Molecular Cloning and Characterization of G Alpha Proteins from the Western Tarnished Plant Bug, *Lygus hesperus*

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**Abstract:** The Ga subunits of heterotrimeric G proteins play critical roles in the activation of diverse signal transduction cascades. However, the role of these genes in chemosensation remains to be fully elucidated. To initiate a comprehensive survey of signal transduction genes, we used homology-based cloning methods and transcriptome data mining to identify Ga subunits in the western tarnished plant bug (*Lygus hesperus* Knight). Among the nine sequences identified were single variants of the Gai, Gao, Gas, and Ga12 subfamilies and five alternative splice variants of the Gaq subfamily. Sequence alignment and phylogenetic analyses of the putative *L. hesperus* Ga subunits support initial classifications and are consistent with established evolutionary relationships. End-point PCR-based profiling of the transcripts indicated head specific expression for LhGaq4, and largely ubiquitous expression, albeit at varying levels, for the other LhGa transcripts. All subfamilies were amplified from *L. hesperus* chemosensory tissues, suggesting potential roles in olfaction and/or gustation. Immunohistochemical staining of cultured insect cells transiently expressing recombinant His-tagged LhGai, LhGas, and LhGaq1 revealed plasma membrane targeting, suggesting the respective sequences encode functional G protein subunits.

**Keywords:** *Lygus hesperus*; plant bug; heterotrimeric G protein; signal transduction; Ga subunit; gene cloning; expression profile
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1. Introduction

Heterotrimeric guanine-nucleotide-binding proteins (G proteins) are molecular switches that mediate many extracellular signaling processes by coupling cell surface receptor activation with the diverse signal transduction effector molecules that drive cellular responses. The heterotrimeric G protein complex is composed of an a-subunit (Ga) that functions in guanine nucleotide binding/hydrolysis and a heterodimer composed of a b and g subunit (Gbg). In the absence of receptor stimulation, the three subunits are associated and GDP is bound to Ga. Receptor activation triggers GDP exchange for GTP and dissociation of Gbg from the Ga-GTP complex. The dissociated Ga and Gbg subunits are then able to modulate the activity of various downstream effector proteins (ion channels, adenylyl cyclases, phospholipase Cbg, etc.). The intrinsic GTPase activity of Ga hydrolyzes GTP to GDP, which promotes reassociation of the heterotrimeric G protein complex and terminates the signal [1–3]. Based on this intermediary molecular role, heterotrimeric G proteins play pivotal roles in determining the specificity and duration of the cellular response to extracellular signals.

The Ga subunits form a large multigene family composed of 39–52 kDa proteins that share 35%–95% sequence identity and have been grouped into four subfamilies (Gas, Gai/o, Gaq, and Ga12) based on structural and functional similarities [1–3]. Gas subfamily members couple receptors to adenylyl cyclase stimulation (i.e., increases in cAMP), whereas the Gai/o subfamily has the opposite effect. The Gaq subfamily regulate the activity of phospholipase Cbg isoforms (i.e., diacylglycerol and inositol triphosphate production) [1,2] and Ga12 has been extensively characterized based on their ability to activate Rho-specific guanine nucleotide exchange factors [4,5].

Chemosensory signaling in many vertebrates and invertebrates relies on canonical G protein-coupled pathways. In insects, however, the role of G proteins in chemosensory transduction has yet to be definitively established [6,7]. Insect olfactory and gustatory receptors have poor homology with canonical G protein-coupled receptors [8], exhibit inverted topologies [9–12], and are activated through an ionotropic mechanism in which the receptors function as ligand-gated ion channels [13–15]. Other studies though have reported a G protein-coupled metabotropic component in olfactory receptor activation [16]. In support of this pathway, Ga subunits are expressed in chemosensory tissues [17–21], G protein dependent effector pathways are activated by odorants [16,22–24], and inhibition of G protein activation negatively affects odorant perception [22,25–27] as does RNAi-mediated knockdown of Ga subunits [28,29]. In addition, G protein-coupled pathways have been implicated in gustatory receptor activation [30–36].

The western tarnished plant bug (Lygus hesperus) is a polyphagous pest of numerous crops [37,38] that utilizes chemosensory signals to aid in identification of host plants and conspecific mates [39–42]. Despite the pest status of the Lygus spp. complex, transcriptional resources have only recently been developed [43–46], and our knowledge of chemosensory signal transduction is limited to odorant binding proteins [45,47] and the olfactory receptor co-receptor (Orco) [12] in L. lineolaris and L. hesperus. Furthermore, while G proteins have been studied in a number of insects with Ga subunits cloned from Drosophila melanogaster [17,21,48–50], Anopheles gambiae [20], Bombyx mori [19,51,52], Manduca sexta [53], Locusta migratoria [54], Lissorhoptrus oryzophilus [55], Helicoverpa assaulta [56], Mamestra brassicaceae [18], Bemisia tabaci [57], and Oncopeltus fasciatus [58], little progress has been made on the role of these genes in mediating chemosensory behaviors in plant bugs such as Lygus. In
this study, we sought to begin to address this lack of knowledge by identifying the molecular sequences and expression profile of Ga subunits in *L. hesperus*. Using homology-based PCR and transcriptome database mining methods, we cloned a group of cDNAs with high sequence homology to each of the Ga subfamilies. In addition, we performed detailed sequence comparisons of the *L. hesperus* transcripts with those from other insects, profiled transcript expression levels, and examined the subcellular localization of a subset of recombinantly expressed *L. hesperus* Ga proteins in cultured insect cells.

2. Experimental Section

2.1. Insect Rearing

*L. hesperus* were obtained from an in-house stock colony (USDA-ARS Arid Land Agricultural Research Center, Maricopa, AZ, USA) periodically outbred with locally caught conspecifics. The colony is fed an artificial diet packaged in Parafilm M [59,60] and maintained under rearing conditions consisting of 27 °C, 40% humidity and a L14:D10 photoperiod. Experimental nymphs were generated from eggs deposited in oviposition packets and maintained as described previously [61].

2.2. Identification and Cloning of L. hesperus Ga Subunits

To identify *L. hesperus* Ga subunits (LhGa), we initially utilized a degenerate PCR approach similar to that reported previously in *B. mori* [51,52] using degenerate primers (Table 1) designed to conserved amino acid stretches identified in protein sequence alignments of known insect Ga sequences. Total RNA was isolated from adult *L. hesperus* female heads and bodies using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Isolated total RNA was quantified based on absorbance at 260 nm using a Take3 multi-volume plate on a Synergy H4 hybrid multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA). First strand cDNA was synthesized from 1 µg of DNase I-treated total RNA in separate Thermoscript or SuperScript III (Life Technologies, Carlsbad, CA, USA) first-strand cDNA synthesis reactions with random hexamers. To minimize primer bias towards particular classes of Ga proteins [62,63], multiple PCR amplifications were performed using ExTaq DNA polymerase (Takara Bio Inc./Clontech, Palo Alto, CA, USA) with 0.7 µL (35 ng) cDNA template and 2.5–3 µL (0.5–0.6 µM) of each primer and varying thermocycler conditions (Figure 1). Nested PCR was performed as above but using a 1-µL aliquot of the previous reaction as the template. PCR products were electrophoresed on 1.7% agarose gels and stained with SYBR Safe (Life Technologies). Amplimers of the expected sizes were gel-excised using an EZNA Gel Extraction kit (Omega Bio-Tek Inc., Norcross, GA, USA), cloned into the pGEM T Easy-TA cloning vector (Promega, Madison, WI, USA) and sequenced at the Arizona State University DNA Core Lab (Tempe, AZ, USA).

The partial fragments amplified above were extended by RACE PCR using templates generated with a SMARTer RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) and 2 µg DNase I-treated RNA. Amplification was performed using ExTaq with 0.5 µL (50 ng) cDNA, primers corresponding to one of the Universal Primers supplied with the SMARTer RACE cDNA Amplification kit, a gene specific primer (Table 1), and touchdown thermocycler conditions (Figure 1). PCR products were electrophoresed on 1.5% agarose gels with amplimers of the expected sizes gel excised and
sequenced. Incorporating the resulting 5' and 3' RACE sequence data with the degenerate PCR derived sequences yielded sufficient data to design gene specific primers encompassing the putative start and stop codons (Table 1). The respective *L. hesperus* Ga open reading frames (ORFs) were amplified in multiple independent reactions using ExTaq DNA polymerase and sequence verified. The consensus nucleotide sequence data are available in the GenBank database under the accession numbers: AEK80438 (LhGai), AEK80436 (LhGas), and AEK80437 (LhGaq1).

**Table 1. Oligonucleotide primers used.**

| Primer          | Sequence (5'–3')          | Primer          | Sequence (5'–3')          |
|-----------------|---------------------------|-----------------|---------------------------|
| Ga deg 1 F      | ACNATNGTNAARCARATG (TIVKQM) | LhGao 679 F     | CGACGTCGATACAGAGGATG      |
| Ga deg 2 F      | GAYTNGNCGNGCNYG (DVGQR)   | LhGao 1,211 R   | TTGTCAGATGCGCTCTTCTTT    |
| Ga deg 3 F      | AARTGGATHCYGTGGYTT (KWIIHF) | LhGao 499 F     | AACTACGTTCCAACTCAGC      |
| Ga deg 1a R     | RTCTTYTTRTINAGRAA (FLNKKD) | LhGai 1,026 R   | ATCAAGTCGACAGCATCGAAG     |
| Ga deg 1b R     | RTCYGTYTRGIRNAGRAA (FLNKKD) | LhGai 331 F     | GTCCGTCGACTATATAC        |
| Ga deg 2 R      | TCNGTACNRCRTGAANAC (VFDAVTD) | LhGai 862 R     | CTTGATCTTCCTCTGCCAG       |
| LhGai sp F2     | CAATGCTTGTTCGAGACTCCC     | LhGaq 474 F2    | GGCAGAATAGAAGACTGCA      |
| LhGai sp R1     | CATCTTCTGGACTAGACGCTG    | LhGaq 1,036 R1  | AAGGTITTGGCTCCGTAGATATT  |
| LhGai sp R2     | TGGTAGGTTCAGTGGCAGGAGT   | LhGaq 1,035 R2  | CTGATTGACTCTTCCTGAGCTGA  |
| LhGai sp F2b    | GGTCCGGATAGGCAAGACGAG    | LhGa12/13 1307 F | TGTGACGAGAATATGGCAAA      |
| LhGa2q F1       | CTTCTGGTTCGCTCTGAAACTG   | LhGa12/13 1303 R | CCACGAGAACTTGTACGAA      |
| LhGa2q F2       | TCNGTACNRCRTGAANAC (VFDAVTD) | LhGa12/13 1384 R | CTTGATCTTCCTCTGCCAG       |
| LhGa2q R1       | GGCGAGAATAGAGAGTCAGG     | LhGa12/13 1307 F | TGTGACGAGAATATGGCAAA      |
| LhGa2q R2       | TTCATTCGGTTCTCATTTCCGAGTCA | LhGa12/13 1303 R | CTTGATCTTCCTCTGCCAG       |
| LhGas F1        | CCGCTTCTTCATCTGGCAGCGCT | LhGai no stop R | TACAGAATCATTATTCGCC      |
| LhGas F2        | AAAGCCCGACGAGAACCGCTCCTCA | LhGai no stop R | AACAGGTGGTGACATCTCTCATCA |
| LhGas R1        | TGGAGCGGTCGGCTGCGGCTTCTT | LhGi no stop R | GAATAGGCCAATTTTTTAAGTCTT |
| LhGas R2        | AAGCCGTTACGGAATATGATGCG  | LhGi no stop R | GAATAGGCCAATTTTTTAAGTCTT |
| LhGas + stop R  | TTATAGCAACTATCATATTGCG    | LhGa no stop R | TACAGAATCATTATTCGCC      |
| LH Gas start F  | AAATCGTCATGCGCGCGTCG     | LhGaq no stop R | AACAGGTGGTGACATCTCTCATCA |
| LH Gaq start F  | AGATGGCGTGCTGCTTTTGG     | LhGa12/13 1303 R | CTTGATCTTCCTCTGCCAG       |
| LhGa end R      | TTAAACAAGGTTACTCTTCTTGA  | LhGa12/1303 | CTTGATCTTCCTCTGCCAG       |
| LhGi start F    | TAATGGGTCAGAAGCCGCTG     | LhGa12/13 1303 R | CTTGATCTTCCTCTGCCAG       |
| LhGi end R      | TTGAATAGGCGACAATTTTTTAAGTGT | LhGa12/13 1303 R | CTTGATCTTCCTCTGCCAG       |
| LhGao start F   | ATGGGCTGTGCAATGCTG       | LhGa stop R    | TTATAGCAACTATCATATTGCG      |
| LhGao stop R    | TTATAGCAACTATCATATTGCG    | LhGa12/13 1303 R | CTTGATCTTCCTCTGCCAG       |
| LhGa12/13 start | ATGGGCGGTCGTAATATATTTT   | LhGa12/13 stop | TTATAGCAACTATCATATTGCG      |
| LhGa12/13 stop  | TCATTGCAACATGAGGATG      | LhGa12/13 stop | TTATAGCAACTATCATATTGCG      |

To identify additional Ga subunits and potential variants of the LhGa subunits identified above, *L. hesperus* transcriptomes [43,46], which became available after the initiation of the LhGa cloning project, were searched using BLASTx (*E* value ≤ 10⁻¹⁰) with queries consisting of the consensus LhGa sequences and other insect Ga subunits. Sequence hits were then re-evaluated against the NCBI nr (non-redundant) database and duplicates removed. This search identified two additional Ga subunits (LhGa0 and LhGa12) and three potential LhGaq variants. Primers were designed to the putative start and stop codons of LhGa0 and LhGa12 and to unique portions of the LhGaq variants (Table 1). The respective sequences were amplified from multiple independent reactions using Sapphire Amp Fast PCR Master Mix (Takara Bio Inc./Clontech), subcloned where possible into a pCR2.1 TOPO TA cloning
vector (Life Technologies) and sequence verified. The nucleotide sequence data are available in the GenBank database under the accession numbers: KM610199-KM610202 (LhGoq2-LhGoq5), KM610203 (LhGα12), and KM610204 (LhGαo).

| Degenerate PCR 1 | Degenerate PCR 2 | Degenerate PCR 3 |
|-------------------|-------------------|-------------------|
| Tm °C  | Time (min) | Tm °C  | Time (min) | Tm °C  | Time (min) |
| 95    | 2:00        | 95    | 2:00        | 95    | 2:00        |
| 94    | 0:30        | 94    | 0:30        | 94    | 0:30        |
| 57    | 0:30        | 55    | 0:30        | 57    | 0:30        |
| 72    | 1:00        | 72    | 1:00        | 72    | 1:00        |
| 94    | 0:30        | 72    | 5:00        | 94    | 0:30        |
| 54    | 0:30        | 55    | 0:30        | 95    | 2:00        |
| 72    | 3:00        | 95    | 2:00        | 94    | 0:30        |
| 55    | 0:30        | 50    | 0:30        | 72    | 1:00        |
| 72    | 0:30        | 72    | 5:00        | 94    | 0:30        |
| 50    | 0:30        | 45    | 0:30        | 72    | 0:30        |
| 72    | 0:30        | 72    | 0:30        | 94    | 0:30        |
| 72    | 3:00        | 95    | 2:00        | 94    | 0:30        |
| 94    | 0:30        | 72    | 3:00        | 94    | 0:30        |
| 45    | 0:30        | 55    | 0:30        | 72    | 0:30        |
| 72    | 0:30        | 72    | 0:30        | 94    | 0:30        |
| 94    | 0:30        | 72    | 3:00        | 94    | 0:30        |
| 45    | 0:30        | 72    | 3:00        | 94    | 0:30        |
| 72    | 3:00        | 95    | 2:00        | 94    | 0:30        |

**Figure 1.** Thermocycler conditions used.

2.3. **Bioinformatic Analyses**

LhGα sequences were evaluated against the NCBI nr database by BLASTx (E value ≤ 10^{-5}). Putative myristoylation sites were predicted using NMT-MYR Predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) and palmitoylation sites with CSS-PALM (http://csspalm.biocuckoo.org/index.php) [64].
To determine potential phylogenetic relationships, multiple sequence alignments of the putative LhGa subunits and other insect Gα subunits (nine per subfamily) were constructed using default settings in MUSCLE [65,66]. Phylogenetic inferences were made using the maximum likelihood, minimum evolution, NJ, and UPGMA modules implemented in MEGA6.06 [67] with bootstrap analysis conducted of 1000 replicates. Data shown are for the maximum likelihood method based on the JTT matrix-based model [68]. Initial tree(s) for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using a JTT model. The analysis involved 53 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 350 positions in the final dataset.

2.4. Transcriptional Profiling of L. hesperus Gα Subunits

The expression profiles of the respective LhGa transcripts were examined across L. hesperus development and within sex-specific adult body tissues. Developmental profiling consisted of eggs, pooled samples from each of the five nymphal instars, and mixed sex adults comprising equal numbers of males and females at 1, 10, and 20 days post-adult emergence. Adult tissue profiling was performed using cDNAs generated from pooled, sex specific virgin 7-day-old adult bodies, heads, midgut/hindgut, Malpighian tubules, antennae, probosci, and legs as well as pooled tissue sets of female ovaries and seminal depositories, and male medial/lateral accessory glands and testes. Samples were homogenized in TRI Reagent Solution (Ambion/Life Technologies) using a TissueLyser (Qiagen, Valencia, CA, USA) with total RNA extracted based on recommendations from the manufacturer. First-strand cDNAs were generated using a Superscript III first-strand cDNA synthesis kit (Life Technologies) with custom-made random pentadecamers (IDT, San Diego, CA, USA) and 500 ng of DNase I-treated total RNAs. End-point PCR amplification was done using Sapphire Amp Fast PCR Master Mix with 0.4 μL (10 ng) cDNA template, sequence-specific primers (Table 1) designed to amplify ~500–600 bp fragments of the LhGa transcripts, and thermocycler conditions described in Figure 1. Both developmental and adult tissue expression profiles were replicated at least three times using cDNA templates prepared from different biological replicates. Differing combinations of primer sets (see Table 1) designed from transcriptomic data were used to profile the LhGaq1–4 variants: LhGaq1 (LhGaq 468 F1/LhGaq 1036 R1), LhGaq2 (LhGaq 468 F1/LhGaq 1035 R2), LhGaq3 (LhGaq 474 F2/LhGaq 1036 R1), and LhGaq4 (LhGaq 474 F2/LhGaq 1035 R2). PCR products were electrophoresed on 1.5% agarose gels and representative amplimers of the expected sizes were sub-cloned and sequence verified.

2.5. Immunocytochemical Localization of L. hesperus Gα in Cultured Insect Cells

To examine the intracellular localization of select LhGa subunits, the respective coding sequences lacking endogenous stop codons were amplified from plasmid DNAs using KOD HotStart DNA polymerase (Toyobo/Novagen, EMD Biosciences, San Diego, CA, USA) and sub-cloned into a pIB/V5-His TOPO TA expression vector (Life Technologies) upstream of the plasmid-derived epitope tag such that the translated LhGa subunits contain a carboxyl terminal 6×-His tag. All resulting expression plasmids were sequence verified. Adherent Trichoplusia ni (Tni) cells (Orbigene Inc., San Diego, CA, USA) attached to 35-mm #1.5 glass bottom dishes (In Vitro Scientific, Sunnyvale, CA, USA) were transfected with 2 μg plasmid DNA using Insect Gene Juice transfection reagent (Novagen) for 5 h. Transfected cells were maintained in serum-free media for 48 h at 28 °C and then fixed for 15 min at 4 °C with 3.5%
formalin/IPL-41. The cells were blocked and permeabilized for 1 h at 25 °C in PBS/10% fetal bovine serum/0.1% Triton X-100. The cells were then incubated for 2 h at 25 °C with 1:50 rabbit polyclonal anti-His antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; #SC-804), which recognizes the plasmid-derived His epitope tag. After washing, the cells were incubated with 1:100 goat anti-rabbit IgG-TRITC (Southern Biotechnology; Birmingham, AL, USA; #4030-03) for 2 h at 25 °C. Fluorescent imaging was performed on an Olympus FSX-100 fluorescence microscope with FSX-BSW imaging software (Olympus, Center Valley, PA, USA). Images were processed for publication with Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

3. Results and Discussion

3.1. Identification of L. hesperus Ga Sequences

To identify Ga proteins expressed in L. hesperus (LhGa), we initially utilized a homology-based approach with degenerate primers designed to conserved regions of Ga proteins and both PCR and nested PCR conditions. Sequence analysis indicated amplimers of the expected sizes were partial fragments of proteins homologous with Gαs, Gαi, and Gαq proteins. Further extension of the partial sequences using conventional RACE PCR methods identified putative start and stop codons. Primers designed to those regions facilitated amplification of the respective open reading frames (ORFs). Based on sequence similarities with known Ga subunits (Table 2), we designated the cloned sequences as LhGαi, LhGαs, and LhGαq. The 1230 nt LhGαi transcript contains a 1068 nt ORF encoding a 355 amino acid residue protein, whereas the 1538 nt LhGαq transcript encompasses a 1062 nt ORF encoding a protein containing 353 amino acids. The 1350 nt LhGαs transcript has a 1137 nt ORF encoding a protein containing 378 amino acids. The predicted molecular masses of the three Ga proteins (LhGαi = 40.6 kDa, LhGαs = 44.2 kDa, and LhGαq = 41.5 kDa) are comparable with previous reports [3].

Because the degenerate primers used in the homology-based PCR approach have the potential to bias toward particular classes of Ga proteins [62,63], we sought to use recently assembled L. hesperus transcriptomes [43,46] to more comprehensively evaluate LhGa expression. The respective databases were queried with the LhGαi, LhGαs, and LhGαq sequences as well as Ga subunits from other insects. All three LhGa transcripts are present in the databases with minimal (>99% nt identity) sequence variation. In addition, complete transcripts for Gαo and Gα12 subunits were identified. The putative LhGαo ORF encodes a 355 amino acid protein with highest sequence similarity to a Gαo subunit cloned from a migratory locust (Locusta migratoria) head cDNA library [54]. While the putative LhGα12 encodes a 368 amino acid protein that has significant sequence identity with genomic sequences annotated simply as Ga subunit-like proteins (Table 2), it is 63% identical (E value = 3e^{-154}) with the D. melanogaster Gα12 homolog, concertina [50]. To confirm correct assembly of the transcriptomic data, the complete coding regions for both LhGαo and LhGα12 were amplified from L. hesperus cDNAs in multiple independent reactions and sequenced. As before, the cloned sequences exhibited >99% nt sequence identity with the transcriptomic sequences.
Table 2. Top five BLASTx hits for LhGα sequences.

| Query   | Description                                                                                                     | Accession     | E Value      | % identity  | % positives  |
|---------|------------------------------------------------------------------------------------------------------------------|---------------|--------------|--------------|--------------|
| LhGα    | Guanine nucleotide-binding protein G(s) subunit alpha [Zootermopsis nevadensis]                                  | KDR14965.1    | 0.00E + 00   | 340/379 (90%) | 359/379 (94%) |
|         | PREDICTED: guanine nucleotide-binding protein G(s) subunit alpha [Diaphorina citri]                               | XP_008468199.1| 0.00E + 00   | 338/380 (89%) | 360/380 (94%) |
|         | PREDICTED: guanine nucleotide-binding protein G(s) subunit alpha [Acyrthosiphon pisum]                           | XP_001944148.1| 0.00E + 00   | 335/380 (88%) | 362/380 (95%) |
|         | guanine nucleotide binding protein, alpha stimulating activity polypeptide [Daphnia pulex]                        | EFX88427.1    | 0.00E + 00   | 330/379 (87%) | 359/379 (94%) |
|         | guanine nucleotide-binding protein G, putative [Pediculus humanus corporis]                                    | XP_002431834.1| 0.00E + 00   | 331/380 (87%) | 355/380 (93%) |
| LhGii   | Guanine nucleotide-binding protein G(i) subunit alpha [Zootermopsis nevadensis]                                 | KDR22153.1    | 0.00E + 00   | 321/355 (90%) | 339/355 (95%) |
|         | PREDICTED: guanine nucleotide-binding protein G(i) subunit alpha-like [Megachile rotundata]                      | XP_003707938.1| 0.00E + 00   | 314/355 (88%) | 333/355 (93%) |
|         | PREDICTED: G protein alpha i subunit [Tribolium castaneum]                                                      | XP_008200240.1| 0.00E + 00   | 313/355 (88%) | 331/355 (93%) |
|         | PREDICTED: guanine nucleotide-binding protein G(i) subunit alpha-like [Apis mellifera]                          | XP_395172.2   | 0.00E + 00   | 311/355 (88%) | 331/355 (93%) |
|         | PREDICTED: guanine nucleotide-binding protein G(i) subunit alpha-like [Bombus terrestris]                       | XP_00339073.1 | 0.00E + 00   | 310/355 (87%) | 330/355 (92%) |
| LhGq1   | GTP-binding protein alpha subunit, gna [Anopheles sinensis]                                                    | KFB50356.1    | 0.00E + 00   | 336/353 (95%) | 343/353 (97%) |
|         | AGAP005079-PI [Anopheles gambiae str. PEST]                                                                       | XP_313956.1   | 0.00E + 00   | 336/353 (95%) | 343/353 (97%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha isoform X1 [Acyrthosiphon pisum]                | XP_001948628.2| 0.00E + 00   | 333/353 (94%) | 346/353 (98%) |
|         | GTP-binding protein alpha subunit, gna [Aedes aegypti]                                                          | XP_001660884.1| 0.00E + 00   | 335/353 (95%) | 343/353 (97%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform 1 [Megachile rotundata]           | XP_003702524.1| 0.00E + 00   | 335/353 (95%) | 344/353 (97%) |
| LhGq2   | PREDICTED: G protein alpha q subunit isoform X2 [Acyrthosiphon pisum]                                          | XP_008178833.1| 0.00E + 00   | 322/353 (91%) | 337/353 (95%) |
|         | AGAP005079-PB [Anopheles gambiae str. PEST]                                                                       | XP_00168493.1 | 0.00E + 00   | 325/353 (92%) | 334/353 (94%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform 5 [Megachile rotundata]           | XP_003702528.1| 0.00E + 00   | 318/353 (90%) | 338/353 (95%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform X5 [Apis mellifera]                | XP_006562642.1| 0.00E + 00   | 319/353 (90%) | 334/353 (94%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform X13 [Apis dorsata]               | XP_006515865.1| 0.00E + 00   | 318/353 (90%) | 333/353 (94%) |
| LhGq3   | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform 2 [Megachile rotundata]           | XP_003702525.1| 0.00E + 00   | 334/353 (95%) | 343/353 (97%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha isoform X3 [Acyrthosiphon pisum]               | XP_008178834.1| 0.00E + 00   | 331/353 (94%) | 345/353 (97%) |
|         | AGAP005079-PF [Anopheles gambiae str. PEST]                                                                       | XP_001688490.1| 0.00E + 00   | 333/353 (94%) | 343/353 (97%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform X8 [Apis mellifera]              | XP_0623211.2   | 0.00E + 00   | 328/353 (93%) | 341/353 (96%) |
|         | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform 1 [Megachile rotundata]         | XP_003702524.1| 0.00E + 00   | 325/353 (92%) | 338/353 (95%) |
Table 2. Cont.

| Query    | Description                                                                 | Accession          | E Value   | % identity | % positives |
|----------|-----------------------------------------------------------------------------|--------------------|-----------|------------|-------------|
| LhGq4    | PREDICTED: guanine nucleotide-binding protein G(q) subunit alpha-like isoform 3 [Megachile rotundata] | XP_003702526.1     | 0.00E+00  | 324/353 (92%) | 334/353 (94%) |
|          | G protein alpha q isoform 2 [Bombyx mori]                                   | NP_001128385.1     | 0.00E+00  | 322/353 (91%) | 335/353 (94%) |
|          | PREDICTED: G protein alpha q subunit isoform X4 [Acyrtosiphon pisum]        | XP_008178835.1     | 0.00E+00  | 320/353 (91%) | 336/353 (95%) |
|          | GTP-binding protein alpha subunit, gna [Aedes aegypti]                      | XP_001660085.1     | 0.00E+00  | 322/353 (91%) | 334/353 (94%) |
|          | AGAP005079-9-PD [Anopheles gambiae str. PEST]                               | XP_001688487.1     | 0.00E+00  | 322/353 (91%) | 334/353 (94%) |
| LhGq5    | PREDICTED: G protein alpha q subunit-like [Diaphorina citri]                | XP_008479779.1     | 4.00E-102 | 145/157 (92%) | 151/157 (96%) |
|          | PREDICTED: G protein alpha q subunit isoform X4 [Acyrtosiphon pisum]        | XP_008178835.1     | 2.00E-99  | 152/193 (79%) | 155/193 (80%) |
| LhGq12   | Guanine nucleotide-binding protein subunit alpha-like protein [Harpegnathos saltator] | EFN86700.1         | 0.00E+00  | 287/367 (78%) | 327/367 (89%) |
|          | PREDICTED: guanine nucleotide-binding protein subunit alpha homolog [Apis mellifera] | XP_394382.2        | 0.00E+00  | 286/367 (78%) | 326/367 (88%) |
|          | Guanine nucleotide-binding protein subunit alpha homolog [Nasonia vitripennis] | XP_001600076.1     | 0.00E+00  | 282/363 (78%) | 324/363 (89%) |
|          | PREDICTED: guanine nucleotide-binding protein subunit alpha homolog [Bombus terrestris] | XP_003402866.1     | 0.00E+00  | 283/367 (77%) | 325/367 (88%) |
| LhGuo    | Guanine nucleotide-binding protein G(o) subunit alpha [Locusta migratoria]  | P38404.1           | 0.00E+00  | 346/354 (98%) | 349/354 (98%) |
|          | Guanine nucleotide-binding protein G(o) subunit alpha [Zootermopsis nevadensis] | KDR16702.1         | 0.00E+00  | 345/354 (97%) | 348/354 (98%) |
|          | Guanine nucleotide-binding protein G(o) subunit alpha [Camponotus floridanus] | EFN66163.1         | 0.00E+00  | 344/354 (97%) | 348/354 (98%) |
|          | PREDICTED: guanine nucleotide-binding protein G(o) subunit alpha-like isoform 1 [Megachile rotundata] | XP_003701784.1     | 0.00E+00  | 342/354 (97%) | 347/354 (98%) |
|          | PREDICTED: guanine nucleotide-binding protein G(o) subunit alpha [Microplitis demolitor] | XP_008545405.1     | 0.00E+00  | 339/354 (96%) | 346/354 (97%) |
Multiple sequence variants have been reported for Ga subunits [20,48,51,52,69,70] with variants/isoforms also predicted in many insect genomes. Furthermore, high throughput sequencing methods, such as those used to construct the *L. hesperus* transcriptome databases, offer the possibility of identifying low representation and/or unique transcripts [71–73]. Consistent with previous findings, our transcriptome database search identified three additional LhGaq variants, which we have designated LhGaq2-4. Sequence identity among the four subunits varies from 89%–96% with all four variants identical through Leu155, at which point identity is maintained between LhGaq1/2 and LhGaq3/4 up to Pro292, with identical residues then shared between LhGaq1/3 and LhGaq2/4 throughout the rest of the protein (Figure 2A). This variation is consistent with the alternative exon splicing described in *A. gambiae* [20] and *D. melanogaster* [70] and is present in a number of species from disparate orders, suggesting that the putative splice sites have been evolutionarily conserved. While characterizing the respective LhGaq variants, we cloned a partial sequence corresponding to a fifth variant (LhGaq5) that is not represented in either of the transcriptomic databases and which lacks residues 291–326 (Figure 2B). While this variant is also present in *A. gambiae* (AAW50316) and *Diaphorina citri* (XP_008479779) (Figure 2B), we were unable to identify it from other insects, which suggests that the splice site is either not conserved or that it is a cryptic site [73]. The Gaq locus in *A. gambiae* spans 11 exons, three of which (identified as D/D*, G/G*, and H/H*) are homologous and undergo alternative splicing [20]. While the genomic structure of the *L. hesperus* Gaq locus has not been determined, we can surmise based on the transcript sequences that similar alternative splicing likely generates the five variants (Figure 2C).

In their characterization of Ga in *A. gambiae*, Rützler et al. [20] identified a sixth Gaq variant (AAW50317) characterized by inclusion of a 43 amino acid insertion that corresponds to two of the exons alternatively spliced in the other variants. Although this variant is a predicted product in a number of insect genomes, it was considered to be a premature transcript as inclusion of the second exon could potentially disrupt the catalytic pocket of the GTP hydrolysis domain. We were unable to detect this variant during characterization of the other LhGaq subunits nor was it represented in the *L. hesperus* transcriptomes [43,46]. No other LhGa sequence variants were identified. The respective transcriptomes, however, may underrepresent the number of Ga transcripts actively expressed in *L. hesperus* due to the exclusion of temporally or spatially restricted transcripts.

### 3.2. Bioinformatic Analysis of LhGa Subunits

Sequence identity across the respective full-length LhGa sequences ranges from 33%–96% (Table 3) with the highest values associated, as expected, with the LhGaq variants. Sequence identity across the subfamilies varied from 35%–67%, with highest identity shared between LhGao and LhGaα, which is consistent with previous studies showing that Gao and Gaα are phylogenetically related [1,3]. BLASTx analyses using the nr database revealed the highest similarities (Table 2) were predominantly with non-hemipteran sequences, indicating the highly conserved evolutionary nature and functional importance of Ga subunits and the current lack of molecular resources for hemipteran pests. Sequence alignment and phylogenetic analyses of the putative LhGa proteins with those from other arthropods (Figure 3) support our initial classifications and are consistent with previously reported evolutionary relationships [20,51,57]. Based on their conserved structural similarities, the respective Ga classes were divided into five central clades with strong bootstrap support for a shared branch point between the Gao and Gaα clades.
Figure 2. Alternative splice variants of LhGαq. (A) MUSCLE-based sequence alignment of LhGαq1-4; (B) MUSCLE-based sequence alignment of LhGαq5 with LhGαq1 and Gαq variants from A. gambiae (AAW50316; AgGαq5) and Diaphorina citri (XP_008479779; DcGαq1). The alignment corresponds to LhGαq1 amino acid residues 187–353. Shading denotes amino acid sequence similarity and is scaled as: 100% similarity (black), 99%–80% (dark grey), 79%–60% (light grey), and less than 59% (white); (C) Proposed alternative splice scheme utilized to generate the cloned LhGαq variants. Similar to A. gambiae and D. melanogaster, the LhGαq locus appears to have three homologous exons (B/B’, C/C’, and D/D’) that are alternatively spliced to generate the five LhGαq variants. Putative exon-intron boundaries are based on observed sequence variations with coding sequences shown as boxes.

Table 3. Percent identity matrix heat map for LhGα proteins.

|       | LhGαq1 | LhGαq2 | LhGαq3 | LhGαq4 | LhGαq5 | LhGas | LhGαi | LhGαo | LhGα12 |
|-------|--------|--------|--------|--------|--------|-------|-------|-------|--------|
| LhGαq1| 100    | 93     | 96     | 89     | 72     | 42    | 49    | 48    | 44     |
| LhGαq2| 93     | 100    | 89     | 96     | 75     | 42    | 47    | 46    | 44     |
| LhGαq3| 96     | 89     | 100    | 93     | 78     | 42    | 50    | 48    | 45     |
| LhGαq4| 89     | 96     | 93     | 100    | 81     | 42    | 48    | 46    | 44     |
| LhGαq5| 72     | 75     | 78     | 81     | 100    | 39    | 47    | 47    | 43     |
| LhGas | 42     | 42     | 42     | 42     | 39     | 100   | 41    | 42    | 35     |
| LhGαi | 49     | 47     | 50     | 48     | 47     | 41    | 100   | 67    | 38     |
| LhGαo | 48     | 46     | 48     | 46     | 47     | 42    | 67    | 100   | 39     |
| LhGα12| 44     | 44     | 45     | 44     | 43     | 35    | 38    | 39    | 100    |
Figure 3. Phylogenetic analysis of Gα subunits from *L. hesperus* and other insects. Phylogenetic relationships were inferred using the maximum likelihood method based on the JTT matrix-based model [68]. The tree with the highest log likelihood is shown. *L. hesperus* Gα sequences are shown in red.

The conserved guanine nucleotide binding/hydrolysis motifs characteristic of Gα subunits are present in the predicted LhGα proteins (Figure 4) including sequences critical for diphosphate binding...
(GXGEGSKS), Mg\textsuperscript{2+} binding (RXXTXGI and DXXG), and guanine ring-binding (NKXD and TCAT) [3]. Deviations from the canonical sequences, however, are present in the TCAT motif in LhG\textsubscript{os} (TCAV), LhGa\textsubscript{12} (TTAV), and LhGq\textsubscript{2/4} (TTAT). These deviations are not specific to the \textit{L. hesperus} sequences as all of the Ga\textsubscript{s} and Ga\textsubscript{12} sequences used in the phylogenetic analysis had the same sequence changes and numerous Gq sequences (e.g., NP_001128385, \textit{B. mori}; ACJ06653, \textit{Spodoptera frugiperda}; CAB76453, \textit{Calliphora vicina}; XP_005180085, \textit{Musca domestica}; XP_004526037, \textit{Ceratitis capitata}) have a TTAT motif. Mutations to the TCAT motif in mammalian Ga subunits mimic an activated receptor by enhancing GDP release [74,75]. Thus, activation of insect Ga subunits with the modified TCAT motif may proceed more readily, which could account for the observed heterogeneity in receptor-G protein interactions and promiscuous activation of multiple Ga subunits by some receptors [1].

Figure 4. Multiple sequence alignment of \textit{L. hesperus} Ga sequences. The respective full length \textit{L. hesperus} Ga sequences were aligned in MUSCLE using default settings. Percent similarity shading is as in Figure 2. Conserved Ga subunit features/motifs are boxed: predicted myristoylation sites (purple M), predicted palmitoylation sites (orange P), diphosphate binding (red), Mg\textsuperscript{2+} binding (blue), guanine ring binding (green), putative cholera toxin-mediated ADP-ribosylation site (Arg186) in LhG\textsubscript{os} (yellow circle), putative pertussis toxin-mediated ADP-ribosylation sites (Cys352/Cys351) in LhGa\textsubscript{i} and LhGao (pink circle).
Further analysis of the LhGa sequences indicated the presence of conserved modification sites for fatty acids and toxin-driven ADP-ribosylation (Figure 4). Palmitoylation of Ga amino terminal Cys residues and/or myristoylation of amino terminal Gly residues in Gai/o subunits can influence cellular localization/membrane targeting, interactions with downstream effector proteins, and secondary structure [2,3,76]. ADP-ribosylation of a carboxyl terminal Cys by pertussis toxin uncouples Gai/o subunits whereas similar modification of an internal Arg in Gas subunits by cholera toxin abolishes GTP hydrolysis activity and leads to constitutive Gas activation [1,3].

The last five residues of the Ga carboxyl terminus are critical for receptor interactions, with minor modifications of this region altering receptor specificity and ADP-ribosylation uncoupling Gai/o subunits from the respective receptor [2]. The identical carboxyl terminal ends shared by LhGaq1/3 and LhGaq2/4 raises questions regarding potentially overlapping functional roles. One possibility is that the respective subunits exhibit different expression profiles (see below), which would limit functional redundancy. A second possibility is that the sequence variations that differentiate the respective LhGaq subunits also function to stabilize receptor interactions. Thus, despite identical carboxyl terminal ends the LhGaq subunits interact with the receptors differently. Consequently, despite the critical role the carboxyl terminus plays, functional specificity is driven by the summation of receptor contact points.

3.3. End Point PCR-Based Transcriptional Expression Profiling

The tissue and/or developmental specificity of transcript expression can provide insights into gene functionality. To begin to assess the potential functional role of the LhGa subunits, we examined their transcriptional expression as ~500–600 bp fragments across L. hesperus development, from eggs through 5th instars and in 1-day-old, 10-day-old, and 20-day-old adults (Figure 5A). While most LhGa subunits were ubiquitously expressed in all stages examined, the expression of LhGaq2 and LhGaq4 was more restrictive. The LhGaq4 product was absent in eggs but was detected throughout nymphal development and in adults (Figure 5A). Even though LhGaq2 and LhGaq4 share identical carboxyl terminal ends (see above), no amplimers were detected for LhGaq2, suggesting little functional redundancy with respect to receptor specificity between the two variants. Despite overlap with the LhGaq4 primer set (as demonstrated by the serendipitous cloning of LhGaq5 while verifying the LhGaq4 sequence), no LhGaq5 amplimers, which would migrate as a lower molecular weight product (i.e., 476 bp vs. 584 bp for LhGaq4) were detected, suggesting low transcript levels for this variant.

We also examined the expression profile of the LhGa subunit fragments in sex-specific adult tissues (Figure 5B). A majority of the LhGa transcripts were amplified from all of the tissue sets from both sexes, albeit to varying degrees. Similarly wide tissue distribution profiles for Ga subunits have been reported in B. mori [19], A. gambiae [20], D. melanogaster [21], B. tabaci [57], and L. oryzophilus [55] and likely reflect the critical role of G proteins in mediating the diverse signal transduction cascades that drive cellular processes. LhGaq4 was the lone LhGa transcript to exhibit tissue specific expression with amplification limited to head-derived cDNAs (Figure 5B). LhGaq4 shares significant sequence identity with D. melanogaster Gaq1 (i.e., Gq-RD), the Ga subunit involved in phototransduction [77,78], and the presumptive A. gambiae ortholog, Agq1 [20]. All three are derived from analogous alternative splice sites and are specifically expressed in adult heads and pre-adult stages with no detectable embryonic expression [20,21,77]. These similarities suggest that functionality may also be conserved, with LhGaq4
likewise mediating phototransduction. This, however, remains to be experimentally verified. No amplimers corresponding to LhGαq2 were detected in any of the tissues examined, which is consistent with the developmental expression profile (Figure 5A).

**Figure 5.** End point PCR-based expression profile of *L. hesperus* Ga transcripts. (A) Expression profile of LhGα sequences in eggs, nymphal development (1st–5th instars), 1-day-old mixed sex adults (d1a), 10-day-old mixed sex adults (d10a), and 20-day-old mixed sex adults (d20a); (B) Expression profile of LhGα sequences in sex-specific adult tissues. Abbreviations are: M. tubule, Malpighian tubule; AG, accessory glands (lateral and medial); SD, seminal depository. In both (A) and (B), amplimers correspond to ~500-600 bp fragments of each transcript with products analyzed on 1.5% agarose gels stained with SYBR Safe. Negative images of the gels are shown for enhanced clarity of low expression transcripts. Numbers to the right of each gel image indicate the number of amplification cycles.
With the exception of LhGa\textsubscript{q2} and LhGa\textsubscript{q4}, all of the LhGa subunits were amplified to varying degrees from chemosensory tissues (antenna, proboscis, and leg) indicating the absence of a chemosensory specific subunit (Figure 5B). While variation in amplification across the chemosensory tissues was observed for LhGa\textsubscript{o} (highest in antennae) and LhGa\textsubscript{q1} (highest in leg), more accurate determinations (e.g., quantitative real-time PCR) of transcript abundance are required to draw definitive conclusions regarding expression. Ga\textsubscript{s} has been reported to be more highly expressed in antennae than other Ga subunits in \textit{A. gambiae}, \textit{D. melanogaster}, and \textit{B. mori} [19–21]. The expression of Ga\textsubscript{s} in olfactory neurons coupled with abnormal olfactory behavior following disruption of the Ga\textsubscript{s} signal transduction cascade [25] has led some to postulate that Ga\textsubscript{s} functions in olfaction. However, elevated levels of Ga\textsubscript{o} and Ga\textsubscript{q} transcripts have been reported in antennae and olfactory neurons of a number of insects [17–19,54,55]. Furthermore, similar to the Ga\textsubscript{s} pathway, downstream effectors of Ga\textsubscript{q} such as Ca\textsuperscript{2+}/calmodulin can also activate adenylyl cyclase [79] and RNAi-mediated knockdown of Ga\textsubscript{q} likewise reduces antennal responses [29]. In contrast, other studies have suggested that Ga proteins have little role in insect olfaction [35]. Given the conflicting conclusions drawn by disparate groups and the critical role of Ga proteins in normal cellular function, it is becoming increasingly clear that simple co-localization of Ga transcripts within chemosensory tissues, while correlational, is not indicative in and of itself of an olfactory function.

3.4. Intracellular Localization of Transiently Expressed LhGa Subunits

Post-translational lipid modifications (i.e., myristolation/palmitoylation) facilitate targeting and subsequent anchoring of Ga subunits to the inner surface of the plasma membrane [76,80]. To further characterize and confirm the sequence validity of the cloned LhGa transcripts, we sought to examine the intracellular localization of a subset of the LhGa proteins (LhGa\textsubscript{q1}, LhGa\textsubscript{s}, and LhGa\textsubscript{i}) following transient expression in cultured insect cells. To facilitate detection, expression vectors were constructed in which a 6×-His tag was incorporated in frame with the carboxyl terminal ends of the respective LhGa sequences. Immunofluorescence analyses were performed in cultured \textit{Trichoplusia ni} cells 48 h after transfection using a polyclonal anti-His antibody in conjunction with a TRITC-tagged anti-rabbit antibody. No fluorescence was observed in non-transfected cells (Figure 6). In contrast, plasma membrane-associated fluorescence was clearly observed in cells transfected with the respective LhGa-His constructs (Figure 6). These results are consistent with previous findings [12] and indicate that intracellular trafficking of the cloned LhGa sequences is as expected. In addition to the clear plasma membrane-associated signal, we also observed a diffuse red fluorescent signal throughout the cytosol of cells transfected with the respective LhGa subunits. The current model of G protein trafficking suggests that interactions between Ga subunits and G\beta\gamma subunits are crucial for plasma membrane localization. Consequently, overexpression of one subunit (e.g., Ga subunits) may disrupt the necessary stoichiometry and lead to inefficient localization [76]. Thus, the intracellular signal we observed might be “free” Ga subunits that lack the apparent G\beta\gamma binding partners that facilitate plasma membrane localization. Alternatively, the signal may represent the normal trafficking profile of Ga subunits as both cell membrane and discrete cytosolic localization for Ga subunits have been reported in both native tissue and cell culture [81–83].
Intracellular localization of transiently expressed *L. hesperus* Ga subunits in cultured insect cells. Fixed *Trichoplusia ni* (Tni) cells transfected with plasmids encoding carboxyl terminal 6×-His tagged LhGa subunits (LhGα1-His, LhGαq1-His, and LhGα4-His) or mock transfected cells (Tni) were probed with a polyclonal mouse anti-His antibody (primary) and a goat anti-mouse IgG-TRITC antibody (secondary). Red fluorescence corresponds to the TRITC signal and denotes localization of the His-tagged Ga subunits. Scale bar = 20 μm.

4. Conclusions

As part of our continuing efforts to further elucidate molecular mechanisms driving signal transduction in *L. hesperus*, we identified nine Ga subunits. Expression analyses and sequence similarities strongly suggest that LhGα4 is orthologous to *D. melanogaster* Gq-RD, which functions in phototransduction. While the presence of multiple LhGa transcripts in chemosensory tissues is consistent with potential roles in olfaction and/or gustation, localization at the tissue level alone does not imply function in chemosensory-based signal transduction. To address that issue, the actual role of each of the LhGa subunits and variants in chemosensory functionality must be established, including demonstration of specific expression of LhGa within olfactory/gustatory receptor neurons and *in vivo* functional studies examining the biological effects of Ga mutations, Ga knockdown, and/or Ga overexpression.

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Author Contributions

Conceived and designed the experiments: J. Joe Hull. Performed the experiments: J. Joe Hull, Meixian Wang. Analyzed the data: J. Joe Hull, Meixian Wang. Wrote the paper: J. Joe Hull, Meixian Wang.

Conflicts of Interest

The authors declare no conflict of interest.

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