for a total of 15 s. The average and standard deviation of the three technical replicates were determined and dose response curves were generated ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\log EC_{50} - X)})$; GraphPad Prism 6).

2.6. Quantitative real time PCR analysis

Quantitative real time PCR (qPCR) was used to determine the expression of AstARs and AstA precursors in the heads of female mosquitoes when fed on sucrose or blood, as described below. To decrease variation in the qPCR analysis, adults were given access to sugar water *ad libitum* for the first three days after emergence, and then only 1 h per day, at 2 h after light onset. This feeding regime ensures similar nutritional conditions for all investigated species, as shown in a previous study [16]. Six days after adult emergence, the males were removed from the cages, and the female mosquitoes were separated into two cohorts, of which one was fed on a 4 % sucrose solution from a filter paper wick, and one was fed on defibrinated sheep blood (Hätuna Lab, Sweden) via a membrane feeding system (Hemotek, Discovery Workshops, Accrington, UK). Heads of fully fed females from each of the two cohorts were dissected 3 h and 27 h post-feeding. The tissue was stored in RNAlater (Thermo Fisher Scientific) at -20°C . Total RNA was extracted using the Nucleospin RNA kit (Macherey-Nagel), and subsequently treated using DNase I (Qiagen, Hilden, Germany). Quality and quantity of the RNA was analyzed by Nanodrop 1000 and Qubit 3.0 (Thermo Fisher Scientific), respectively. The RNA (250 ng) for each

sample was used as a template for cDNA synthesis, using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories Ltd.). The synthesized cDNA was diluted 5-fold, and used as a template for qPCR in a Mx3005P qPCR System, using the Brilliant III Ultra-Fast SYBR Green Low ROX qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), which contains the fluorescent ROX™, as a passive reference. Primers for qPCR were designed, whenever possible, to bridge exon boundaries and for similar annealing temperatures (60°C), using the IDT PrimerQuest Tool (<https://eu.idtdna.com/PrimerQuest/Home/Index>; Supplementary Tables 2 and 3). Three reference genes per species (*eIF-1A*: eukaryotic translation initiation factor 1A; *RpS7*: 40S ribosomal protein S7; *RpL8*: 60S ribosomal protein L8; in *Cx. quinquefasciatus*, 40S ribosomal protein S4; *RpL4* was used instead of *RpL8*) were used [48,49]. Primer efficiency for the genes-of-interest and the three reference was tested and primers were subjected to melting curve analysis to verify their specificity and efficiency of amplification. Three technical replicates for three biological replicates were performed for each treatment. For each sample, 'no template' controls were run alongside the samples. The following two-step amplification program was used: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. To investigate fold-change differences between treatments, the $\Delta\Delta C_t$ method was used to normalize against the three reference genes [50]. Data were plotted and statistical differences between blood fed and sugar fed animals at the two time points were determined using an unpaired *t*-test assuming unequal variance in Graph Pad Prism 6 (GraphPad Software).

3. Results

3.1. Gene structure and phylogenetic analyses of mosquito AstARs

To identify and verify the mosquito AstARs, we manually curated sequences from genomic databases, followed by cloning, isolation and characterization of the transcripts for AstAR1 and AstAR2 in *An. coluzzii* (Anoco-AstAR), *Ae. aegypti* (Aedae-AstAR) and *Cx. quinquefasciatus* (Culqu-AstAR). The Aedae-AstAR1 (AAEL007169) has previously been described as an AstAR2 orthologue [23], but its gene structure and amino acid sequence is more homologous to that of the AstAR1 genes (see below), and is thus renamed in this study. The Aedae-AstAR2 consists of exon 1, located at AAEL006076, exons 2 and 3, located at AAEL006077, previously described as an AstAR pseudo-gene [23], and exon 4 in a previously non-annotated region located 3' to AAEL006077 (Supplementary Table 4). The cloned Culqu-AstAR1 and Culqu-AstAR2 each contained parts of two putative AstARs, incompletely annotated from the genome of *Cx. quinquefasciatus*, in addition to previously non-annotated regions. Exon 1 of Culqu-AstAR1 is located in the region of the genome with CPIJ016162, exons 2–4 are located in a non-annotated region between CPIJ016162 and CPIJ016163, while CPIJ016163

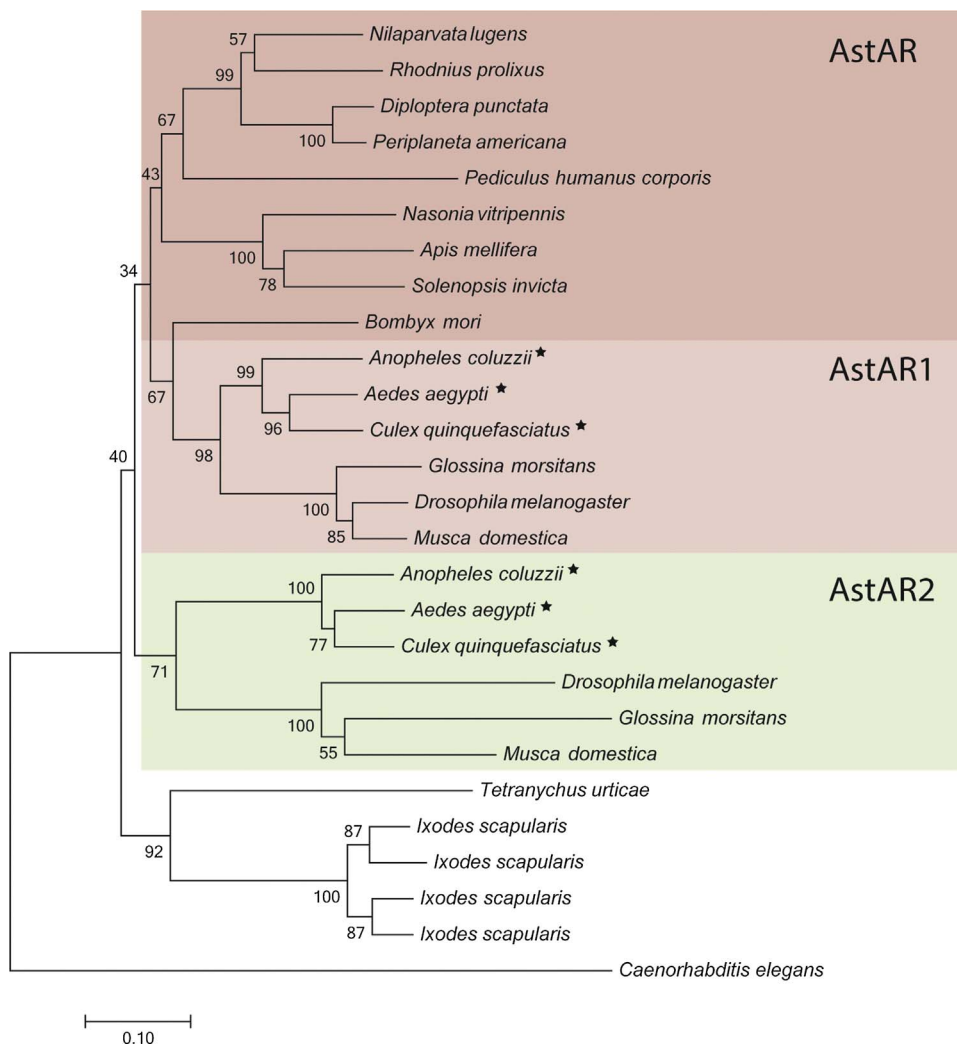


Fig. 2. Phylogeny of allatostatin-A receptors (AstarARs). Phylogenetic analysis of the dipteran AstarAR1s (light red) and AstarAR2s (green), as well as insect AstarARs (red), inferred using the neighbor-joining method. Numbers at branches are percent bootstrap values based on 2000 replicates. Receptors investigated in this study are marked with a star. Scale bar represents a mutation rate of 10 substitutions per 100 residues.

contains exons 5–7, and exon 8 is found in a non-annotated region 3' to CPIJ016163. The exon 1 of *Culqu-AstarAR2* is located in CPIJ013095 and exons 2 and 3 are found in CPIJ011118 followed by the previously not annotated exon 4 (Supplementary Table 4).

A phylogenetic analysis, based on the aligned amino acid sequences derived from cloned mosquito and annotated insect AstarARs, revealed that orthologues of the AstarAR1s are highly conserved and share a distinct branch with AstarARs of other insects (Fig. 2). In contrast, the AstarAR2s form a distinct monophyletic cluster together with the Drosophila AstarAR2 and AstarAR2s from other dipteran species (Fig. 2). The mosquito AstarAR1 and AstarAR2 genes are characterized by a highly conserved structure and amino acid sequence (Figs. 3 and 4; Supplementary Table 5). The dipteran AstarAR1s are comprised of 7–8 exons, whereas AstarAR2s consist of 4 exons, each with introns at similar positions and with a similar phasing. The AstarAR1s of all three species show an amino acid sequence similarity of between 75 % and 85 % (Supplementary Table 5). Similarly, AstarAR2s were found to share high similarity, ranging from 84 % to 90 %. In contrast, AstarAR1s and AstarAR2s only share between 60 % and 65 % amino acid similarity.

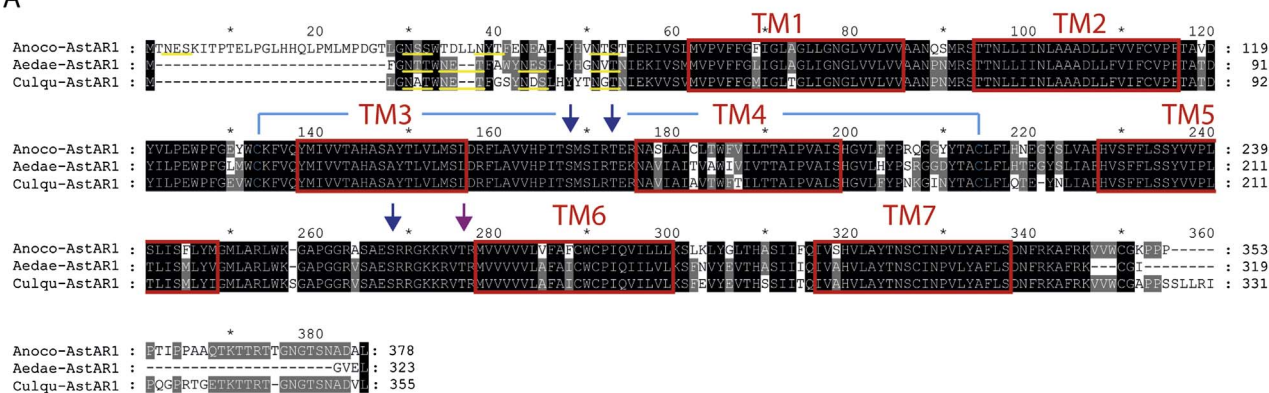
Alignment of the AstarAR amino acid sequences of the three mosquito species were used to indicate the seven transmembrane domains and conserved motifs, including several putative N-glycosylation sites in the amino (N)-terminus and two conserved cysteines, predicted to form a disulfide bond between extracellular loop (ECL) 1 and ECL2 (Figs. 3 and 4). In addition, several recognition sites for protein kinase C, as well as for the cAMP- and cGMP-dependent protein kinases were identified (Figs. 3 and 4). The differences in amino acid sequence of the identified

AstarAR1s and AstarAR2s, in all three species, are found mainly in the N-termini, and for AstarAR1s at the C-termini (Figs. 3–5). However, amino acid substitutions are also found in the intracellular loops (ICLs), ECLs, and in the transmembrane (TM) regions, as highlighted in the predicted receptor structures (Figs. 3–5). Between orthologues of the AstarAR1s, one cluster of non-conserved amino acid substitutions was found in ECL2 and a second in TM4 (Fig. 3). A similar cluster of non-conserved amino acid substitutions in ECL2 was observed in the AstarAR2s (Fig. 4). Comparisons among the AstarAR1s and AstarAR2s for all three species confirmed low sequence similarity between paralogues, especially in ECL2 and TM4, with additional clusters of non-conserved substitutions found in ECL3 and ICL3 (Fig. 5).

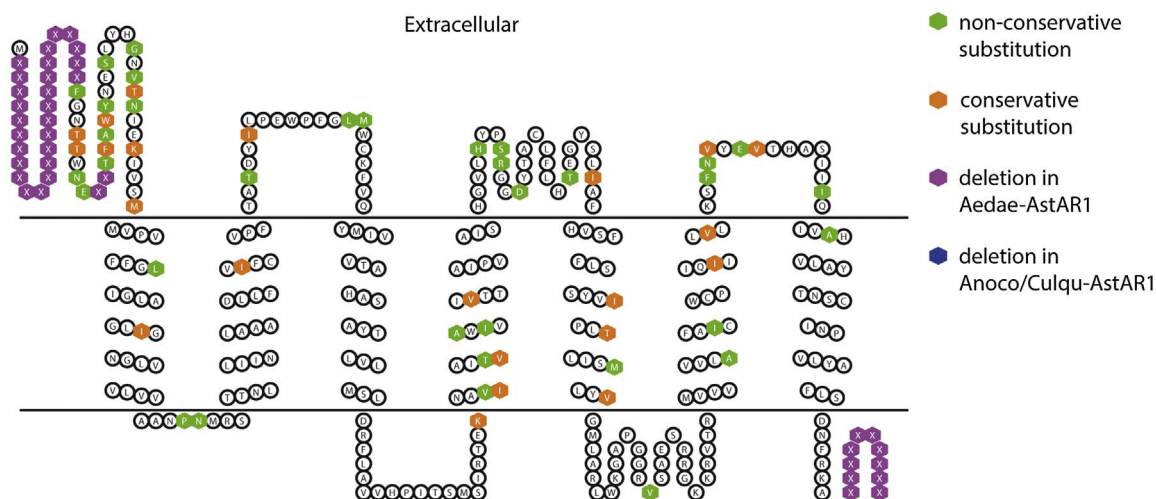
3.2. Functional receptor analysis

The mosquito AstarARs were functionally characterized using a bioluminescence assay with stably transfected CHO/G16 cells (Fig. 6). All of the synthetic AstarARs tested activated the AstarARs in a differential dose-dependent manner, an example of which (AstarA-5) is shown in Fig. 6A. Differences in potency (EC_{50}) were observed among the AstarARs activating the AstarAR1 (Fig. 6B). The *Culqu-AstarAR1* displayed the lowest affinity for the tested AstarARs with EC_{50} values ranging from 300 nM to 2700 nM, while *Aedae-AstarAR1* displayed EC_{50} values ranging from 150 nM to 500 nM. In contrast, *Culqu-AstarAR2* and *Aedae-AstarAR2* displayed a higher affinity to the AstarARs tested, with EC_{50} values generally ranging from 1 to 4 nM and 5–25 nM, respectively. Interestingly, both *Anoco-AstarAR1* and 2 showed similar activation by all isoforms, with

A



B Anoco-AstAR1 onto Aedae-AstAR1



C Culqu-AstAR1 onto Aedae-AstAR1

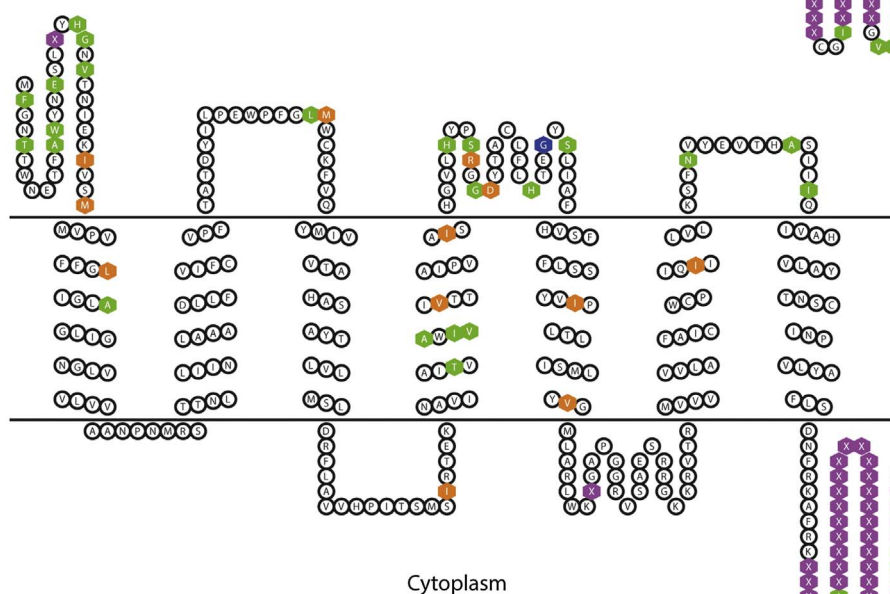


Fig. 3. Structural comparison of *Anopheles coluzzii* (Anoco), *Aedes aegypti* (Aedae) and *Culex quinquefasciatus* (Culqu) allatostatin-A receptors (AstAR1s). (A) Sequence alignment of mosquito AstAR1s. Identical and conserved amino acids across sequences are highlighted in black and gray, respectively. Predicted seven transmembrane domain regions are boxed in red, N-linked glycosylation sites are underlined (yellow) and phosphorylation sites are marked with arrows for locations recognized by protein kinase C (blue) and cAMP- and cGMP-dependent protein kinases (purple). Two conserved cysteines, potentially forming a disulfide bridge, are marked in blue. The chevron indicates a deletion of the 3 amino acids in the Suakoko strain compared to the Vectorbase annotation. (B, C) Protein structure models of AstAR1s indicate differences between the AstAR1 orthologues Anoco-AstAR1 mapped onto Aedae-AstAR1 (B) and Culqu-AstAR1 mapped onto Aedae-AstAR1 (C). Models show protein orientation and the seven transmembrane domains. Colors code for conservative substitutions (orange), non-conservative substitutions (green) and deletions in the sequence of *Ae. aegypti* (purple), and *An. coluzzii* or *Cx. quinquefasciatus* (blue), respectively.

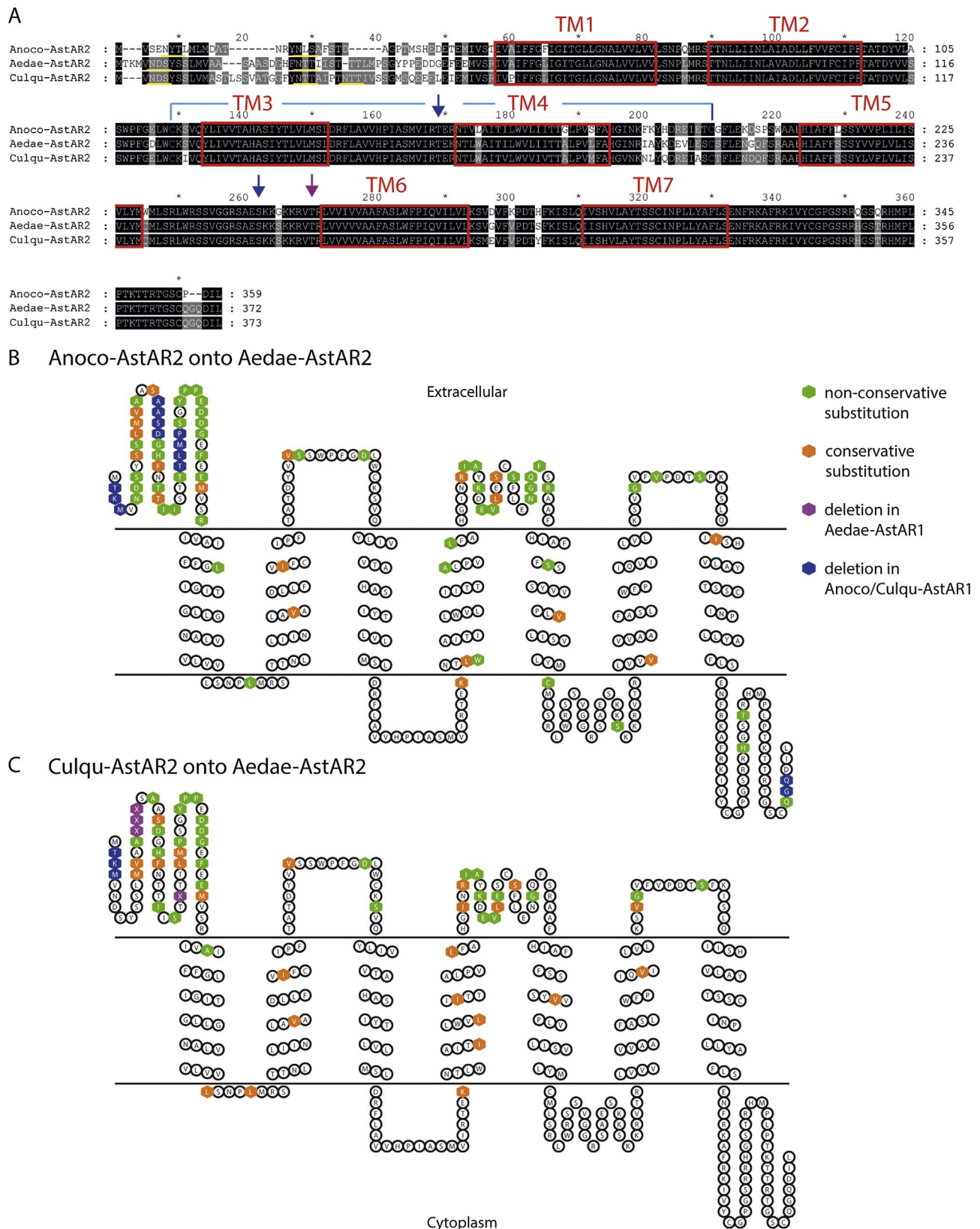


Fig. 4. Structural comparison of *Anopheles coluzzii* (Anoco), *Aedes aegypti* (Aedae) and *Culex quinquefasciatus* (Culqu) allatostatin-A receptors (Astar2s). (A) Sequence alignment of mosquito Astar2s. Identical and conserved amino acids across sequences are highlighted in black and gray, respectively. Predicted seven transmembrane domain regions are boxed in red, N-linked glycosylation sites are underlined (yellow) and phosphorylation sites are marked with arrows for locations recognized by protein kinase C (blue) and cAMP- and cGMP-dependent protein kinases (purple). Two conserved cysteines, potentially forming a disulfide bridge, are marked in blue. (B, C) Protein structure models of Astar2s indicate differences between the Astar2 orthologues. Anoco-AstAR2 mapped onto Aedae-AstAR2 (B) and Culqu-AstAR2 mapped onto Aedae-AstAR2 (C). Models show protein orientation and the seven transmembrane domains. Colors code for conservative substitutions (orange), non-conservative substitutions (green) and deletions in the sequence of *Ae. aegypti* (purple), and *An. coluzzii* or *Cx. quinquefasciatus* (blue), respectively.

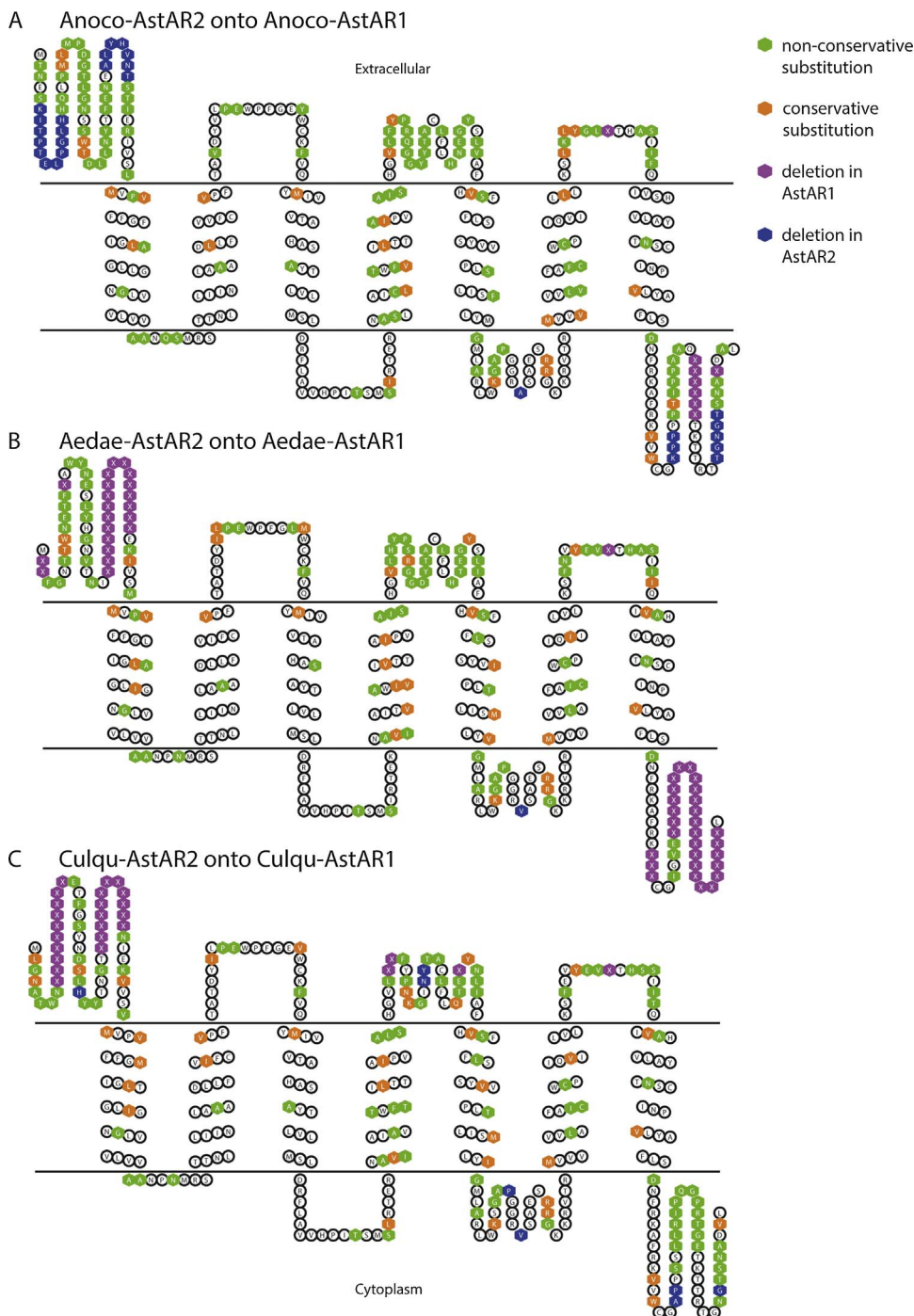


Fig. 5. Protein structure models of paralogue allatostatin-A receptors (AstARs) in *Anopheles coluzzii* (Anoco), *Aedes aegypti* (Aedae) and *Culex quinquefasciatus* (Culqu). Differences between AstAR1s and AstAR2s are shown, mapping the AstAR2 onto the AstAR1 for *An. coluzzii* (A), *Ae. aegypti* (B) and *Cx. quinquefasciatus* (C). Sequence based models show protein orientation and the seven transmembrane domains. Colors code for conservative substitutions (orange), non-conservative substitutions (green) and deletions in the sequence of the AstAR2 (purple) or AstAR1 (blue), respectively.

EC₅₀ values ranging from 70 nM to 170 nM and 30–130 nM, respectively (Fig. 6B).

The amino acid sequence of the AstA isoforms affected the activation of the AstARs (Fig. 6B). The AstA-3 isoforms showed low potency (blue in Fig. 6B) to activate the AstARs in all three species. The Aedae-AstA-3, however, generally demonstrated a higher potency than its counterparts in the other two species (Fig. 6B). All three AstA-3 isoforms have a unique histidine in the 4th position from the C-terminus (Fig. 6B). Moreover, Culqu-AstA-3 has a unique glycine in the 7th position from the C-terminus, and Anoco-AstA-3 is much longer than the other isoforms with several glycines within the sequence. Interestingly, the endogenous AstA-2 isoform of both *Ae. aegypti* and *An. coluzzii* (Aedae/Anoco-AstA-2), was found to activate both Anoco-AstAR1 and Anoco-AstAR2 with high EC₅₀ values (red in Fig. 6B). The only isoform

to consistently activate the AstARs at low EC₅₀ values was AstA-5 (red in Fig. 6B), which is conserved across all three species. Allatostatin-A-5 is different from the other neuropeptides as it has an arginine in the 6th position from the C-terminus, a feature only shared with the AstA-3s (Fig. 6B).

3.3. Effect of blood feeding on gene transcripts

For both *An. coluzzii* and *Ae. aegypti*, no difference in *AstAR* or *AstA* transcript levels was found 3 h and 27 h post-blood feeding (Fig. 7). In contrast, the abundance of *Culqu-AstAR2*, but neither *Culqu-AstAR1* nor *Culqu-AstA* transcripts, was found to be approximately 2-fold lower 3 h post-blood meal, compared to sugar fed controls. This effect was restored 27 h after blood feeding (Fig. 7).

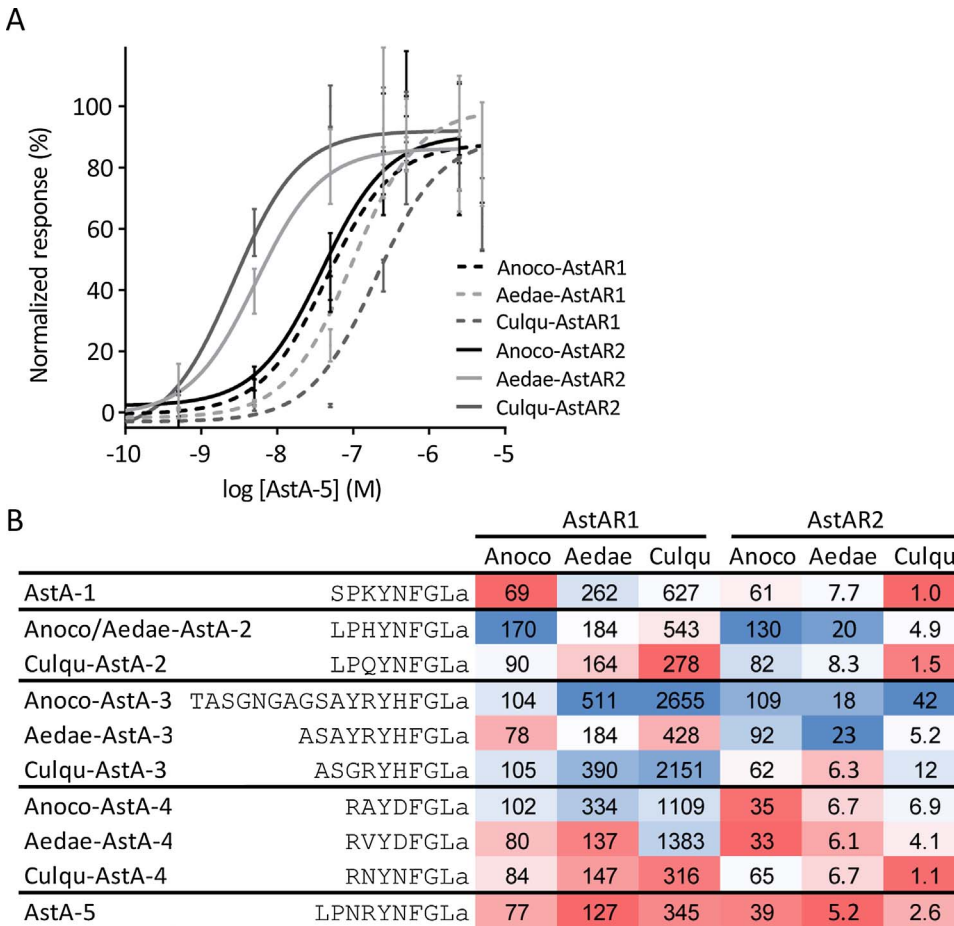


Fig. 6. Functional analysis of *Anopheles coluzzii* (Anoco), *Aedes aegypti* (Aedae) and *Culex quinquefasciatus* (Culqu) allatostatin-A receptors 1 and 2 (AstAR1s and AstAR2s), in response to allatostatin-A isoforms (AstAs). (A) Dose-dependent activation of mosquito AstARs, stably expressed in CHO/G16 cells, and challenged with various concentrations of AstA-5. Bioluminescence was normalized to the lowest and highest values, respectively, for each replicate (n = 3). The AstAR1s are indicated with dashed and AstAR2s with solid lines. Error bars represent the standard deviation. (B) Heat map showing the EC₅₀s (n = 3; nM) of the AstAR1s and AstAR2s tested with the endogenous AstA isoforms of *An. coluzzii*, *Ae. aegypti* and *Cx. quinquefasciatus*. EC₅₀s are highlighted in colors ranging from red (low EC₅₀s) to blue (high EC₅₀s) for each receptor (column) individually.

4. Discussion

4.1. Evolution of AstA and its receptors

Recently, ancestral bilaterian peptidergic signaling systems have been identified [23,51,52]. These studies demonstrate a common lineage between the receptors in the arthropod AstA and the vertebrate galanin and kisspeptin peptidergic signaling systems [23,51], all of which are involved in the regulation of feeding, metabolism and reproduction. Within the AstAR family, there are two distinct subfamilies: AstAR1, which forms a monophyletic branch across all of the insect species investigated; and AstAR2, which appears to form a dipteran-specific branch [23]. The general structure of the AstAR genes is conserved at the 3' end, with the second last intron of all AstARs being conserved (Fig. 8). In addition, the final intron is conserved across the dipteran AstAR1s and 2s, and in the lepidopteran Bommo-AstAR. In the dipteran AstAR1s, the open reading frame is composed of 7–8 exons, whereby six of the seven introns are conserved, where the seventh is mosquito specific (Fig. 8). The AstAR2s have 4 exons, of which the introns are conserved across all dipterans (Supplementary Table 1; Fig. 8) [23,28,30]. The AstARs in other insects have three to four exons, more closely resembling the dipteran AstAR2s, however the placement of the introns is variable (Supplementary Table 1) [34–36]. In dipterans, AstAR1s appear to have undergone structural changes that have since been conserved throughout the lineage, while AstAR2s appears to have maintained a more ancestral gene structure, despite a reduction in the overall sequence similarity to the other insect AstARs. In both *D. melanogaster* and *An. coluzzii*, the AstAR1 and the AstA peptide precursor each derive from unique genes on the same chromosome, suggesting that both the receptor and peptide are linked and have evolved via lineage specific events [51]. In both of these species, the ligand

affinity of AstAR1 for the AstAs is high [23,24], supporting the argument for the co-evolution of the receptor and ligands. Since the chromosomal location of the peptide precursors and receptors in *Ae. aegypti* and *Cx. quinquefasciatus* are not yet known, it is not possible to speculate on whether this hypothesis includes a broader range of species.

The origin of two AstARs in dipterans is not known, but might have arisen from a gene duplication event early in the dipteran lineage followed by an adaptive selection after its divergence [23]. In *D. melanogaster* and *An. coluzzii*, both AstARs demonstrate a conserved sensitivity to the AstA ligands, suggesting that there has been no directional selection on the ligand specificity of these two receptors (this study; [29]). In contrast, the culicine AstARs demonstrate differential sensitivity to the AstA ligands, with the AstAR2s displaying a higher sensitivity than the AstAR1s. As the chromosome sequences for *Ae. aegypti* and *Cx. quinquefasciatus* have yet to be assembled, we are unable to confirm whether either AstAR is linked with the AstA precursor. However, available structural and functional evidence suggest that AstAR2 in culicine mosquitoes has maintained the ancestral role as a high sensitivity AstA receptor, while AstAR1 has reduced its sensitivity to AstA. Since purifying selection has not removed AstAR1 from these genomes, it is possible that AstAR1 may have gained a novel, yet unknown, functional role. Alternatively, the receptors may show differential tissue expression patterns, as shown in *D. melanogaster* [53].

4.2. Function of AstA receptors

Mosquito AstAR2s are activated by lower concentrations of all endogenous AstA isoforms compared to AstAR1s (this study; [23]). One exception to this was noted in *An. coluzzii*, in which only a slight difference in sensitivity between the two AstAR types were observed. These results are different from that of Félix et al. (2015), which

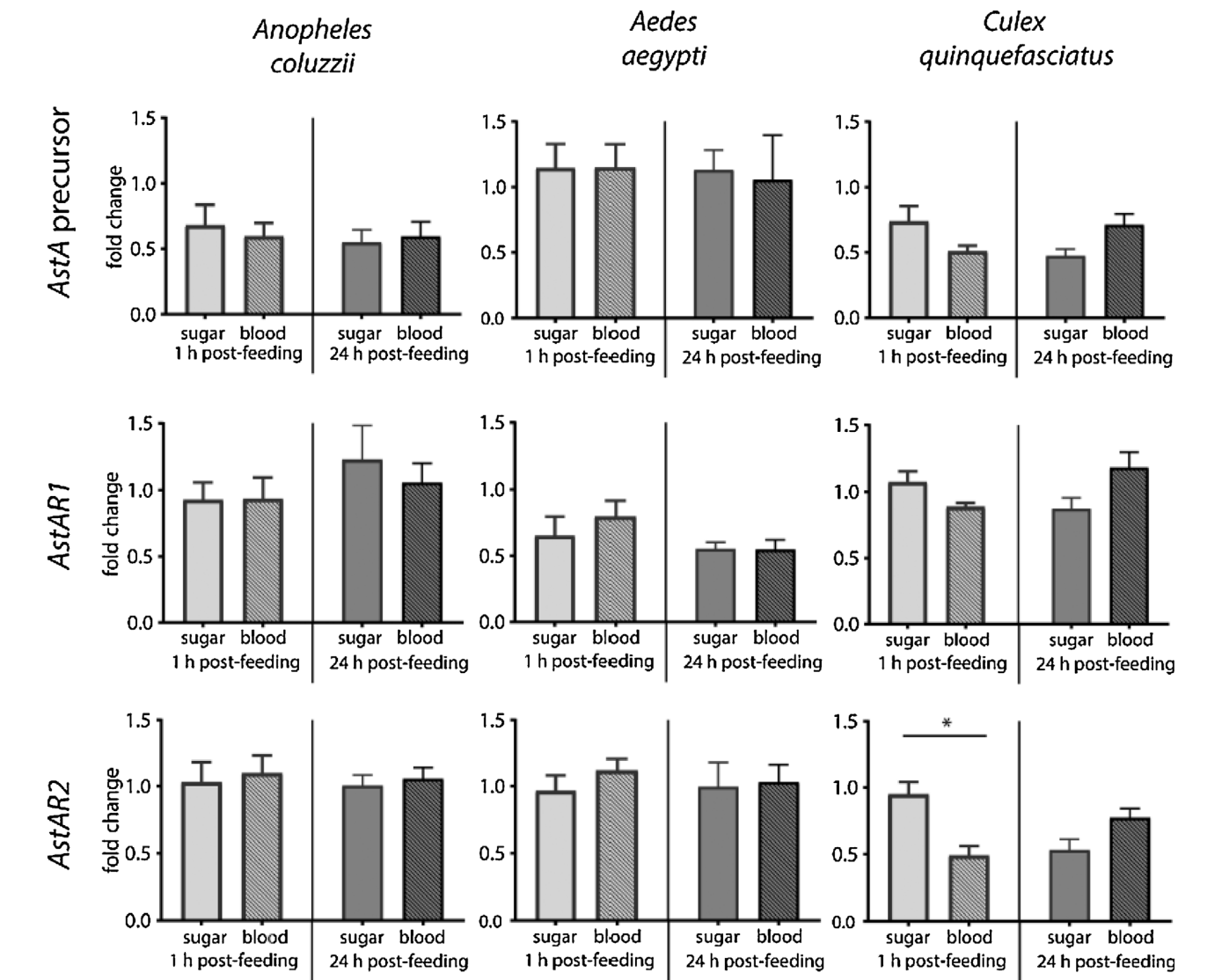


Fig. 7. Expression of allatostatin-A receptor (AstAR) and allatostatin A precursor (AstA) transcripts in the head tissue of *Anopheles coluzzii*, *Aedes aegypti* and *Culex quinquefasciatus* pre- and post-blood meal. Expression was analyzed using quantitative PCR, 1 h (light gray) and 24 h (dark gray) post-feeding on sugar (solid) or blood (hatched). Bars represent the mean $\pm$ SEM of three biological replicates. Significant differences between treatments were calculated using a non-paired Student's *t*-test. *: $P < 0.05$.

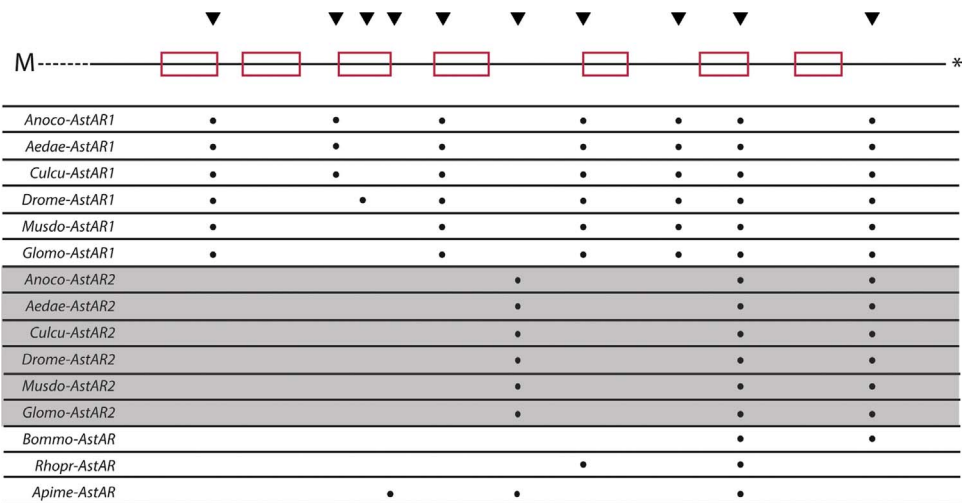


Fig. 8. Intron analysis of insect allatostatin-A receptor (AstAR) genes. The intron positions (arrow heads) are shown on a schematic diagram based on the amino acid sequence alignment (top) and marked by a dot to indicate the presence in specific receptors (below). For orientation, transmembrane regions are boxed in red. Note, that the second *Apis mellifera* (Apime)-AstAR intron is shifted by one amino acid compared to the dipteran AstAR2s. The *Glossina morsitans* (Glomo) and *Musca domestica* (Musdo)-AstAR2 have been manually curated. For accession numbers, please see Supplementary Table 1. Anoco: *Anopheles coluzzii*, Aedae: *Aedes aegypti*, Bommo: *Bombyx mori*, Culcu: *Culex quinquefasciatus*, Drome: *Drosophila melanogaster*, Rhopr: *Rhodnius prolixus*.

described the AstAR2 as more sensitive than AstAR1. The reason for this discrepancy is at present unknown, as the AstAR1 and AstAR2 sequences were identical in both studies (J.C.R. Cardoso, *personal communication*). Comparison between paralogues of AstARs showed that receptor sensitivity is likely to be affected by differences in the putative ligand binding domains, including ECL2 and 3, which differ substantially in structure between AstAR1s and AstAR2s. A summary of crystal chromatography data for G-protein coupled receptors (GPCRs) reveals ECL2 as an important region for the initial steps in determining ligand specificity and in forming a lid to the ligand binding pocket in many class A GPCRs [54]. Aside from one of the cysteines essential for forming a disulfide bridge and maintaining receptor stability, only a motif of phenylalanine and leucine, one residue after the cysteine, and a motif of alanine, phenylalanine and histidine, directly before TM5, are conserved in all three species. Sequence comparison with other species confirms that these sequence motifs (CXFL/S; XF/YH/Q) are conserved throughout insect AstARs (this study; [23]), but are not conserved in other neuropeptide receptors [51]. We therefore suggest that these residues may be essential for AstA neuropeptide recognition. Further analysis is required to elucidate how ECL2 and ECL3, as well as other structurally variable regions such as TM4, affect the sensitivity and specificity of the AstARs.

The mosquito AstARs share a similar pharmacological profile, as well as activation with peptides sharing the conserved C-terminal YXFGL-amide motif, with the insect AstARs, with the exception of the culicine AstAR1s [29,34–36,38,40]. Allatostatin-A isoforms display inter- and intraspecific variation within the N-terminal region [24]. In general, the endogenous mosquito AstAs were found to be the most potent ligands for their cognate AstARs compared with the interspecific isoforms. The two isoforms AstA-1 and AstA-5, which are conserved in all three species, have a high potency for all receptors, especially AstA-5, supporting the argument for the co-evolution of neuropeptide precursor and receptors [51]. The AstA-3 isoforms, the most diverse isoforms in both structure and length, demonstrate a low potency for both AstARs in all three species. The histidine in the 4th position from the C-terminus in AstA-3 s is a rare substitution (i.e. honey bee; [24]), and may play a role in the decreased affinity with the receptors. Our data suggest that, in mosquitoes, AstA isoforms containing this histidine and glycines near the N-terminus, are less potent than other AstAs. This scheme appears to be dipteran-specific (this study; [31]), as neither sequence length, nor N-terminal glycine residues, seem to affect the potency in cockroaches [38,40]. In fact, no other characteristic relationship between structural features of the AstA isoforms and their potency have been found, suggesting that there is only a weak selection on the precursor to retain a specific sequence apart from the core motif FXGLFa [29,38,40].

4.3. AstA receptor expression

In the head tissue of non-blood fed *An. coluzzii* (Suakoko strain), *Ae. aegypti* and *Cx. quinquefasciatus*, the abundance of AstAR transcripts was similar. These results contradict those from *An. coluzzii* (Yaoundé strain), in which AstAR1 transcripts in head tissue were found to be approximately 30 times less abundant compared to *Anoco-AstAR2* transcripts [23]. The difference in expression between the two *An. coluzzii* strains reflects the observed difference in AstAR1 sensitivity (this study; [23]) and suggests a change in function of the AstAR1 in the Yaoundé strain, but not in the Suakoko strain. A comparison between both non-blood fed and blood fed mosquitoes in the transcript abundance of the AstARs and the AstA precursor in heads, revealed that the expression was not regulated by blood feeding in *An. coluzzii* and *Ae. aegypti*, in line with that which was previously reported [22,23]. In contrast, AstAR2 in *Cx. quinquefasciatus* demonstrated a transient reduction in its abundance 1 h post-blood meal, which recovered by 24 h post-blood feeding, similar to what has been reported for *R. prolixus* [17], while that of the *Culqu-AstAR1* and the *Culqu-AstA* precursor

abundance was unaltered. The observation that there is a lack of regulation of the receptors and precursor transcripts may be an artifact of differential expression in various brain compartments. In support of this, a previous study has demonstrated that at least one isoform of AstA in *Ae. aegypti* (Aedae-AstA-5) was found to be differentially abundant in the antennal lobes 24–48 h post-blood feeding [16]. However, while regulation of transcription may not necessarily lead to a differential peptide expression, the correlation between these two studies is suggestive. Further analysis of the expression of the receptor and the precursor is required to further verify the role of AstA in various tissues.

5. Conclusion

While the AstA pathway in mosquitoes appears to be conserved, both species- and strain-specific selection pressures on both of the AstARs have likely led to the observed differential expression, as well as differential ligand specificity and sensitivity. In mosquitoes, feeding to completion on a protein-rich blood meal transiently inhibits both carbohydrate- and protein-seeking foraging behaviors [16,19,20,55,56]. Systemic injection of AstA reproduces the effect of a blood meal on non-blood fed *Ae. aegypti* [16]. Both the neural circuitry regulating AstA signaling and the transient reduction in carbohydrate foraging post-protein meal thus appears to be conserved in *Ae. aegypti* and *D. melanogaster* [57–60], and correlates with a change in AstA levels [3–6,16]. As feeding behavior in mosquitoes brings them into direct conflict with humans, further investigation into the regulation of such vector-related activities by AstA signaling is warranted.

Conflicts of interest

None

Author contributions

All authors planned and designed the study. FH provide technical support and resources. PC carried out the experiments and data analysis, and together with SH and RI wrote the manuscript. All authors critically revised the manuscript and approved the final article.

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Data statement

All sequences reported here have been deposited in Genbank as follows:

BankIt2029530 Aedae-AstAR2 MF443907
BankIt2030246 Culqu-AstAR2 MF443908
BankIt2030248 Aedae-AstAR1 MF443909
BankIt2030251 Culqu-AstAR1 MF443910
BankIt2030257 Anocu-AstAR2 MF443911
BankIt2030379 Anocu-AstAR1 MF443912

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.peptides.2017.10.016>.

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Supplementary table 1: Accession numbers of orthologues of the allatostatin A (AstA) precursor, and receptors 1 and 2 (AstAR1 and AstAR2, respectively).

<u>Arthropoda</u>	AstA precursor	AstAR1	AstAR2
<i>Aedes aegypti</i>	AAEL015251-PA	AAEL007169-PA	AAEL006076-PA AAEL006077-PA
<i>Anopheles coluzzii</i>	AGAP003712-PA	AGAP003658-PA	AGAP001773-PA
<i>Culex quinquefasciatus</i>	CPIJ008017-PA	CPIJ016162-PA CPIJ016163-PA	CPIJ013095-PA CPIJ011118-PA
<i>Drosophila melanogaster</i>		NP_524700.1	NP_524544.1
<i>Glossina morsitans</i>		GMOY005179-PA	GMOY012001-PA
<i>Musca domestica</i>		MDOA007526-PA	MDOA015456-PA
<i>Bombyx mori</i>		AAG44631.1	
<i>Apis mellifera</i>		XP_006560262.1	
<i>Nasonia vitripennis</i>		XP_008216092.1	
<i>Solenopsis invicta</i>		XP_011170315.1	
<i>Nilaparvata lugens</i>		BAO01051.1	
<i>Rhodnius prolixus</i>		KM283241.1	
<i>Pediculus humanus corporis</i>		XP_002427757.1	
<i>Diploptera punctata</i>		AIS40016.1	
<i>Periplaneta americana</i>		AAK52473.1	
<u>Arachnida</u>		AstAR	
<i>Ixodes scapularis</i>		XP_002403852.1 XP_002433372.1 XP_002433373.1 XP_002414997.1	
<i>Tetranychus urticae</i>		XP_015789363.1	
<u>Nematoda</u>		AstAR	
<i>Caenorhabditis elegans</i>		NP_509896.1	

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2 Supplementary table 2: List of primers used for cloning (*AstARs*) and the real-time PCR
 3 analysis of the *AstARs*, *AstA* precursors and reference genes in *Aedes aegypti* (*Aedae*), *Culex*
 4 *quinquefasciatus* (*Culqu*), and *Anopheles coluzzii* (*Anoco*). T_A indicates the annealing tem-

Cloning Primer	Forward	Reverse	T _A
<i>Aedae-AstAR1</i>	CTCAAGCTTCGAATTATCATCATGTTTGGAAATACAA	GTCGACTGCAGAATTCAATTACAACCTCACTCCG	62
<i>Aedae-AstAR2</i>	CTCAAGCTTCGAATTATGACGAAGATGGTGAATGA	GTCGACTGCAGAATTTTATAAAATGTCCTGGCCCT	62
<i>Anoco-AstAR1</i>	CTCAAGCTTCGAATTATGACGAACGAGTCTAAAATT	GTCGACTGCAGAATTTTATAAGGCGTCCGCGTT	60
<i>Anoco-AstAR2</i>	CTCAAGCTTCGAATTATGGTAAGCGAGAACTACAC	GTCGACTGCAGAATTTTATAAAATGTCCTGGACAGCT	60
<i>Culqu-AstAR1</i>	CTCAAGCTTCGAATTATGTTGGGAAACGCTACATG	GTCGACTGCAGAATTTTACAAAACATCCGCATTTG	62
<i>Culqu-AstAR2</i>	CTCAAGCTTCGAATTATGGTCAACGATAGCTACAG	GTCGACTGCAGAATTTTATAAAATGTCCTGGCCCT	62

qPCR Primer	Forward	Reverse	
<i>Aedae-AstA</i> precursor	CGTGCCAGGAACAATCTG	ATTTGTCCTTGTGCGCCATC	60
<i>Aedae-AstAR1</i>	GGTCGTAAGTCTGTTGTC	GAAAGGCCATTCTGGTAGG	60
<i>Aedae-AstAR2</i>	GCTCATGGAATAAACAGAACTCG	AAGAACGCGATATGGAAGG	60
<i>Aedae-eIF-1A</i>	GCTGATCTTCAAGGAAGACG	ACACAATCGCTTGACACC	60
<i>Aedae-RpS7</i>	CTTTCGAGGGACAAATCGG	GATCAGCATAGCCTTCTTGC	60
<i>Aedae-RpL8</i>	GCCTACCACAAGTACAAGG	CCTTACCGATATGCTGATGG	60
<i>Anoco-AstA</i> precursor	AGTACAACCTTGGGCTTGG	TCCACCCATAGGGCTTC	60
<i>Anoco-AstAR1</i>	GGTTGCCGCGAACCAGAG	CCAATACTCTCCGAAGGGC	60
<i>Anoco-AstAR2</i>	AATCGGTACAATCTGTCTGC	CCCAGTAATACCGATAAAGCC	60
<i>Anoco-eIF-1A</i>	GACTACCAGGACTCGAAGG	TCACCGTTTCGTTGATGC	60
<i>Anoco-RpS4</i>	TGATCAACATCCACAAGACC	TTCAGCAGCTTGTACTTGG	60
<i>Anoco-RpS7</i>	TGGAGGATCTGGTATTCCC	AAAGTGCAACTTTGTGTTTCG	60
<i>Anoco-RpL8</i>	GGGTGTCGTGAAGCAAAT	AACAGCTGCTTGCTCAG	62
<i>Culqu-AstA</i> precursor	GCAAGCGAGGAAGGATG	TTCCGCAGATTTGTCCTG	60
<i>Culqu-AstAR1</i>	CGCGACTGTGGAATCG	AACAAGAATGACCTGAATAGGG	60
<i>Culqu-AstAR2</i>	TTTCGCTCCAAATCATCTCC	CCACAGTACACGATCTTTCG	60
<i>Culqu-eIF-1A</i>	GCCGAAGAATAAGGGAAAGG	TTCCGAGCATTTTGTGACC	60
<i>Culqu-RpS4</i>	AAGAAGGTGCCGTTTCATCC	GGTGATCATGCACAGATTGC	60
<i>Culqu-RpS7</i>	CCTCGAGGATCTGGTCTTCC	CTTGACACCGACGTGAAGG	60

Sequencing Primer	Forward	Reverse	
<i>pIRES</i>	GTGGGAGGTCTATATAAGCAG	CACACCGGCTTATTCCAAG	62

5 perature of the respective primer pairs.

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	<i>eIF-1A</i>	<i>RpS4</i>	<i>RpS7</i>	<i>RpL8</i>
<i>Anopheles coluzzii</i>	<u>AGAP003119</u>		<u>AGAP010592</u>	<u>AGAP005802</u>
<i>Aedes aegypti</i>	<u>AAEL004378</u>		<u>AAEL009496</u>	<u>AAEL000987</u>
<i>Culex quinquefasciatus</i>	<u>CPIJ016188</u>	<u>CPIJ003013</u>	<u>CPIJ006763</u>	

Supplementary table 3: Reference genes used for the quantitative real-time PCR

Supplementary table 4: Exon positions of the two newly identified allatostatin-A type 2 receptors (AstAR2s) in *Aedes aegypti* and *Culex quinquefasciatus*.

Species	Strain	Scaffold	Predicted transcripts	Exon1	Exon2	Exon3	Exon4
<i>Aedes aegypti</i>	Liverpool	supercont1.188	AAEL006076-RA AAEL006077-RA	311339 - 311920	564736 - 565005	621540 - 621698	673864 - 673971
<i>Culex quinquefasciatus</i>	Johannesburg	supercont3.442 supercont3.318	CPIJ013095-RA CPIJ011118-RA	361631 - 362216	369472 - 369741	383905 - 384063	414386 - 414493

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Anoco-AstAR1						
Aedae-AstAR1	76.1 %					
Culqu-AstAR1	78.8 %	84.1 %				
Anoco-AstAR2	61.5 %	66.5 %	69.3 %			
Aedae-AstAR2	59.9 %	62.5 %	65.2 %	83.9 %		
Culqu-AstAR2	61.7 %	64.5 %	66.8 %	84.9 %	90.4 %	
	Anoco-AstAR1	Aedae-AstAR1	Culqu-AstAR1	Anoco-AstAR2	Aedae-AstAR2	Culqu-AstAR2

2 Supplementary table 5: Sequence similarities between mosquito AstARs

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