Regulation of P450-mediated permethrin resistance in *Culex quinquefasciatus* by the GPCR/Gαs/AC/cAMP/PKA signaling cascade

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ABSTRACT

This study explores the role of G-protein-coupled receptor-intracellular signaling in the development of P450-mediated insecticide resistance in mosquitoes, *Culex quinquefasciatus*, focusing on the essential function of the GPCRs and their downstream effectors of Gs alpha subunit protein (Gαs) and adenylyl cyclase (ACs) in P450-mediated insecticide resistance of *Culex* mosquitoes. Our RNAi-mediated functional study showed that knockdown of Gαs caused the decreased expression of the downstream effectors of ACs and PKAs in the GPCR signaling pathway and resistance P450 genes, whereas knockdown of ACs decreased the expression of PKAs and resistance P450 genes. Knockdown of either Gαs or ACs resulted in an increased susceptibility of mosquitoes to permethrin. These results add significantly to our understanding of the molecular basis of resistance P450 gene regulation through GPCR/Gαs/AC/cAMP-PKA signaling pathways in the insecticide resistance of mosquitoes. The temporal and spatial dynamic analyses of GPCRs, Gαs, ACs, PKAs, and P450s in two insecticide resistant mosquito strains revealed that all the GPCR signaling pathway components tested, namely GPCRs, Gαs, ACs and PKAs, were most highly expressed in the brain for both resistant strains, suggesting the role played by these genes in signaling transduction and regulation. The resistance P450 genes were mainly expressed in the brain, midgut and malpighian tubules (MTs), suggesting their critical function in the central nervous system and importance for detoxification. The temporal dynamics analysis for the gene expression showed a diverse expression profile during mosquito development, indicating their initially functional importance in response to exposure to insecticides during their life stages.

1. Introduction

Insecticides, as primary components in mosquito vector control, play a critical role in preventing mosquito-borne diseases [1–3]. Pyrethroids, the class of insecticides recommended by the WHO for bed-net treatment and indoor residual spraying, are used for disease vector management [4], but the development of resistance to pyrethroids in mosquitoes now poses a serious threat to mosquito control and mosquito-borne disease efforts worldwide [5–7]. Innovative strategies and/or insecticides are needed to sustain mosquito control effectively [8–10], hence a good understanding of the molecular basis underlying insecticide resistance is essential [7,11].

Although the involvement of the G protein–coupled receptor (GPCR) in the insecticide resistance of *Culex quinquefasciatus* through the regulation of resistance-related P450 gene expression is now clear [12–14], precisely how the GPCR-regulatory pathway affects insecticide resistance is not yet fully understood [15]. Li et al. [14] identified the involvement of 3′,5′-cyclic adenosine monophosphate

(cAMP) in P450 gene regulation and permethrin resistance in the *Culex* mosquitoes. Cellular cAMP is produced by G-protein subunit-stimulated adenylyl cyclase (AC) in both mammals and insects [16] and an interaction between the Gs alpha subunit protein (Gαs) and AC produced cAMP has been implicated in diverse intracellular signal transduction pathways [17], including the cell development in fungus [18], gene expression regulation in human cells [19], and the cAMP-PKA regulation pathway in multiple organisms [20], as well as the adenosine signaling pathway in *Drosophila* [21]. Nevertheless, the regulatory function of the Gα-protein and AC in insecticide resistance is still unclear. In the current study, we took a new approach by utilizing RNA-interference (RNAi) to both characterize the roles of Gαs and AC genes in the G-protein-coupled receptor regulation pathway, which has been shown to be involved in the development of insecticide resistance in *Culex* mosquitoes [13,14], and investigate the pivotal roles played by Gαs and ACs in P450 gene expression regulation and permethrin resistance. In order to focus specifically on the potential cellular functions of specific genes and pinpoint the gene expression characters in the

GPCR regulatory pathway, we also characterized the expression profile of GPCR, Gαs, AC, PKA and the P450 genes throughout the life cycle (egg, 1st/2nd instar larva, 3rd/4th instar larva, pupa, adult) of *Culex quinquefasciatus* mosquitoes and the specific tissue expression of the genes in their brain, midgut, malpighian tubules, ovary and thorax.

2. Materials and methods

2.1. RNA extraction, cDNA preparation and quantitative real-time PCR (qRT-PCR)

Two insecticide resistance mosquito strains, HAmCq^{G8} and MAmCq^{G6}, with ~2700 and ~570-fold resistance to permethrin, respectively [22] were used for this study. Total RNAs were extracted from different mosquito tissues (brain, midgut, malpighian tubules, ovary, and thorax) and mosquito life cycle stages (egg, 1st/2nd instar larva, 3rd/4th instar larva, pupa, adult) using the acidic quinidine thiocyanate-phenol-chloroform method [23]. Prior to cDNA synthesis, DNA was removed from the total RNA using a TURBO DNA-free kit (Ambion) following the manufacturer's instructions. cDNA was synthesized by SuperScript® IV Reverse Transcriptase (Invitrogen) following the method specified in the kit manual. The qRT-PCR was performed using the PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and ABI 7500 Real Time PCR system. Each qRT-PCR reaction (25ul) consisted of SYBR Green master mix, specific primer pairs for genes (Table 1) at final concentrations of 3–5 μM, and a 1 μg cDNA template from each mosquito sample site. The negative control was a "no-template" reaction. The reaction cycle applied the following PCR program: a melting step of 50 °C for 2 min, then 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the PCR reactions was assessed by a melting curve analysis using Dissociation Curves software [24]. All the reactions were run on 3 technical replicates. Relative expression levels of target genes were analyzed by the $2^{-\Delta\Delta CT}$ method using SDS RQ software [25]. The 18S ribosomal RNA (rRNA) gene served as an endogenous control because of its constitutive expression in all samples [13]. Each experiment was repeated on more than 3 independent isolated RNA mosquito samples. The statistical data analysis was conducted using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (Statistical Package for the Social Sciences-SPSS software with both Least Significant Difference-LSD and Tukey tests for analysis of significance of means); a value of P ≤ 0.05 was considered statistically significant. Significant up-regulation or down-regulation was determined using a cut-off value of a "2-fold change in expression" [26].

2.2. Double-strained RNA (dsRNA) synthesis and microinjection

The dsRNA with length ~300–600 bp was synthesized using the MEGAscript T7 High Yield Transcription Kit (Ambion). The primers were designed for each gene based on the T7 promotor sequences (Table 1). Synthesized dsRNA was purified by phenol/chloroform extraction and then ethanol precipitation. A dsRNA of a green fluorescent protein (GFP) gene complementary to a pMW1650 plasmid served as

Table 1
Oligonucleotide primers used in qRT-PCR and PCR reactions.

Primer Description	Primer Name	Primer Sequence
18S Ribosomal RNA	18S rRNA F1	5′CGCGGTAATTCCAGCTCCACTA3′
	18S rRNA R1	5′GCATCAAGCGCCACCATATAGG3′
GPCR020021 Real-time PCR	qPCR GPCR020021 F	5′ACTACCTCACCGACACCTTCTC3′
	qPCR GPCR020021 R	5′GCCTTGATGATGAAGATG3′
AC020210 Real-time PCR	qRT AC020210 F	5′GCCGTAGCGTTAGGTCTATTT3′
	qRT AC020210R	5′CGATGAGCGAGGTGGAAATTA3′
AC015189 Real-time PCR	qRT AC015189 F	5′AGCGAACAAGAGCAACTTCT3′
	qRT AC015189R	5′GCGGTGGTCGAGGTTTAAT3′
AC004739 Real-time PCR	qRT AC004739 F	5′GATTCTCGGCGATTGCTACT3′
	qRT AC004739 R	5′CTTCGCGGACGAACCTTATAG3′
AC007240 Real-time PCR	qRT AC007240 F	5′GCGAGAAGAACATCGAGGAA3′
	qRT AC007240 R	5′GCCCTCCAGTTCGATGTAAA3′
AC007059 Real-time PCR	qRT AC007059 F	5′CTACTCGGCCCAGGATTTG3′
	qRT AC007059R	5′AGCAATCACCCAGGATCTTTAT3′
AC015155 Real-time PCR	qRT AC015155 F	5′GGCTAGAGCTGGAACGAAAG3′
	qRT AC015155 R	5′GTAGACCACCTGATTGGTGAAG3′
PKA000798 Real-time PCR	qPCRPKA000798F	5′TTGATTGGTGGGCATTAGGCGTTC3′
	qPCRPKA000798R	5′AGCAGCTTCTTGACCAGGTCCTTT3′
PKA018257 Real-time PCR	qPCRPKA018257F	5′ATACCGTGACTTGAAGCGGAGAA3′
	qPCRPKA018257R	5′AATTTGTATTGGCTGATCAGC3′
CYP9M10 Real-time PCR	qPCRP4509M10F	5′ATGCAGACCAAGTGCTTCCTGTAC3′
	qPCRP4509M10R	5′AACCCACTCAACGTATCCAGCGAA3′
CYP9J40 Real-time PCR	qPCRP4509J40F	5′ACCCGAATCCGGGCAAGTTTGAT3′
	qPCRP4509J40R	5′AACTCCAAACGGTAAATACGCCGC3′
CYP6AA7 Real-time PCR	qPCRP4506AA7F	5′ATGACGCTGATTCCCGAGACTGTT3′
	qPCRP4506AA7R	5′TTCATGGTCAAGGTCTCACCCGAA3′
CYP9J34 Real-time PCR	qPCRP4509J34F	5′ATCCGATGTCGGTAAAGTGCAGGT3′
	qPCRP4509J34R	5′TGTACCTCTGGGTTGATGGCAAGT3′
Gαs006458 Real-time PCR	qPCRGαs006458F	5′CATCCGGTCCTGACTTCAATTA3′
	qPCRGαs006458R	5′TTCGTTTGATCGCTCGTAGG3′
GPCR020021dsRNAsynthesis	dsRNAGPCR020021F	5′TAATACGACTCACTATAGGGGCCATCTTCTTCCTGTGC3′
	dsRNAGPCR020021R	5′TAATACGACTCACTATAGGGCGGGGCGAAGTACACGAA3′
Gαs006458 dsRNAsynthesis	dsRNAGαs006458F	5′TAATACGACTCACTATAGGGTCGCTGGAGCTGTTCAAA3′
	dsRNAGαs006458R	5′TAATACGACTCACTATAGGGTGTCTACCGCACAGGTAAAG3′
AC007240dsRNA synthesis	dsRNAAC007240 F	5′TAATACGACTCACTATAGGGCAAGGTGTGCGTGATGTTTG3′
	dsRNAAC007240 R	5′TAATACGACTCACTATAGGGCGTTCACGTCCGTAATCTTCT3′
AC004739dsRNA synthesis	dsRNAAC004739F	5′TAATACGACTCACTATAGGGCCTGTACTACCGCATCATGTC3′
	dsRNAAC004739R	5′TAATACGACTCACTATAGGGCTTCGCGGACGAACCTTATAG3′
GFP gene dsRNA synthesis	dsRNAGFPF	5′TAATACGACTCACTATAGGGAGAAGAACTTTTCACTGG3′
	dsRNAGFPR	5′TAATACGACTCACTATAGGGCTTCTACCTAGGCAAGTT3′

the negative control. Non-injected mosquitoes were used for the calibration. The embryo microinjection method utilized in this study was as previously described [13,14]. In brief, ~1000 fresh grey embryos of HAmCq^{G8} and MAmCq^{G6} were individually injected with dsRNA of GPCR, Gαs, ACs, and the GFP control using the Picospritzer III injector system (Parker Instrumentation) under a Nikon Eclipse TS100 microscope (Nikon Instruments). The survival ratio was ~50% and the hatched mosquitoes developed to 2nd instar larvae that were then subjected to the larva bioassay and gene expression. Each experiment was repeated at least 3 times with independent injection and RNA extraction.

2.3. Permethrin insecticide bioassay

The bioassay method used for the larvae was as described in previous studies [13,14,22]. Stock and serial dilutions of permethrin (94.34%, supplied by FMC Corp., Princeton, NJ) for the insecticide bioassays were prepared in acetone. Each bioassay consisted of 2nd instar mosquito larvae of Gαs-, AC-dsRNA-, GFP-injected or non-injected mosquitoes in a 1% insecticide solution in acetone at the required concentration in regular tap water at four to eight concentrations that resulted in > 0 and < 100% mortality. LC$_{50}$ was analyzed using a standard probit analysis. Control groups received only 1% acetone. Mortality was assessed after 24 h. All tests were run at 25 °C and each assay was replicated at least 3 times. Bioassay data were pooled and analyzed by a standard probit analysis [22]. The statistical analysis for the insecticide bioassay was conducted by examining the LC$_{50}$ values based on non-overlapping 95% confidence intervals (CI). Resistance ratios (RRs) were calculated by dividing the LC$_{50}$ of the specific gene injected or GFP-injected mosquitoes by the LC$_{50}$ of the non-injected mosquitoes.

3. Results

3.1. Impact of GPCRs on the expression of ACs in insecticide resistant mosquitoes

The GPCR gene GPCR020021 (CPIJ020021) has been implicated in the development of permethrin resistance through its role in regulating resistance-related P450 gene expression in permethrin resistance *Culex* mosquito strains [14]. Functional studies have revealed that two PKA genes also play key roles in the permethrin resistance of mosquitoes through the GPCR020021 (CPIJ020021) regulatory pathway in both MAmCq^{G6} and HAmCq^{G8} mosquitoes [14]. To further determine the involvement of GPCR downstream effectors in insecticide resistance, in this study we investigated the impact of GPCR020021 on the expression and function of six ACs that have been annotated in *Cx. quinquefasciatus* genome by knockdown of the GPCR gene. The results show a significant decrease in the expression of the AC004739 (CPIJ004739) gene in GPCR020021 dsRNA injected HAmCq^{G8} (Fig. 1A) compared to no change in the expression in either dsRNA-GFP- or non-injection mosquitoes; the expression of the AC004739 andAC007240 (CPIJ007240) genes both decreased significantly in dsRNA-GPCR020021 injected MAmCq^{G6} (Fig. 1B). These results suggest that these downstream effectors AC007240 and AC004739 play essential roles in GPCR regulatory pathway-mediated insecticide resistance of *Culex* mosquitoes.

3.2. Impact of ACs on GPCR pathway-mediated insecticide resistance

Since these two PKA genes, PKA018257, and PKA000798, are known to be involved in the development of permethrin resistance in *Culex* mosquitoes [14], we focused on a functional test of the two AC genes, AC007240 and AC004739, in permethrin resistance and their regulatory effects on PKA and resistance P450 gene expression. The dsRNA of AC007240 was injected into the embryos of resistant MAmCq^{G6} mosquitoes. Second instar larvae were then tested for any

changes in their gene expression and permethrin sensitivity. The qRT-PCR and larva bioassay results revealed that knockdown of AC007240 gene expression (3.2-fold decrease in gene expression compared to non-injected mosquitoes, Table 2) in the resistant strain MAmCq^{G6} caused a ~3-fold decrease in resistance level to permethrin (resistance ratio was 0.37 ± 0.01 compared to non-injected mosquitoes), demonstrating a strong correlation between AC007240 gene expression and insecticide resistance in this mosquito. The dynamic changes in the expression of two down-stream PKAs (PKA000798 and PKA018257) and three resistance P450s (CYP9M10, CYP 6AA7, and CYP 9J34) were further investigated in the AC007240 knockdown mosquitoes, showing that the expression of both PKAs and all the P450 genes tested had decreased significantly in the MAmCq^{G6} mosquitoes (Fig. 2A). Knockdown of the AC004739 gene in both HAmCq^{G8} and MAmCq^{G6} with 2.3 and 3.0 fold decreases, respectively, resulted in decreased levels resistance to permethrin with resistance compared to non-injected mosquitoes (Table 2). Interestingly, the expression of two PKAs (PKA000798 and PKA018257) and two P450 genes (CYP6AA7 and −9J40) were decreased following the knockdown of the AC004739 gene in HAmCq^{G8} (Fig. 2B), while the expression of only PKA000798 and two P450s (CYP6AA7 and -9J34) decreased in MAmCq^{G6} (Fig. 2C). These results strongly suggest the functional importance and common involvement of AC genes (AC007240 and AC004739) in insecticide resistance in *Culex* mosquitoes.

3.3. Impact of Gαs on GPCR downstream effectors and insecticide resistance

The G-protein plays multiple critical roles in GPCR regulatory pathways. In particular, one of the subunits, Gαs, has been identified as an important regulation factor in the GPCR/AC/PKA-intracellular transduction pathway [17]. We therefore tested the function of *Culex* mosquito Gαs006458 (CPIJ006458) in the two insecticide resistant strains using RNAi, finding a strong correlation between knockdown of the Gαs006458 gene in HAmCq^{G8} and MAmCq^{G6} (with 1.3- and 1.6-fold decreases, respectively) and decreased levels of resistance to permethrin with decreased resistance ratios of 0.57 ± 0.04 and 0.29 ± 0.04, respectively, compared to non-injected mosquitoes (Table 2). To confirm the involvement of Gαs006458 in the GPCR regulatory pathways, the impact of Gαs006458 on the expression of two downstream PKAs (PKA018257 and PKA000798) implicated in a previous study [14] and two ACs (AC007240 and AC004739) that were also involved in the GPCR020021 pathway was investigated in Gαs006458 knockdown mosquitoes. The results showed that suppression of the Gαs006458 gene in the dsRNA of Gαs injected mosquitoes caused a significant decrease in the gene expression of the ACs (AC007240 and AC004739), PKAs (PKA018257 and PKA000798), and resistance-related P450s (CYP9M10, -9J34, -6AA7 and -9J40) in both HAmCq^{G8} and MAmCq^{G6} mosquitoes (Fig. 3A, B). These results indicate that Gαs is indeed engaged in both the GPCR/G-protein/AC/PKA molecular pathway and in the P450-mediated insecticide resistance in *Cx. quinquefasciatus*.

3.4. Developmental expression of GPCR, Gαs, ACs, PKAs, and P450 genes

Since very little is known regarding the developmental expression of the GPCR, Gαs, ACs and PKAs genes in mosquitoes, we tested the dynamic changes in the expression of these genes throughout the *Culex* mosquito life cycle stages, including egg, 1st/2nd instar larva, 3rd/4th instar larva, pupa, and adult for both HAmCq^{G8} and MAmCq^{G6} mosquito strains. We chose the gene expression level in the egg as the base line (relative gene expression = 1) to make the gene expression comparison between the different development stages clear. The GPCR020021 gene showed a similar expression pattern throughout the developmental stages of both the HAmCq^{G8} and MAmCq^{G6} strains, with the highest expression being in the 3rd/4th instar larva (about 500- and
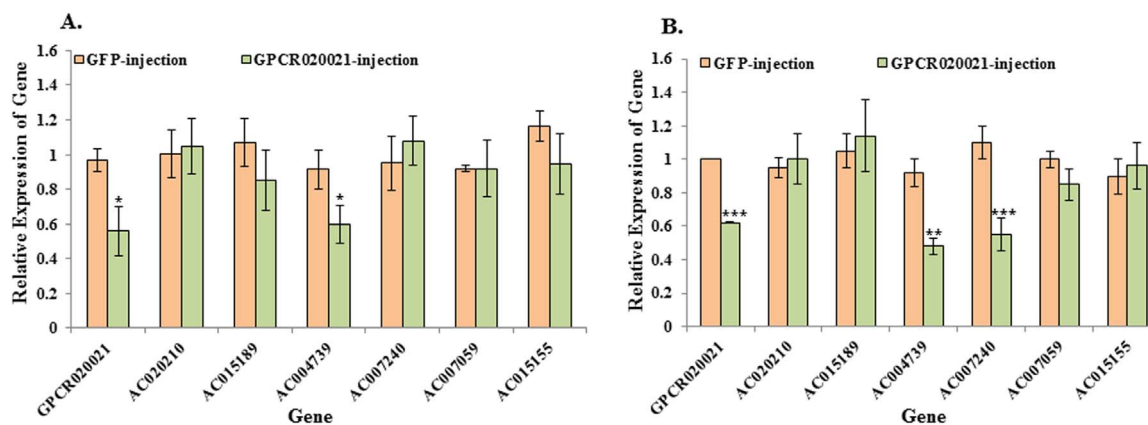
Fig. 1. Gene expression of ACs and PKAs in dsRNA-GPCR injection mosquitoes. A. The relative expression of GPCR020021, six AC genes in the GPCR020021-dsRNA injection and GFP-dsRNA injection mosquitoes are shown along the Y-axis. B. The relative expression of GPCR020021, six AC genes in the GPCR020021-dsRNA injection and GFP-dsRNA injection MAmCq^{G6} mosquitoes are shown along the Y-axis. All the gene expression levels were measured in 2nd instar larvae, 3-days-post injection of the mosquito embryos. The relative expression of genes in non-injected mosquitoes is served as calibrator (defined as 1), which is not shown in figures. The results are shown as the mean ± S.E (n ≥ 3). Statistical significance of the gene expression among GPCR-, GFP- and non-injection mosquitoes was analyzed using One-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 2
Relative gene expression and insecticide resistance in RNAi injected and non-injected mosquitoes.

Target gene	RNAi	HAmCq^{G8}			MAmCq^{G6}		
		LC$_{50}$ (ppm)	Resistance Ratio[a]	Gene expression	LC$_{50}$ (ppm)	Resistance Ratio[a]	Gene expression
AC007240	Non-injection	–	–	–	0.16 ± 0.01a[b]	1.00a	1.00a
	GFP-injection				0.15 ± 0.01a[b]	0.94 ± 0.02a	0.94 ± 0.04a
	AC007240-injection				0.06 ± 0.01b[b]	0.38 ± 0.01b	0.31 ± 0.13b
AC004739	Non-injection	0.35 ± 0.01a	1.00a	1.00a	0.14 ± 0.09a	1.00a	1.00a
	GFP-injection	0.37 ± 0.02a	1.05 ± 0.02a	1.08 ± 0.12a	0.17 ± 0.02a	1.21 ± 0.11a	0.96 ± 0.03a
	AC004739-injection	0.07 ± 0.01b	0.21 ± 0.01b	0.43 ± 0.05b	0.07 ± 0.01b	0.52 ± 0.09b	0.33 ± 0.09b
Gαs006458	Non-injection	0.31 ± 0.05a	1.00a	1.00a	0.10 ± 0.06a	1.00a	1.00a
	GFP-injection	0.29 ± 0.01a	0.94 ± 0.02a	1.07 ± 0.04a	0.09 ± 0.01a	0.94 ± 0.09a	1.03 ± 0.03a
	Gαs006458-injection	0.17 ± 0.01b	0.55 ± 0.04b	0.75 ± 0.03b	0.03 ± 0.01b	0.29 ± 0.04b	0.60 ± 0.05b

[a] Resistance Ratios were calculated by dividing the LC$_{50}$ of the GFP or target gene injection mosquito groups by the LC$_{50}$ of the non-injection mosquito group.
[b] Statistical significance is presented by P ≤ 0.05 in the level of the gene expression or resistance ratio/LC$_{50}$ among the target gene-injected, GFP-injected and no-injected mosquitoes with different alphabet letters (a or b) using One-way ANOVA.

50-fold, respectively, higher than in the eggs) and the lowest expression in adults and/or eggs (Fig. 4A, B). Unlike the significant expression diversity of GPCR020021 across the developmental cycle, the Gαs006458 gene presented quite similar expression levels throughout the development cycle in both the HAmCq^{G8} and MAmCq^{G6} strains, with only 5- to 2-fold differences across all the life stages tested (Fig. 4A, B). Both AC genes, AC004739 and AC007240, also showed similar expression patterns in both HAmCq^{G8} and MAmCq^{G6} strains, with the highest expression in adults followed in descending order by pupa ≥ larva stages ≥ egg (Fig. 4A, B). Both PKA genes, PKA000798 and PKA018257, showed their highest expression levels in the adult and egg stages in both the HAmCq^{G8} and MAmCq^{G6} strains (Fig. 4A, B), except that PKA000798 was lower in the eggs of HAmCq^{G8} mosquitoes (Fig. 4A). Apart from *CYP9M10*, whose expression was developmentally regulated and specifically overexpressed in the larval stages (~50-fold in 3rd/4th instar larva > ~20-fold higher expression in 1st/2nd instar larva > 2-fold in egg > pupa/adult in both strains), the expression of the other three P450 genes, *CYP6AA7*, *CYP9J34*, *CYP9J40*, showed relatively similar expression levels across the development stages. CYP6AA7 showed similar expression levels in both adults and larval stages with a ~3-fold higher expression compared to the pupa and egg stages in both strains (Fig. 4A, B). *CYP9J34* showed an expression order of ~10-fold and ~15-fold in 1st/2nd instar larva > ~4-fold and 5-fold in 3rd/4th instar larva and egg > pupa and adult in the HAmCq^{G8} and MAmCq^{G6} strains, respectively (Fig. 4A, B). *CYP9J40*, which is specifically overexpressed in HAmCq^{G8} [27], presented a 3-fold higher expression in the larva and egg stages compared with the pupa and adult

stages in HAmCq^{G8} (Fig. 4A).

3.5. Tissue-specific expression of GPCR, Gαs, ACs, PKAs, and P450

To investigate whether the expression of GPCR, Gαs, ACs, PKAs, and P450 is tissue specific, RNAs from the brain, midgut, malpighian tubules (MTs), ovary, and thorax of female HAmCq^{G8} and MAmCq^{G6} adults were subjected to qRT-PCR analyses. Here, the expression level in the thorax was used as the base line (relative gene expression = 1) for the gene expression comparison in different tissues. The results of the relative gene expression test by qRT-PCR revealed that GPCR020021 was predominantly expressed in the brain with levels ~100- and ~20-fold higher than in other tissues (Fig. 5A, B) in HAmCq^{G8} and MAmCq^{G6}, respectively. The expression level of the Gαs006458 gene was different in the two strains, following a descending order of ~20-fold in the brain > ~5-fold in MTs and ovary > midgut and thorax in HAmCq^{G8} and ~8-fold in the brain > ~2-fold in midgut and ovary > thorax and MTs in MAmCq^{G6} (Fig. 5A, B).

The two AC genes (AC004739 and AC007240) also showed their highest expression in the brain in both the HAmCq^{G8} and MAmCq^{G6} strains, with levels ~70 to ~140-fold higher than those found in the midgut (Fig. 5A, B). While the two ACs in HAmCq^{G8} showed similar expression levels in the other tissues tested, the expression of AC007240 in MAmCq^{G6} exhibited the following expression order: ~40-fold in ovary > ~20-fold in thorax > ~6-fold in MTs > midgut; the AC004739 expression followed the order: thorax (~15-fold) > ovary
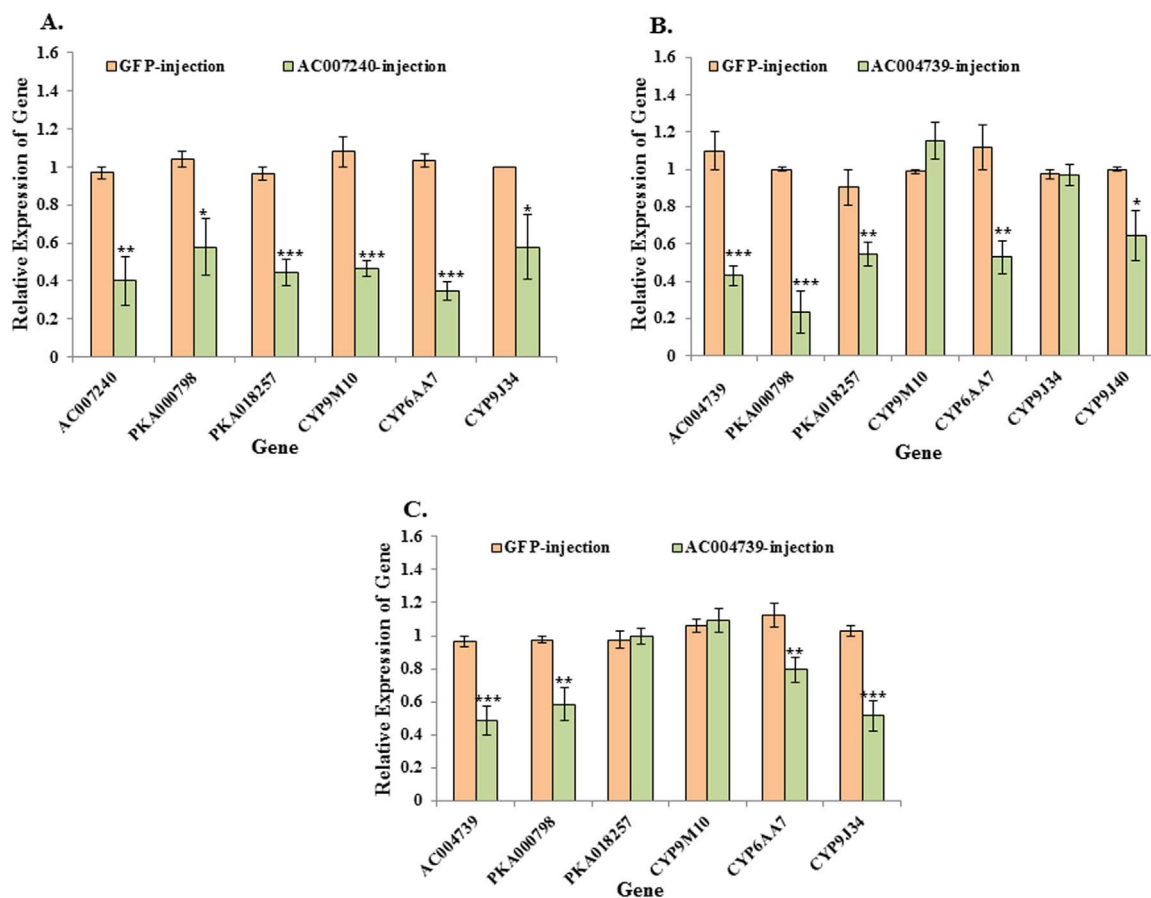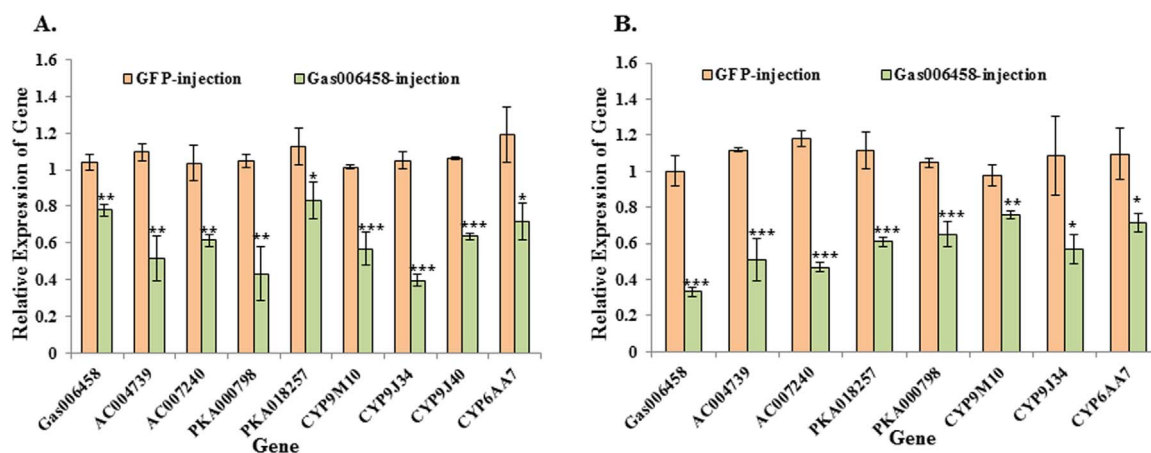
Fig. 2. Gene expression of ACs, PKAs and P450s in dsRNA-ACs injection mosquitoes. A. The relative expression of AC007240, two PKA and three P450 genes in dsRNA-AC007240- and GFP-injection MAmCq^{G6} mosquitoes is shown along the Y-axis. B. The relative expression of AC004739, two PKA and four P450 genes in dsRNA-AC004739- and GFP-injection HAmCq^{G8} mosquitoes is shown along the Y-axis. C. The relative expression of AC004739, two PKA and three P450 genes in dsRNA-AC004739- and GFP-injection MAmCq^{G6} mosquitoes is shown along the Y-axis. All the gene expression levels were measured in 2nd instar larvae, 3-days-post injection of the mosquito embryos. The relative expression of genes in non-injection mosquitoes is served as calibrator (defined as 1), which is not shown in figures. The results are shown as the mean ± S.E (n ≥ 3). Statistical significance of the gene expression among AC007240-, GFP- and non-injection mosquitoes was analyzed using One-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001.

(~4-fold) > midgut and MTs. Similarly, the expression levels of two PKA genes, PKA000798 and PKA018257, were highest in the brain in both mosquito strains, followed by a decreasing expression order of thorax ≥ ovary/MTs > midgut for PKA000798 and an equal expression level in thorax, ovary, MTs, midgut tissues for PKA018257 (Fig. 5A, B).

Two P450 genes, *CYP9M10* and *CYP6AA7*, were expressed at levels ~2-fold higher in the brain than in other tissues in HAmCq^{G8} and similarly expressed (except for being 1.5-fold higher in the brain, ovary,

Fig. 3. Gene expression of Gαs, ACs, PKAs and P450s in dsRNA-Gαs injection mosquitoes. A. The relative expression of Gαs006458, two AC, two PKA and four P450 genes in dsRNA-Gαs006458- and GFP-injection HAmCq^{G8} mosquitoes is shown along the Y-axis. B. The relative expression of Gαs006458, two AC, two PKA and four P450 genes in dsRNA-Gαs006458- and GFP-injection MAmCq^{G6} mosquitoes is shown along the Y-axis. All the gene expression levels were measured in 2nd instar larvae, 3-days-post injection of the mosquito embryos. The relative expression of genes in non-injection mosquitoes is served as calibrator (defined as 1), which is not shown in figures. The results are shown as the mean ± S.E (n ≥ 3). Statistical significance of the gene expression or resistance ratio among Gαs006458-, GFP- and non-injection mosquitoes was analyzed using One-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001.
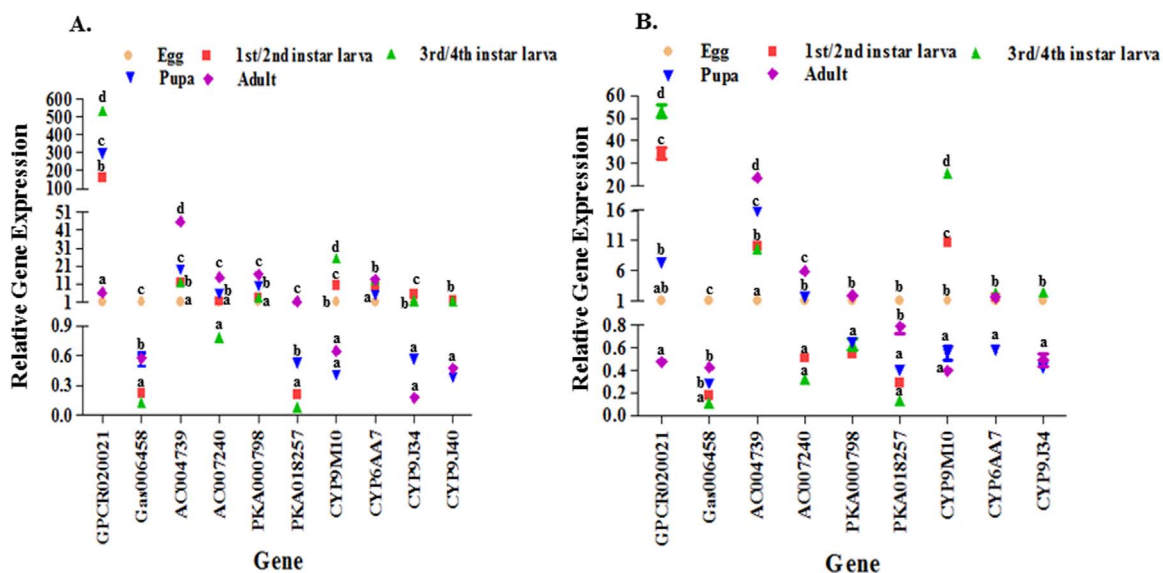
Fig. 4. Relative expression of genes in the *Culex* mosquito life stages. Total RNAs were extracted from mosquito life cycle stages including egg, 1st/2nd instar larva, 3rd/4th instar larva, pupa, and adult. The relative gene expression shown along the Y-axis was tested by qRT-PCR. Specific gene names are shown along the X-axis. Each figure legend presents different development stages; gene expressions were based on > 3 independent RNA extractions. A. Temporal dynamic data for the gene expression of GPCR020021, Gαs006458, AC007240, −004739, PKA000798, −018257, *CYP9M10*, −6AA7, −9J34, and −9J40 for different life cycle stages of HAmCq^{G8}. B. Temporal dynamic data for the gene expression of GPCR020021, Gαs006458, AC007240, −004739, PKA000798, −018257, *CYP9M10*, −6AA7, and -9J34 for different life cycle stages of MAmCq^{G6}. The results are shown as the mean ± S.E (n ≥ 3). There were significant differences (P < 0.05) in the levels of gene expression among the samples labeled with different letters (a, b, c, or d).

and midgut than that in MTs for *CYP6AA7*) in all the tissues tested in MAmCq^{G6} (Fig. 5A, B). *CYP9J34* showed its highest expression level in the midgut (~10-fold) than in other tissues in HAmCq^{G8} and higher expression levels in the midgut and thorax (~5-fold) in MAmCq^{G6} (Fig. 5A, B). *CYP9J40*, which was specifically overexpressed in HAmCq^{G8} [27], showed a ~60-fold higher expression level in the midgut and was ~10-fold higher in the MTs compared to other tissues (Fig. 5A).

4. Discussion

Previous studies have shown that GPCRs play an essential role in the development of insecticide resistance in mosquitoes in regulating the resistance-related P450 gene expression via the cAMP/PKA regulatory pathway [13,14]. The current study explored further to understand precisely how the GPCR pathway functions in resistance by investigating two additional GPCR downstream effectors, Gαs and ACs, their relationship with GPCR, their impact on both PKAs and resistance P450s, and their involvement in the permethrin resistance of
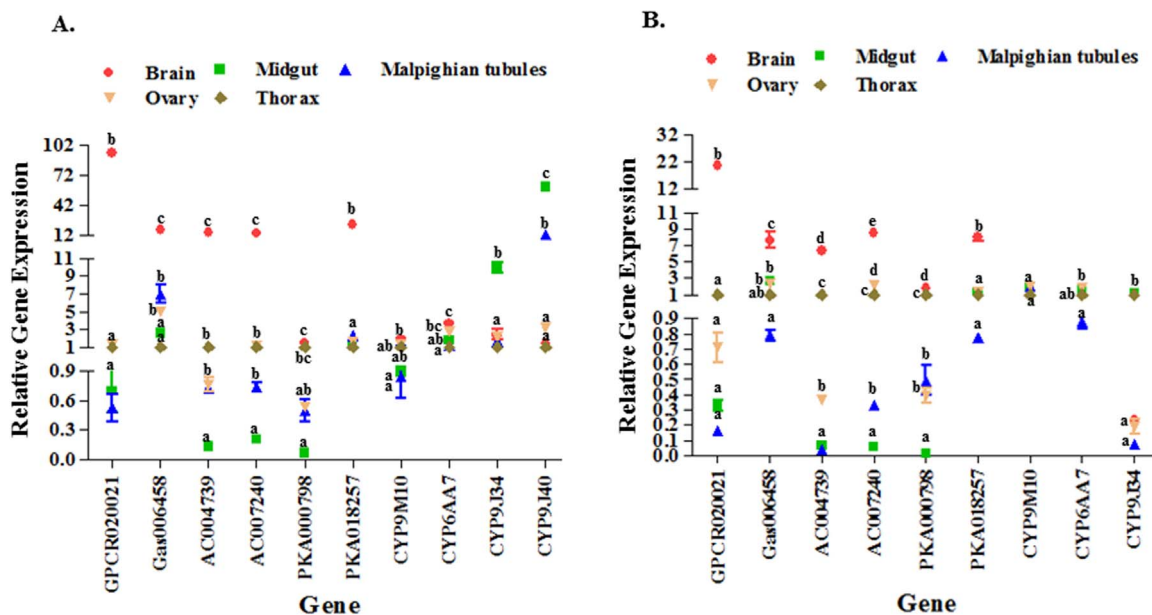

Fig. 5. Relative expression of genes in the tissues of *Culex* mosquitoes. Specific gene names are shown along the X-axis. Each figure legend presents a different tissue; gene expressions were based on > 3 independent RNA extractions. A. Relative gene expression of GPCR020021, Gαs006458, AC007240, −004739, PKA000798, −018257, *CYP9M10*, -6AA7, -9J34, and -9J40 in different tissues of HAmCq^{G8}. B. Relative gene expression of GPCR020021, Gαs006458, AC007240, −004739, PKA000798, −018257, *CYP9M10*, −6AA7, and −9J34 in different tissues of MAmCq^{G6}. The results are shown as the mean ± S.E (n ≥ 3). There were significant differences (P < 0.05) in the levels of gene expression among samples labeled with different letters (a, b, c, d, or e).

mosquitoes. ACs have been extensively characterized in the GPCR regulatory pathway and have been considered potential medical therapy targets in mammalian research because of their important function as a "producer" for cAMP, which is known to be a critical regulator or second messenger in several intracellular networks [28]. The involvement of cAMP and PKAs in the GPCR regulation of insecticide resistance has been reported [14], leading us to speculate on the roles of the ACs in this GPCR regulatory pathway. In this study, knocking down the GPCR020021 by RNAi in resistant mosquitoes decreased the expression of both the AC004739 and AC007240 genes, suggesting that these ACs are indeed involved in the GPCR regulatory pathway. Subsequently, knocking down these two ACs in insecticide resistant mosquitoes resulted in not only decreasing the expression of two downstream PKA genes (PKA000798 and PKA018257) and four resistance P450 genes (*CYP9M10, -6AA7, -9J34*, and *9J40*) genes, but also an increased susceptibility of the resistant mosquito strains to permethrin. It is interesting to note that different ACs regulated the expression of different resistance P450 genes in the two strains, suggesting the joint action of these ACs on the regulation of resistance P450 gene expression in mosquitoes.

In contrast, G-proteins, the upstream regulator of AC, are critical signal transducers in the GPCR signaling pathways. They are responsible for dealing with the outside signals arriving through GPCRs and interacting with other intracellular factors to form networks within the cell [17]. In particular, Gαs in the GPCR complex are essential for several physiologic regulation pathways based on the conformational changes observed in the G-protein and GPCRs [29,30], as well as mediating the cell response to stress and regulated cell death [31]. In the current study, the knockdown Gαs006458 in resistant *Culex* mosquitoes triggered an increased susceptibility of the mosquitoes to permethrin, implying the functional potency of Gαs in permethrin resistant mechanisms. Downstream genes were also affected, with two ACs, two PKAs and four P450s showing decreased expression following Gαs knockdown in the permethrin resistant mosquitoes. Taken together, this strongly indicates the essential role of Gαs in insecticide resistance through the GPCR mediated regulation pathway in the regulation of P450 mediated resistance in the mosquito *Cx. quinquefasciatus*.

Spatial expression patterns in genes have been frequently characterized in order to highlight the potential physiological functions of the genes in many insect species [32–35]. As a further step towards drawing inferences regarding the precise function of the GPCR-regulatory pathway, the functional relationships among gene families, and possible linkages between the genes and their consequent resistance phenotypes, tissue-specific expression patterns in GPCRs, Gαs, ACs, PKAs, and P450s in two insecticide resistant mosquito strains were investigated. All the GPCR signaling pathway components tested in this study, namely GPCRs, Gαs, ACs and PKAs, had their highest expression in the brain tissue of both the *Cx. quinquefasciatus* strains when compared with the levels in the midgut, MTs, ovary, and thorax, indicating the essential roles of these genes in the insect's central nervous system and corresponding to their signal transduction functions. Indeed, over 90% of the GPCRs have also been reported as being specifically expressed in the human brain [36]. In addition to their specific expression in the brain tissue of mosquitoes, these genes also showed different expression levels in all the tissues tested, suggesting their wide range of initial functions in the different mosquito organs. While resistance related P450 genes in mosquitoes were found to be differentially distributed in different tissues, we also found that *CYP9M10* and *CYP6AA7* were expressed at higher levels in the brain, whereas *CYP9J34* and *CYP9J40* showed higher expression levels in the midgut, thorax and/or MT tissues, suggesting their critical function in the central nervous system and importance for detoxification. Similar findings have also been reported in *Drosophila*, where numerous P450 genes were found to be expressed in tissues such as the malpighian tubules, midgut, and brain [37]. Given the diverse physiological functions that cytochrome P450s fulfill, especially in response to physiological and environmental

stimulators and in xenobiotic detoxification, this is no doubt why insect P450s are expressed differently in different tissues. In insects, the midgut and fat body tissue are generally considered to be the primary detoxification organs where most insect detoxification P450s are expressed [38]. Nevertheless, other tissues such as the brain [39] and nervous system [40] may also be important for P450 gene expression and the insect's response to insecticides.

Author contributions

Performed the experiments: T.L. Analyzed the data: N.L., T.L. Contributed reagents/materials/analysis tools: N.L., Wrote the paper: N.L., T.L.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.08.010.

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