1) GENETIC CHARACTERIZATION OF PUTATIVE NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT TRANSCRIPTS. 2) INITIAL EXPLORATION OF THE USE OF THE CRISPR/Cas9 GENE EDITING SYSTEM IN CODLING MOTH, *CYDIA POMONELLA* (L.) (LEPIDOPTERA: TORTRICIDAE)

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Jessica A. Martin

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1) GENETIC CHARACTERIZATION OF PUTATIVE NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT TRANSCRIPTS. 2) INITIAL EXPLORATION OF THE USE OF THE CRISPR/Cas9 GENE EDITING SYSTEM IN CODLING MOTH, *CYDIA POMONELLA* (L.) (LEPIDOPTERA: TORTRICIDAE)

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Putative nicotinic acetylcholine receptor subunits express differentially through the life cycle of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae)

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Abstract Nicotinic acetylcholine receptors (nAChRs) are the targets of neonicotinoids and spinosads, two insecticides used in orchards to effectively control codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Orchardists in Washington State are concerned about the possibility of codling moth field populations developing resistance to these two insecticides. In an effort to help mitigate this issue, we initiated a project to identify and characterize codling moth nAChR subunits expressed in heads. This study had two main goals; (i) identify transcripts from a codling moth head transcriptome that encode for nAChR subunits, and (ii) determine nAChR subunit expression profiles in various life stages of codling moth. From a codling moth head transcriptome, 24 transcripts encoding for 12 putative nAChR subunit classes were identified and verified by PCR amplification, cloning, and sequence determination. Characterization of the deduced protein sequences encoded by putative nAChR transcripts revealed that they share the distinguishing features of the cys-loop ligand-gated ion channel superfamily with 9 α-type subunits and 3 β-type subunits identified. Phylogenetic analysis comparing these protein sequences to those of other insect nAChR subunits supports the identification of these proteins as nAChR subunits. Stage expression studies determined that there is clear differential expression of many of these subunits throughout the codling moth life cycle. The information from this study will be used in the future to monitor for potential target-site resistance mechanisms to neonicotinoids and spinosads in tolerant codling moth populations.

Key words codling moth; nAChR expression; nicotinic acetylcholine receptor subunits

Introduction

The codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a major pest of apple, pear, and walnut worldwide, has historically been controlled by aggressive chemical treatments using insecticides (Knight, 2010). While effective, organophosphates and pyrethroids cause serious environmental and agricultural worker safety issues (Brown & Brix, 1998; Chen, 2012). The United States Environmental Protection Agency (EPA) has cancelled the registration of several organophosphates, including azinphos-methyl in September 2012, forcing orchardists to seek alternatives. Since the early 2000s, a growing number of orchardists have turned to neonicotinoids and spinosads as safer alternatives to control codling moth in Integrated Pest Management (IPM) programs (Agnello *et al.*, 2009; Casida & Durkin, 2013). Neonicotinoids and spinosads have been successful in controlling codling moth in the orchard, with these insecticides being highly selective for insect nicotinic acetylcholine receptors (nAChRs) while displaying low selectivity for mammalian nAChRs (Tomizawa & Casida, 2005). However, there is evidence to suggest that neonicotinoids could be having a negative impact on some beneficial

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insect species, including the honey bee, *Apis mellifera* (L.) (Hymenoptera: Apidae) (Blacquiere *et al.*, 2012).

Neonicotinoids and spinosads target nAChRs, interfering with their function in the nervous system. Functional nAChRs exist in the nerve membrane as homo- or heteropentamers composed of α and β subunits, the combination of which determines both the functional and pharmacological properties of these ligand-gated ion channels (Jones *et al.*, 2007). The actions of nAChRs have been implicated to have roles in learning, memory, and neurodegenerative disorders (Buckingham *et al.*, 2009; Gauthier, 2010). Normally closed, nAChRs are opened by the binding of acetylcholine, the main excitatory neurotransmitter in the insect central nervous system, for the purpose of rapid neurotransmission. Neonicotinoids competitively bind in the acetylcholine pockets within the nAChRs, while spinosads bind outside of these receptors' active site (Watson *et al.*, 2010; Casida & Durkin, 2013). Both insecticides cause hyperexcitation of the central nervous system, leading to paralysis and death (Salgado, 1998; Gervais *et al.*, 2010).

While progress has been made in understanding how neonicotinoids function at the nAChR binding sites (Lansdell & Millar, 2000; Shimomura *et al.*, 2003; Shimomura *et al.*, 2004; Shimomura *et al.*, 2005; Li *et al.*, 2010; Li *et al.*, 2012), relatively little is known about the molecular properties of nAChRs and the diversity of subunits available to assemble into functional pentameric ion channels. Within the last decade, nAChR subunit gene families have been described for several insect species including the honey bee, *A. mellifera* (Jones *et al.*, 2006), the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Shao *et al.*, 2007), the fruit fly, *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (Sattelle *et al.*, 2005), the red flour beetle, *Tribolium castanneum* (Herbst) (Coleoptera: Tenebrionidae) (Jones & Sattelle, 2007), the malaria mosquito, *Anopheles gambiae* (Giles) (Diptera: Culicidae) (Jones *et al.*, 2005), and the parasitoid wasp, *Nasonia vitripennis* (Ashmead) (Hymenoptera: Pteromalidae) (Jones *et al.*, 2010). Characterization of nAChRs has led to some important discoveries, including target-site point mutations, mis-splicing and truncated transcripts that are implicated in resistance to neonicotinoids and spinosads. Point mutations in 2 nAChR α-subunits, nAChRα1 and nAChRα3, were implicated in imidacloprid resistance detected in Chinese field populations of the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Liu *et al.*, 2005). A point mutation in a β-subunit, nAChRβ1, has been implicated in neonicotinoid resistance in peach–potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), in Southern France (Bass *et al.*, 2011), and an amino acid sequence similar to the resistant form of nAChRβ1 is present in the black-legged tick, *Ixodes scapularis* (Say) (Acari: Ixodidae) which is thought to confer natural resistance to neonicotinoids in this pest (Erdmanis *et al.*, 2012). For spinosads, a point mutation in nAChRα6 has been implicated in resistance in western thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), in Spain (Puinean *et al.*, 2013). In a field population of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), mis-splicing of nAChRα6 leading to a truncated transcript is implicated in resistance of this insect to spinosads (Baxter *et al.*, 2010; Rinkevich *et al.*, 2010). Likewise, truncated transcripts of nAChRα6 have been found to confer resistance to spinosads in both the oriental fruit fly, *Bactrocera dorsalis* (Handel) (Diptera: Tephritidae) (Hsu *et al.*, 2012) and fruit fly, *D. melanogaster* (Perry *et al.*, 2007).

Orchardists using neonicotinoids and spinosads to control codling moth are concerned that target-site resistance in this insect could render these highly successful classes of insecticides ineffective. To help mitigate this issue, the first goal of this study was to identify transcripts encoding putative nAChR subunits from an assembled codling moth head transcriptome. The presence of each transcript encoding for nAChR subunits was confirmed by PCR and validated by DNA sequence determinations. The second goal of this study was to determine the temporal expression profiles of nAChR subunits encoding transcripts. The results of this study provide information that will allow for monitoring of codling moth populations for potential target-site resistance to neonicotinoids and spinosads.

Materials and Methods

Insects

Codling moth collected from field sites in the Yakima Valley were reared and maintained at the Yakima Agricultural Research Laboratory, Wapato, WA, USA. Larvae resulting from adult matings were reared on artificial diet (Southland Products, Lake Village, AR, USA). All insect rearing was done at 26 °C, 70% RH with a 16 : 8 light : dark cycle. Once the codling moth colony was generating a constant source of insects, the heads and bodies of the various larval, pupal, and adult stages were collected and stored at 4 °C in RNAlater® (Ambion, Austin, TX, USA) until use for RNA extraction.

RNA extraction and transcriptome generation

Head tissue from above was recovered from RNAlater® and total RNA was extracted from 30 mg of each larval, pupal and adult stage using the RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol for purification of total RNA from animal tissues. In the final step, total RNA was eluted from the RNeasy spin column with 40 μL of RNase-free water (supplied with kit). The RNA was then used in construction of the Illumina HiSeq (Illumina, Inc., San Diego, CA, USA) library for sequencing following HiSeq 2000 platform-specific protocols. The sequence reads were assembled into contigs and uploaded to ViroBLAST, a standalone BLAST web server (Deng *et al.*, 2007), for identification of transcripts encoding putative nAChR subunits (see below). The transcriptome was generated in the laboratory of Dr. Amit Dhingra (Washington State University) and details, including methods and results, will be presented elsewhere (Hendrickson *et al.*, manuscript in preparation).

Identification of transcripts encoding putative nAChR subunits

Amino acid sequences of nicotinic acetylcholine receptor subunits (nAChRs) identified in *B. mori* (Shao *et al.*, 2007) were used as queries in tBLASTn searches of the codling moth transcriptome. Codling moth transcript sequences with significant homology to the querries were uploaded to Geneious (version 6.8 created by Biomatters, available from http://www.geneious.com/) and oligonucleotide primers were designed using the Primer3 function contained within Geneious. Primers used in this study can be found in Tables S1 and S2.

cDNA synthesis, transcript amplification, and cloning

Aliquots of RNA from each insect stage were pooled for use as template in first-strand cDNA synthesis reactions. Total RNA (1–5 μg) was converted to cDNA using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) according to manufacturer's conditions with modifications in primers used. For cDNAs used for internal amplifications, RNA was primed with oligo $d(T)_{12-18}$ or the CDSIII/3' PCR Primer (Clontech, Mountain View, CA, USA); for 5' RACE, total RNA was primed with oligo dT (supplied with SuperScript® III SuperMix) and the SMART IV oligonucleotide (Clontech). The cDNA reactions were carried out in a final volume of 20 μL and incubated 50 min at 50 °C.

Amplifications of putative nAChR subunits were done in 20 μL reactions containing 0.4 μL Titanium Taq (Clontech, Mountain View, CA, USA), 1× PCR buffer (supplied with Taq), 0.25 μmol/L each primer, 200 μmol/L each dNTP and 1 μL of 10× diluted head cDNA template. Standard PCR conditions were: initial denaturation for 3 min at 94 °C, then amplification steps of 20 sec at 94 °C, 10–15 sec at annealing temperature, and extensions of 90 sec to 5 min at 72 °C for 40 cycles, followed by a final 5-min 72 °C polishing step (specific annealing and amplification conditions are given in Table S1). For Touchdown PCR, conditions were: initial denaturation for 3 min at 94 °C, then amplification for 20 sec at 94 °C, 10 sec at 60–72 °C, and 90 sec to 5 min at 72 °C for 40 cycles, ending with a final 5-min 72 °C polishing step (specific annealing and amplification conditions are given in Table S1). For 5' RACE reactions, 10× Universal Primer A Mix (10× UPM; Clontech) composed of a long oligonucleotide (5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3') and short oligonucleotide (5'–CTAATACGACTCACTATAGGGC–3') were used as forward primer with conditions as described above. PCR products were separated by agarose gel electrophoresis and resultant products were excised for cloning. DNA in the excised bands were extracted and purified using GenElute Minus EtBr Spin columns (Sigma, St. Louis, MO, USA), and the purified PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA) with TOP 10 *Escherichia coli* chemically competent cells according to manufacturer's protocol. At least 4 clones from each TA cloning were grown overnight at 37 °C, and plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) using the manufacturer's protocol. The cDNA clones were sequenced at MC Laboratories (MCLab, San Francisco, CA, USA). Sequences of cloned nAChR subunits are deposited in GenBank (Accession numbers: KP101240-KP101251).

Characterizations, phylogenetics, and alignments of nAChR subunit proteins

Uploaded transcript sequences were translated in Geneious and then trimmed to generate the open reading frame of the encoded putative nAChR subunit. Amino acid sequences of putative nAChR subunits were downloaded in FASTA format and

signal sequences were predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP; Bendtsen *et al.*, 2004), transmembrane regions were predicted using TMPred on the ExPASy server (http://www.ch.embnet.org/software/TMPRED_form.html), and cys-loop regions, putative N-glycosylation sites, and GEK motifs were predicted using the ExPASy Prosite server (http://prosite.expasy.org/). For alignments of putative nAChR subunits, amino acid sequences for *B. mori*, *D. melanogaster*, and *A. mellifera* were retrieved from the NCBI database. Deduced amino acid sequences of putative codling moth nAChR subunits and those from the above-mentioned insects were aligned using Clustal W (http://www.genome.jp/tools/clustalw/; Thompson *et al.*, 1994) with default settings. EvolView (http://evolgenius.info/evolview/; Zhang *et al.*, 2012) was used to display the phylogenetic tree. Statistical calculations for the identity and similarity table were determined using the Sequence Manipulation Suite (http://www.ualberta.ca/~stothard/javascript/ident_sim.html; Stothard, 2000).

Temporal expression

Thirty milligrams of tissue was collected from each insect stage (larvae, pupae, and adults), separating heads from bodies (starting at instar 3) and males from females (starting at instar 4) and stored in RNALater® until RNA extraction. RNA was extracted as described above, and then residual genomic DNA was removed from 3 μg RNA with the TurboDNA-free kit (Ambion). For expression profiles, cDNA was prepared as described above using 0.4 μg DNA-free RNA as template, and the resultant cDNA was diluted 1 : 5 before use in PCR reactions. Standard and Touchdown PCR reactions were performed as described above (specific annealing and amplification conditions are given in Table S2). The nAChR subunit was considered expressed in a stage if the appropriate band was detected by agarose gel electrophoresis. Select bands were cloned and sequenced to verify their identities.

Results and Discussion

Identification of transcripts encoding putative CpomnAChR subunits

A codling moth head transcriptome was mined using tBLASTn searches to identify transcripts encoding nAChR subunit orthologs of those previously identified in *B. mori*. Twenty-four transcripts encompassing 12 putative nAChR subunit classes were identified (see Supplementary Text file for nucleotide and deduced amino acid sequences). From the transcriptome, complete ORFs were obtained for 8 of the subunits: CpomnAChRα1 (1539 nt encoding 512 aa), CpomnAChRα2 (1644 nt encoding 547 aa), CpomnAChRα6 (1500 nt encoding 499 aa), CpomnAChRα7 (1530 nt encoding 509 aa), CpomnAChRα8 (1605 nt encoding 534 aa), CpomnAChRα9 (1293 nt encoding 430 aa), CpomnAChRβ1 (1545 nt encoding 514 aa), and CpomnAChRβ2 (1067 nt encoding 398 aa); and partial sequences were obtained for the remaining 4 subunits (CpomnAChRα3, CpomnAChRα4, CpomnAChRα5, and CpomnAChRβ3; See Supplementary Data 1). RT-PCR and 5′ RACE were used to confirm and build upon the information obtained from the transcriptome (Sequences deposited in GenBank, Accessions Nos. KP101240-KP101251).

Characterization of putative nAChR subunit protein products

Characterization of the predicted proteins encoded by the putative nAChR transcripts revealed that they share the distinguishing features of the cys-loop ligand-gated ion channel superfamily. Predicted N-terminal signal peptides were identified for all subunits with the exception of CpomnAChRβ3, for which sequence data for the start codon was not obtained (Fig. 1, underlined). Predicted N-glycosylation sites in the N-terminal extracellular domain were identified in all twelve of the subunits (Fig. 1, **NXS/T**). The N-terminal extracellular domain is involved in ligand binding, and the N-glycosylation sites found in this region may play a role in the regulation of ACh receptor desensitization and ion permeability (Nishizaki, 2003). Vicinal cysteines were identified in CpomnAChRα1–9 (Fig. 1, **CC**), but were not present in CpomnAChRβ1–3. The vicinal cysteines in α-subunits are required for ACh binding (Kao & Karlin, 1986), and β-subunits lack this feature. A dicysteine loop separated by thirteen amino acids was identified in all 12 subunits (Fig. 1, *). The dicysteine loop consists of 2 disulphide-bond forming cysteines separated by 13 amino acids, and is necessary for complete receptor assembly (Green & Wanamaker, 1997). Four transmembrane regions were predicted for 11 of the 12 subunits (Fig. 1, gray background). Three transmembrane regions were predicted for CpomnAChRα5, however, complete sequence through the 3rd transmembrane domain region was not obtained. It is expected that the 3rd transmembrane domain will be identified in CpomnAChRα5 once

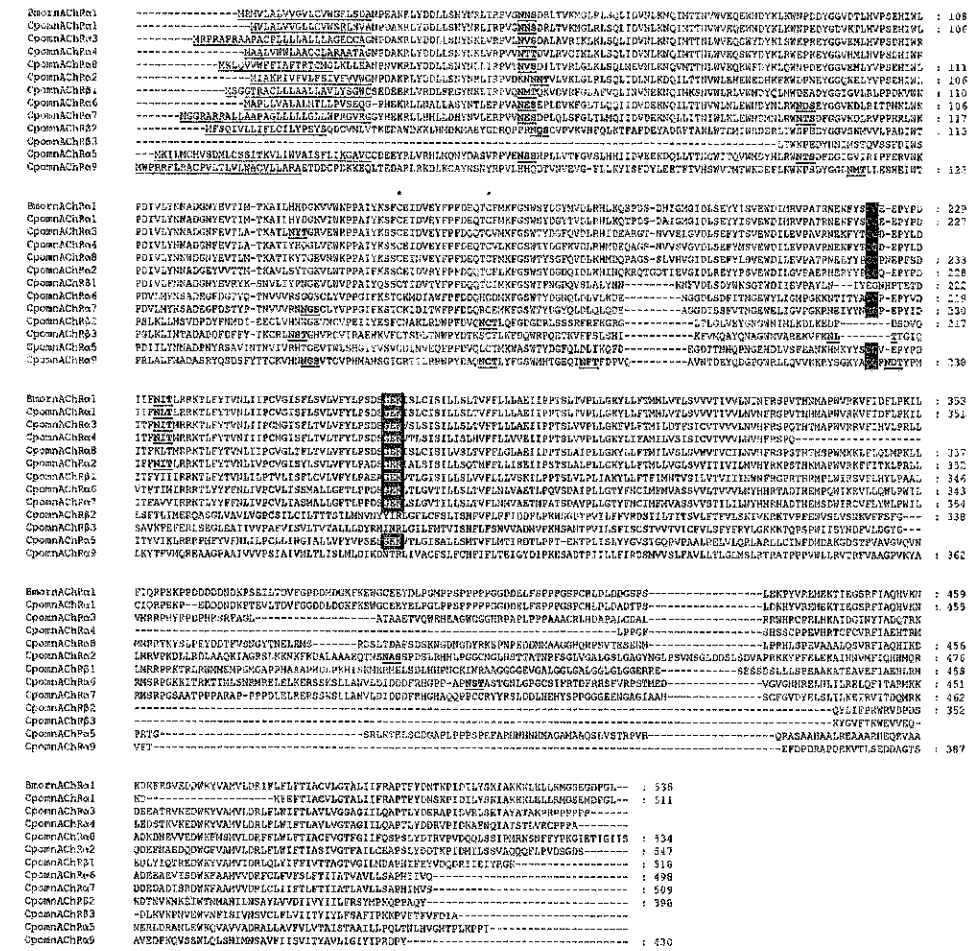

Fig. 1 Protein sequence alignment of codling moth putative nAChR (CpomnAChR) subunits. N-terminal signal peptides are underlined, transmembrane motifs are highlighted in gray, and vicinal cysteines are labeled **CC**. Cys-loops are indicated by *. **GEK** motifs are in black boxes. Predicted N-glycosylation sites are denoted by **NXS/T**. BmornAChRα1 of silkworm is included for comparison.

this region is fully cloned (see Supplementary Data 2 and 3 for nAChRα5 alignments). The 4 transmembrane regions are responsible for how the subunit assembles within the cell membrane (Dani & Bertrand, 2007). The glycine-glutamic acid-lysine (GEK) motif was identified in all of the subunits except CpomnAChRα9, CpomnAChRβ2, and CpomnAChRβ3 (Fig. 1, **GEK**). The second transmembrane region is the main lining along the ionic pore, and the GEK motif, typically located between transmembrane regions 1 and 2, contributes to the cation selectivity of the pore (Dani & Bertrand, 2007). It is not unusual to find that CpomnAChRα9, CpomnAChRβ2, and CpomnAChRβ3 lack a GEK motif as nAChRα9 and nAChRβ2 in the honey bee, as well as the nAChRα9, nAChRβ2, and nAChRβ3 in the silkworm also lack this motif (Jones *et al.*, 2006; Shao *et al.*, 2007).

Comparison of CpomnAChR subunits with those identified in the silkworm

The number of nAChR subunit groups (12) identified in codling moth is identical to those found in the silkworm. Comparison of the codling moth and silkworm nAChR amino acid sequences indicate that CpomnAChRα1–8 and CpomnAChRβ1 all had clear silkworm orthologs showing 85%–99% sequence identity and 89%–100% similarity (Table 1, Supplementary Data 2). CpomnAChRα9, CpomnAChRβ2, and CpomnAChRβ3 did not have clear silkworm orthologs. With only 55% sequence identity and 68% similarity, CpomnAChRα9 is most similar to BmornAChRα9 (Table 1). Additionally, as mentioned above, the CpomnAChRα9 lacks the GEK motif, as do the BmornAChRα9 and the AmelnAChRα9 subunits (Jones *et al.*, 2006; Shao *et al.*, 2007). CpomnAChRβ2 and CpomnAChRβ3 have below 30% sequence identity and below 50% similarity to all silkworm nAChR subunits. For CpomnAChRβ2, the highest sequence identity (26%) and highest similarity (43%) was to BmornAChRβ2, and for CpomnAChRβ3, the highest sequence identity (24%) and highest similarity (45%) was to BmornAChRβ3. CpomnAChRβ2 and CpomnAChRβ3 also lack GEK motifs, as do BmornAChRβ2 and BmornAChRβ3.

Putative CpomnAChR subunits cluster with other insect nAChR subunit groups

To provide further support that putative codling moth nAChR subunits were appropriately classified, a phylogenetic tree with putative nAChR subunits of codling moth and those of the fruit fly (Dmel), the honey bee (Amel), and the silkworm (Bmor) was constructed. Representative subunits from the other 4 cys-loop ligand-gated ion channels (glycine receptor, GABA-gated chlorine channel, glutamate receptor, and 5-HT receptor) found in silkworm were used as an "out group" in the tree. The phylogenetic tree supports the identification of the proteins encoded by codling moth transcripts as cys-loop ligand-gated ion channel subunits in the nAChR subunits family (Fig. 2). The phylogenetic tree also makes apparent the relationships between the putative nAChR subunits of codling moth and those of the fruit fly (Dmel), the honey bee (Amel), and the silkworm (Bmor) (Fig. 2). The CpomnAChRα1, CpomnAChRα2, CpomnAChRα3, CpomnAChRα4, CpomnAChRα6, CpomnAChRα7, and CpomnAChRβ1 subunits group together with their respective nAChR subunits in the other 3 insects considered, indicating a high degree of conservation for each of these subunits. The CpomnAChRα5 subunit groups with the nAChRα5 of honey bee and silkworm, but not with that of the fruit fly. The CpomnAChRα8 subunit groups with the nAChRα8 of honey bee and silkworm, and the nAChRβ2 of the fruit fly. This suggests that the nAChRα5 and nAChRα8 subunits are also fairly conserved. CpomnAChRα9, CpomnAChRβ2, and CpomnAChRβ3 cluster with a group made up of AmelnAChRα9 and AmelnAChRβ2, as well as BmornAChRα9, BmornAChRβ2, and BmornAChRβ3. The nAChRα9/β2/β3 group of subunits clusters separately from the most highly conserved nAChR subunit groups. The groups in our phylogenetic tree correlate with the groups of insect nAChR subunits identified by Jones & Sattelle (2010) based on amino acid sequence homology amongst honey bee, silkworm, fruit fly, malaria mosquito, and red flour beetle. In that study, 7 core groups were identified as highly conserved between species (Dα1, Dα2, Dα3, Dα4, Dα5–7, Dβ1, Dβ2) with an 8th divergent subunit category representing those subunits with low sequence identity to the other nAChR subunits (Jones & Sattelle, 2010). In accordance with these categories the CpomnAChR subunits cluster appropriately supporting their identification as members of the nAChR subunit family (Fig. 2).

CpomnAChR subunits express differentially throughout codling moth life cycle

A second goal of this study was to determine life stage expression profiles of transcripts encoding codling moth nAChR subunits. Transcripts encoding codling moth nAChR subunits were initially identified in a head transcriptome, but it was of interest to determine which subunits are expressed in the body since nerves are also present there. Differential expression of the various nAChR subunit transcripts was apparent in the codling moth life cycle with a head-biased profile (Fig. 3). The CpomnAChRα1 transcript was detected in heads of all larval stages and the pupal stage in both genders, but in males, it is also detected in the bodies of 4th and 5th instar stages and in the adult heads and bodies. CpomnAChRα1 was not detected in adult females. The CpomnAChRα2 and CpomnAChRα3 transcripts were detected in heads at all stages and in both genders, but are also detected in the bodies of the pupal and adult stages. The CpomnAChRα4 transcript was detected in heads of both genders through all larval stages and female pupae, as well as in the bodies of 5th instar females. CpomnAChRα4 was not detected in adult heads or bodies. The CpomnAChRα5 transcript was detected in the heads at all stages and in both

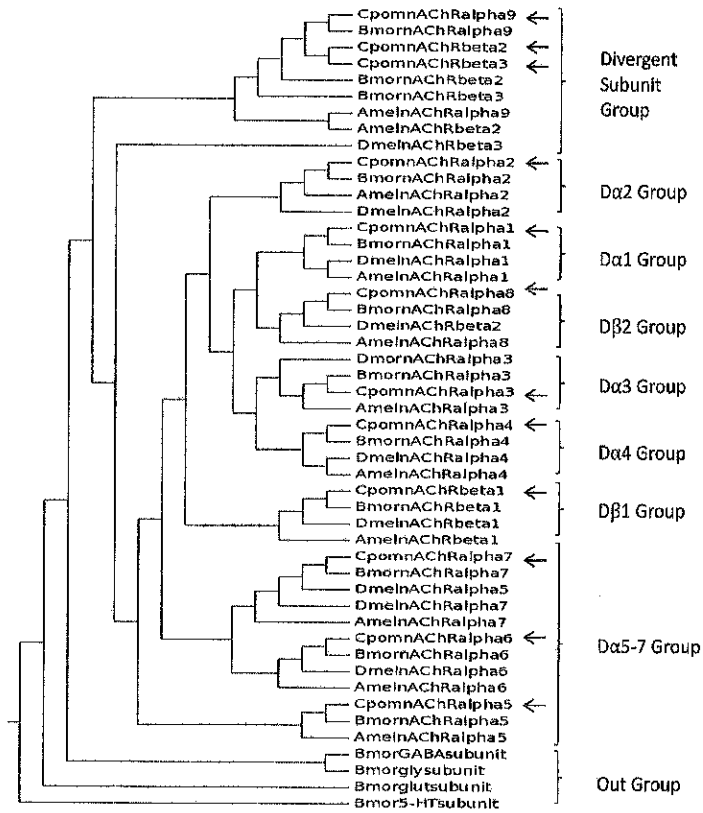

Fig. 2 Phylogenetic tree showing relationship between codling moth (Cpom) nAChR subunits (sequences given in Supplementary Data 1) and silkworm (Bmor), fruit fly (Dmel) and honey bee (Amel) nAChR subunits. The silkworm glycine receptor subunit (Bmorglysubunit; GenBank XM_004926998), GABA-gated chlorine channel subunit (BmorGABAsubunit; GenBank NM_001099824), glutamate receptor subunit (Bmorglutsubunit; GenBank XM_004932681), and 5-HT receptor subunit (Bmor5-HTsubunit; GenBank NM_001044037) are used as out group elements representing the other 4 members of the ligand-gated ion channel superfamily. The nAChR subunits shown in the tree (GenBank Accession nos.) are as follows: BmornAChRα1 (ABV45511), BmornAChRα2 (ABV45512), BmornAChRα3 (ABV45513), BmornAChRα4 (ABV45514), BmornAChRα5 (NP_001166811), BmornAChRα6 (ABV45518), BmornAChRα7 (ABV45520), BmornAChRα8 (ABV45521), BmornAChRα9 (ABV45523), BmornAChRβ1 (ABV45508), BmornAChRβ2 (ABV45509), BmornAChRβ3 (ABV45510), DmelnAChRα1 (CAA30172), DmelnAChRα2 (CAA36517), DmelnAChRα3 (CAA75688), DmelnAChRα4 (CAB77445), DmelnAChRα5 (AAM13390), DmelnAChRα6 (AAM13392), DmelnAChRα7 (ABO26063), DmelnAChRβ1 (CAA27641), DmelnAChRβ2 (AAF56304), DmelnAChRβ3 (CAC48166), AmelnAChRα1 (AAY78790), AmelnAChRα2 (AAS48080), AmelnAChRα3 (AAY87891), AmelnAChRα4 (AAY87892), AmelnAChRα5 (AAS75781), AmelnAChRα6 (AAY87895), AmelnAChRα7 (AAR92109), AmelnAChRα8 (AAM51823), AmelnAChRα9 (AAY87896), AmelnAChRβ1 (AAY87897), and AmelnAChRβ2 (AAY87898). Codling moth putative nAChR subunits are indicated with black arrows.

genders, except for 5th instar males, but is also found in the bodies of 3rd instar and adults. The CpomnAChRα7 transcript was detected in heads at all stages except 2nd instar and is present in both genders. CpomnAChRα7 is also present in bodies at 4th instar and male pupae, as well as in both genders of adults. CpomnAChRα8 transcript was detected in heads of both genders, and in the bodies of adult males. The CpomnAChRα9 transcript

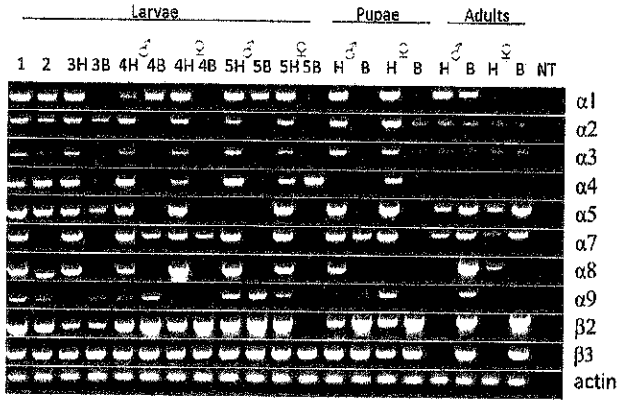

Fig. 3 Temporal expression profiles of 10 nAChR subunits in codling moth. PCR reactions were done to determine nAChR subunit expression in the various codling moth life stages (larval, pupal and adult). For each stage (except 1st, 2nd, and 3rd instar), we separated males (♂) from females (♀). Also, for each stage (except 1st and 2nd instar), we separated heads (H) from bodies (B). NT indicates "no cDNA template" control. CpomnAChR subunits tested are listed to the right of each panel. Expression is deemed positive if amplification products are present (the white bands in each panel).

was detected in bodies of both genders through 4th instar, and then continues to be present in male bodies through adulthood. CpomnAChRα9 is also detected in heads of 4th instar males, 5th instar males and females, and female pupae. The CpomnAChRβ2 and CpomnAChRβ3 transcripts appear to be expressed in both heads and bodies at all stages in both genders, except they do not appear in heads of either gender at the adult stage.

There are 3 differences in nAChR subunit expression between codling moth and the honey bee. First, AmelnAChRα9 and AmelnAChRβ2 appear in both heads and bodies of adults in the honey bee (Jones *et al.*, 2006), while CpomnAChRα9 and CpomnAChRβ2 only appear in the bodies of adults in codling moth (Fig. 3). Second, AmelnAChRα4 exon 4 (Jones *et al.*, 2006) is identical to CpomnAChRα4 (Fig. 4), yet AmelnAChRα4 appears in all stages of the life cycle of the honey bee (Jones *et al.*, 2006), while CpomnAChRα4 appears only in codling moth immature stages. Third, the honey bee does not possess a nAChR subunit homologous to CpomnAChRβ3. Identifying these types of differences between pest and beneficial insect species may be useful in developing pest control agents that are not harmful to nontarget species.

Conclusion

The results of this study indicate that codling moth possesses 12 classes of nAChR subunits, including 9 α-type subunits and 3 β-type subunits. These 12 classes of subunits cluster together in a phylogenetic analysis in a way similar to those of other insects. Temporal expression studies indicate that the diversity of nAChRs is increased by the transcription of varying classes of subunits at different points throughout the life cycle. This information establishes a foundation for future studies of nAChRs in codling moth, including investigation of the role of various subunits in the neurotransmission required for learning and behavior in the insect. Mutations found in nAChR subunit transcripts have been implicated in insect resistance to neonicotinoids and spinosads (Liu *et al.*, 2005; Perry *et al.*, 2007; Baxter *et al.*, 2010; Rinkevich *et al.*, 2010; Bass *et al.*, 2011; Hsu *et al.*, 2012; Puinean *et al.*, 2013). The results of this current study provide sequence information that will be useful in monitoring for target-site resistance in codling moth field populations that become tolerant to neonicotinoids and spinosads. Finally, a greater understanding of the diversity and complexity of nAChRs within multiple insect species can lead to identification of subunits unique to target pests. With this information, more targeted control agents can be designed to spare beneficial species while managing agricultural pests.

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                                                    *               *
AmelnAChRα4V1 (exon 4)      ....ADGNFEVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFK....
CpomnAChRα4V1               ....ADGNFEVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFK....
```

Fig. 4 Predicted protein sequence for codling moth (Cpom) and honey bee (Amel) in exon 4 region. Cys-loop region is indicated by asterisks. AmelnAChRα4 sequence was obtained from GenBank (accession no. AAY87892).

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Disclosure

This article reports the results of research only. Mention of commercial or proprietary products does not constitute an endorsement by the United States Department of Agriculture.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Table 1. Sequences of PCR primers used in nested RT-PCR reactions to detect transcripts of codling moth nAChR subunits. All primers shown 5′ to 3′.

Supplementary Table 2. Sequences of PCR primers used in RT-PCR reactions for temporal expression of codling moth nAChR subunits. All primers shown 5′ to 3′.

Supplementary Data 1. Codling moth head transcriptome sequences encoding putative nicotinic acetylcholine receptors and translations.

Supplementary Data 2. Alignments of nAChR subunits of codling moth *C. pomonella* (Cpom) and silkworm *B. mori* (Bmor). Accession numbers for *B. mori* sequence used are listed in the description of Figure 2.

Supplementary Data 3. Alignment of nAChRα5 sequences from honey bee *A. mellifera* (Amel) with that of codling moth *C. pomonella* (Cpom). Accession numbers for *A. mellifera* sequence is listed in the legend of Figure 2.

Supplementary Table 1. Sequences of PCR primers used in nested RT-PCR reactions to detect transcripts of codling moth nAChR subunits. All primers shown 5' to 3'.

	Run 1			Run 2		
Subunit	Forward	Reverse	Anneal Temp, Time/Ext Time	Forward	Reverse	Anneal Temp, Time/Ext Time
CpomnAChRα1	CTAAGATGGTGTTGGCG CTGG	CTATAAAGACCTTCCGG ACCCAAGG	TD 72°-68°, 62°, 10 sec/3 min	GTCCAACTACAACCGAC TGATCC	CTATAAAGACCTTCCGG ACCCAAGG	TD 72°-68°, 62°, 10 sec/3 min
CpomnAChRα2	10X UPM	CAAGCCATACGTTGGTG GTCAAG	STD 65°, 10 sec/1 min 30 sec	GTGGTGACAACGATGAC AAAGGC	CTAATCGTGTTTAAGAA TCACGACTATCCAC	TD 67°-62°, 10 sec/2 min
CpomnAChRα3	GGCTCCTGGACTTACGA TGGC	CCAGTATCATGGTGAAG AGCACAAAC	STD 64.0°, 15 sec/1 min	ND		
CpomnAChRα4	CTCTCCAACTACAACAA GCTGGTCC	CCACGTCTATTTCACAG GAAGACTTG	TD 70°-66°, 65°, 10 sec/ 1 min 30 sec	CTCTCCAACTACAACAA GCTGGTCC	GGAAAGAACTCGACGTC GATCG	TD 70°-66°, 65°, 10 sec/ 1 min 30 sec
CpomnAChRα5	TGAAGAGTATCGCTTAG TACGCCAC	TTCATGTGTTTATTGGC CTCGAAGC	TD 72°-68°, 62°, 10 sec/2 min	CATGAAATGGGCTTCGT GGACTTAC	TTCATGTGTTTATTGGC CTCGAAGC	TD 72°-68°, 62°, 10 sec/2 min
CpomnAChRα6	GGCGCTGATGACCTTGC TGC	GTGCGTCCTCACTGTAC GATGATGTG	TD 65°-61°, 60°, 10 sec/90 sec	GACCAACACTGTGACAT GAAGTTCG	GGAGAGCCATCCAAGAG ATAAGGAC	TD 70°-65°, 64°, 10 sec/30 sec
CpomnAChRα7	GAACATCTGGCTCAAAC TGGAATGG	GTAGTTGAGGATGAGGA TGGTGGAC	TD 72°-68°, 62°, 10 sec/2 min	ND		
CpomnAChRα8	CAATATGAAGTTGCAAG TGGTGTGG	CTTAACTAATAATCCCA ATAGTCTCAATGCC	TD 72°-68°, 10 sec/2 min	CAATATGAAGTTGCAAG TGGTGTG	CTAATAATCCCAATAGT CTCAATGCCTTTCG	TD 72°-68°, 10 sec/2 min
CpomnAChRα9	GAATGTGGCCCAGAAGG TTCC	ACGTTAACATGACGATG GCTATGGA	TD 72°-68°, 62°, 10 sec/3 min	GAATGTGGCCCAGAAGG TTCC	AGGATGATACTTGCTTT GGGTCCTC	TD 72°-68°, 62°, 10 sec/3 min
CpomnAChRβ1	CCGGTGCAAAACATGAC ACAG	GATTGTCGTACAGTTAT ACGCTGTTCGC	TD 67°-63°, 62°, 10 sec/2 min	CTGTCTGACTTGCATCA CCCTAACT	CGCTGTTCGCAATTAGT ATTTACTTCC	TD 70°-65°, 64°, 10 sec/30 sec
CpomnAChRβ2	10X UPM	GTTGCCTGTCACCATAA CTTGCCAT	TD 72°-68°, 62°, 10 sec/2 min	10X UPM	CGACCATCTTGCTGACT CCTCCATA	TD 72°-68°, 62°, 10 sec/2 min
CpomnAChRβ3	CTGATCAATACGGCAGA TGCGG	GGAAGCTACTTAAACTT CCTTAAGCGATATCG	TD 68°-63°, 10 sec/3 min	ND		

	Run 3		Anneal Temp, Time/Ext Time	Run 4		Anneal Temp, Time/Ext Time
Subunit	Forward	Reverse		Forward	Reverse	
CpomnAChRα1	CTAAGATGGTGTTGGCG CTGG	TTCCATATCACTTTGCC GTCGTAGT	TD 72°-68°, 62°, 10 sec/3 min	GTCCAACTACAACCGAC TGATCC	TGTTTGCTTCAGATGCC TCAAATCC	TD 72°-68°, 62°, 10 sec/3 min
CpomnAChRα2	CCTCAAAGCGAAGGTAA AATGATTGCAAAGCG	AGGGAAAGTACTCGACG TCAATCTC	STD 60.7°, 15 sec/1 min	CCTCAAAGCGAAGGTAA AATGATTGCAAAGCG	GCCTTTGTCATCGTTGT CACCAC	STD 60.7°, 15 sec/1 min
CpomnAChRα3	ND			ND		
CpomnAChRα4	ND			ND		
CpomnAChRα5	ND			ND		
CpomnAChRα6	GGCGCTGATGACCTTGC TGC	ATAAGAACGTCTGGCTT CCACAGTT	TD 70°-65°, 64°, 10 sec/30 sec	GAGCTGCAGTTCATCAC AGCGAGGATG	GTGCGTCCTCACTGTAC GATGATGTG	TD 70°-65°, 64°, 10 sec/30 sec
CpomnAChRα7	ND			ND		
CpomnAChRα8	ND			ND		
CpomnAChRα9	ND			ND		
CpomnAChRβ1	CCGGTGCAAAACATGAC ACAG	CGGGCACTTCTATGATA TCCCATGT	TD 70°-65°, 64°, 10 sec/30 sec	ND		
CpomnAChRβ2	GAGCCATGTTCTCGCAA ATAGTCC	GTGATTTTTCAGTTAGT ATTGTGCTGGTGG	TD 70°-66°, 65°, 10 sec/2 min	ND		
CpomnAChRβ3	ND			ND		

Supplementary Table 2. Sequences of PCR primers used in RT-PCR reactions for temporal expression of codling moth nAChR subunits. All primers shown 5' to 3'.

	Forward	Reverse	Annealing Temp, Time/Extension Time
CpomnAChRα1	GTCCAACTACAACCGACTGATCC	TGTTTGCTTCAGATGCCTCAAATCC	Touchdown 69°-64°, 10 sec/1 min
CpomnAChRα2	CCTCAAAGCGAAGGTAAAATGATTGCAAAGCG	GCCTTTGTCATCGTTGTCACCAC	Standard 60.7°, 15 sec/1 min
CpomnAChRα3	GGCTCCTGGACTTACGATGGC	CCAGTATCATGGTGAAGAGCACAAAC	Standard 66.9°, 15 sec/1 min
CpomnAChRα4	CTCTCCAACTACAACAAGCTGGTCC	CCACGTCTATTTCACAGGAAGACTTG	Touchdown 70°-66°, 65°, 10 sec/30 sec
CpomnAChRα5	TGAAGAGTATCGCTTAGTACGCCAC	TTCATGTGTTTATTGGCCTCGAAGC	Touchdown 72°-68°, 62°, 10 sec/2 min
CpomnAChRα7	GAACATCTGGCTCAAACTGGAATGG	GTAGTTGAGGATGAGGATGGTGGAC	Touchdown 68°-63°, 10 sec/2 min
CpomnAChRα8	CAATATGAAGTTGCAAGTGGTGTG	CTAATAATCCCAATAGTCTCAATGCCTTTCG	Touchdown 68°-63°, 10 sec/2 min
CpomnAChRα9	GAATGTGGCCCAGAAGGTTCC	ACGTTAACATGACGATGGCTATGGA	Touchdown 68°-63°, 10 sec/2 min
CpomnAChRβ2	GAGCCATGTTCTCGCAAATAGTCC	GTGATTTTTCAGTTAGTATTGTGCTGGTGG	Touchdown 68°-63°, 10 sec/2 min
CpomnAChRβ3	CTGATCAATACGGCAGATGCGG	GGAAGCTACTTAAACTTCCTTAAGCGATATCG	Touchdown 68°-63°, 10 sec/2 min

Supplementary Data 1. Codling moth head transcriptome sequences encoding putative nicotinic acetylcholine receptors and translations.

>CpomnAChRα1_Wapato_GAGTGG_L005_R1_001_(paired)_contig_75622_(reversed)
ATGGTGTTGGCGCTGGTGGTCGGACTCCTGTGCGTGTGGAGTCGCCTGAGCGTCGCCAACCCTGATGCGAAGCGCTTGTAC
GACGACCTGCTGTCCAACTACAACCGACTGATCCGGCCCGTCGGCAACAACTCGGACCGACTCACCGTCAAGATGGGGCTG
CGGCTCAGTCAGCTTATCGATGTTAACCTTAAGAATCAAATCATGACAACGAACGTTTGGGTAGAACAGGAGTGGAACGAT
TACAAACTGAAGTGGAACCCGGAGGACTACGGTGACGTCAAAACTCTCCACGTGCCTTCTGAACACATATGGCTGCCAGAC
ATCGTGCTTTATAACAACGCGGACGGTAACTACGAAGTGACGATAATGACGAAAGCCATTCTACACTACGACGGCAAAGTG
ATATGGAAGCCCCCGGCCATCTACAAGTCCTTCTGCGAGATCGACGTGGAGTACTTCCCCTTTGACGAGCAGACTTGCTTC
ATGAAATTCGGTAGTTGGAGCTACGACGGTTATACGGTGGATTTGAGGCATCTGAAGCAAACACCAGATTCTGACGCGATA
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ATACCAAAGAAGCCAGTTTTCACATTTGTTTTCGATATCGCTTAA

>CpomnAChRβ3_contig_136596_translation
LTWKPEDYNNIMSTQVSSFDIWSPGLKLINTADADDFDFFYTKCRLNSTGHVRCVIRAEHKVFCTSDLTNWPYDTKSCTL
KFDQWRPQETKVFFSLSHIKFVMQAYQNAGWMVAREKVFKNLTTGIQSAVKFEFERLSEGLEAIIVVPAFVISVLTVTAL
LLDYRNINRLGILFMTVISHFLFSNVVADNVPKHSANTPVILSFISCSTVVTVICFVLSFYFRYLGKKKTQPSPWIISYN
DFVLDGYGKYGVFTKWEVVEQDLKVKPNVEWVNFISIVNSVCLFLVIITYIYLFSAFIPKKPVFTFVFDIA*

Supplementary Data 2. Alignments of nAChR subunits of codling moth *C. pomonella* (Cpom) and silkworm *B. mori* (Bmor). Accession numbers for *B. mori* sequence used are listed in the description of Figure 2.

```
CpomnAChRα1     MVLALVVGLLCVWSRLSVANPDAKRLYDDLLSNYNRLIRPVGNNSDRLTVKMGLRLSQLI
BmornAChRα1     MVLALVVGVLCVWGRLSDANPEAKRLYDDLLSNYNRLIRPVGNNSDRLTLKMGLRLSQLI
                ********:****.*** ***:****************************:**********

CpomnAChRα1     DVNLKNQIMTTNVWVEQEWNDYKLKWNPEDYGDVKTLHVPSEHIWLPDIVLYNNADGNYE
BmornAChRα1     DVNLKNQIMTTNVWVEQEWNDYKLKWNPDDYGGVDTLHVPSEHIWLPDIVLYNNADGNYE
                ****************************:***.*.************************

CpomnAChRα1     VTIMTKAILHYDGKVIWKPPAIYKSFCEIDVEYFPFDEQTCFMKFGSWSYDGYTVDLRHL
BmornAChRα1     VTIMTKAILHHDGKVVWKPPAIYKSFCEIDVEYFPFDEQTCFMKFGSWSYDGYMVDLRHL
                **********:****:************************************ ******

CpomnAChRα1     KQTPDSDAIGMGIDLSEYYISVEWDIMRVPATRNEKFYSCCEEPYPDIIFNLTLRRKTLF
BmornAChRα1     KQSPDSDHIGMGIDLSEYYISVEWDIMRVPATRNEKFHSCCEEPYPDIIFNITLRRKTLF
                **:**** ****************************:*************:*********

CpomnAChRα1     YTVNLIIPCVGISFLSVLVFYLPSDSGEKISLCISILLSLTVFFLLLAEIIPPTSLTVPL
BmornAChRα1     YTVNLIIPCVGISFLSVLVFYLPSDSGEKISLCISILLSLTVFFLLLAEIIPPTSLTVPL
                ************************************************************

CpomnAChRα1     LGKYLLFTMMLVTLSVVVTIVVLNVNFRSPVTHHMAPWVRKVFIDFLPKILCIQRPEKP-
BmornAChRα1     LGKYLLFTMMLVTLSVVVTIVVLNINFRSPVTHNMAPWVRKVFIDFLPKILFIQRPEKPP
                ************************:*********:****************** *******

CpomnAChRα1     -EDDDNDKPTEVLTDVFGGDDLDGKFKEWGCEEYELPGLPPSPPPPPGGDDELFSPPPGS
BmornAChRα1     DDDDDNDKPSEILTDVFGPDDMDGKFKEWGCEEYDLPGMPPSPPPPPGGDDELFSPPPGS
                 :********:*:****** **:************:***:**********************

CpomnAChRα1     PCHLDLDADTPSLDKHYVREMEKTIEGSRFIAQHVKNKD--------------------K
BmornAChRα1     PCRLDLDDGSPSLEKPYVREMEKTIEGSRFIAQHVKNKDKFESVEDDWKYVAMVLDRIFL
                **:****  .:***:* ************************

CpomnAChRα1     FEFTIACVLGTALIIFRAPTFYDNSKPIDILYSKIAKKKLELLRMGSEMDPGL
BmornAChRα1     FLFTIACVLGTALIIFRAPTFYDNTKPIDILYSKIAKKKLELLRMGSEGDPGL
                * **********************:********************** ****
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```
CpomnAChRα2     MIAKRIVFVLFSIVFVVWGNPDAKRLYDDLLSNYNRLIRPVDKNNNTVLVKLGLRLSQLI
BmornAChRα2     -MSKAIFVVFFCSFTVCYANPDAKRLYDDLLSNYNRLIRPVDKNNNTVLVKLGLRLSQLI
                 :;* *..*;*. . * :.****************************************

CpomnAChRα2     DLNLKDQILTTNVWLEHEWEDHKFKWDPNEYGGQKSLYVPSEHIWLPDIVLYNNADGEYV
BmornAChRα2     DLNLKDQILTTNVWLEHEWEDHKFKWDPLEYGGVRELYVPSEHIWLPDIVLYNNADGEYV
                ***************************** **** ;************************

CpomnAChRα2     VTTMTKAVLSYTGKVLWTPPAIFKSSCEIDVRYFPFDQQTCFLKFGSWSYDGDQIDLKHI
BmornAChRα2     VTTMTKAVLHHNGKVLWTPPAIFKSSCEIDVRYFPFDQQTCFLKFGSWSYDGDQIDLKHI
                ********* :.***************************************************

CpomnAChRα2     NQKRQTGDTIEVGIDLREYYPSVEWDILGVPAERHERYYPCCQEPYPDIFFNITLRRKTL
BmornAChRα2     NQKK--GDMVEVGIDLREYYPSVEWDILGVPAERHERYYPCCQEPYPDIFFNITLRRKTL
                ***;  ** :***************************************************

CpomnAChRα2     FYTVNLIVPCVGISYLSVLVFYLPADSGEKIALSISILLSQTMFFLLISEIIPSTSLALP
BmornAChRα2     FYTVNLIVPCVGISYLSVLVFYLPADSGEKIALSISILLSQTMFFLLISEIIPSTSLALP
                ************************************************************

CpomnAChRα2     LLGKYLLFTMLLVGLSVVITIVILNVHYRKPSTHKMAPWVRKFFITKLPRLLLMRVPKDL
BmornAChRα2     LLGKYLLFTMLLVGLSVVITIIILNVHYRKPSTHKMAPWVRKFFITKLPKLLLMRVPKDL
                ********************;***************************;**********

CpomnAChRα2     LRDLAAQKIAGRSLK-KNKFKDALAAAEQTNSNASSPDSLRHHLPGGCNGLHSTTATNRF
BmornAChRα2     LRDLAAQKIAGRSMKNKNKFKDALAAADQTHSNASSPDSLRHHMPGGCNGLHTTTATNRF
                *************;* ***********;**;***********;********;*******

CpomnAChRα2     SGLVGALGSLGAGYNGLPSVMSGLDDSLSDVAPRKKYPFELEKAIHNVMFIQHHMQRQDE
BmornAChRα2     SGLVGALGSLGAGYNGLPSVMSGLDDSLSDVVPRKKYPFELEKAIHNVMFIQHHMQRQDE
                ******************************.*****************************

CpomnAChRα2     FNAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAILCEAPSLYDDTKPIDMILSSVAQQQF
BmornAChRα2     FNAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAILCEAPSLYDDTKPIDMMLSSVAQQQY
                ************************************************;********;

CpomnAChRα2     LPVDSGDS
BmornAChRα2     LPVDSGDS
                ********
```

```
CpomnAChRα3     MRPRAPRAAPACPLLLLALLLAGECCAGNPDAKRLYDDLLSNYNKLVRPVLNVSDALAVR
BmornAChRα3     -----MPRRAPPLAAPLLLLLALAGCAGNPDAKRLYDDLLSNYNKLVRPVLNISDALTVR
                          ..     * ****   ***************************:****:**

CpomnAChRα3     IKLKLSQLIDVNLKNQIMTTNLWVEQSWYDYKLSWEPREYGGVEMLHVPSDHIWRPDIVL
BmornAChRα3     IKLKLSQLIDVNLKNQIMTTNLWVEQTWYDYKLSWEPREYGGVEMLHVPSDHIWRPDIVL
                ************************:**********************************

CpomnAChRα3     YNNADGNFEVTLATKATLNYTGRVEWRPPAIYKSSCEIDVEYFPFDQQTCVMKFGSWTYD
BmornAChRα3     YNNADGNFEVTLATKATLNYTGRVEWRPPAIYKSSCEIDVEYFPFDQQTCVMKFGSWTYD
                ************************************************************

CpomnAChRα3     GFQVDLRHIDEARGTNVVELGVDLSEFYTSVEWDILEVPAVRNEKFYTCCDEPYLDITFN
BmornAChRα3     GFQVDLRHIDEVRGTNVVELGVDLSEFYTSVEWDILEVPAVRNEKFYTCCDEPYLDITFN
                ***********.************************************************

CpomnAChRα3     ITMRRKTLFYTVNLIIPCMGISFLTVLVFYLPSDSGEKVSLSISILLSLTVFFLLLAEII
BmornAChRα3     ITMRRKTLFYTVNLIIPCMGISFLTVLVFYLPSDSGEKVSLSISILLSLTVFFLLLAEII
                ************************************************************

CpomnAChRα3     PPTSLVVPLLGKFVLFTMILDTFSICVTVVVLNVHFRSPQTHTMAPWVRRVFIHVLPRLL
BmornAChRα3     PPTSLVVPLLGKFVLFTMILDTFSICVTVVVLNVHFRSPQTHTMSPWVRRVFIHVLPRLL
                *******************************************:****************

CpomnAChRα3     VMRRPHYRPDPHRSRFAGLATAAETVQWEHEAGWCGGHRPAPLPPPAAACRLHDAPALCD
BmornAChRα3     VMRRPHYRLDPHRSRFAGLVSGVEAPWEEVPH----AEVPAPPAPTCAVCAPEEAPVLCD
                ******** **********.:..*:   *       .. *** .*..*.*  .:**.***

CpomnAChRα3     ALRRWHRCPELHKAIDGINYIADQTRKDEEATRVKEDWKYVAMVLDRLFLWIFTLAVLVG
BmornAChRα3     TLRRWHRCPELHKAIDGINYIAEQTRKEEESTRVKEDWKYVAMVLDRLFLWIFTLAVVVG
                :*********************:****:**:*************************:**

CpomnAChRα3     SAGIILQAPTLYDERAPIDVRLSEIAYATAKPRPPPPPR
BmornAChRα3     SAGIILQAPTLYDERAPIDVRLSEIAYATAKPRPPPPR-
                ************************************
```

```
CpomnAChRα4     MAALVWWLAACCLARAATAGNPDAKRLYDDLLSNYNKLVRPVVNTTDVLRVCIKLKLSQL
BmornAChRα4     MAAFVWWLAACCLLRAATAGNPDAKRLYDDLLSNYNKLVRPVVNTTDVLRVCIKLKLSQL
                ***:********* **********************************************

CpomnAChRα4     IDVNLKNQIMTTNLWVEQSWYDYKLRWEPKEYGGVHMLHVPSDHIWRPDIVLYNNADGNF
BmornAChRα4     IDVNLKNQIMTTNLWVEQSWYDYKLRWEPKEYGGVHMLHVPSDHIWRPDIVLYNNADGNF
                ************************************************************

CpomnAChRα4     EVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFKVDLRH
BmornAChRα4     EVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFKVDLRH
                ************************************************************

CpomnAChRα4     MDEQAGSNVVSVGVDLSEFYMSVEWDILEVPAVRNEKFYTCCDEPYLDITFNITMRRKTL
BmornAChRα4     MDEQAGSNVVSVGVDLSEFYMSVEWDILEVPAVRNEKFYTCCDEPYLDITFNITMRRKTL
                ************************************************************

CpomnAChRα4     FYTVNIIIPCMGISFLTVLTFYLPSDSGEKVTLSISILISLHVFFLLVVEIIPPTSLVVP
BmornAChRα4     FYTVNIIIPCMGISFLTVLTFYLPSDSGEKVTLSISILISLHVFFLLVVEIIPPTSLVVP
                ************************************************************

CpomnAChRα4     LLGKYLIFAMILVSISICVTVVVLNVHFRSP----------------------------
BmornAChRα4     LLGKYLIFAMILVSISICVTVVVLNVHFRSPQTHRMAPWVKRVFIHILPRLLFMKRPQYK
                ********************************

CpomnAChRα4     ------------------------------------------------------------
BmornAChRα4     FDTTRSRYTACGVVVRCGGTARPLYPYRLAAADDDCCAPGAWPPPAAPPPRPLTRTPSKE

CpomnAChRα4     ----------------------------------------------QLPPGFSHSSCP
BmornAChRα4     DLTHTTYLNSGIDSNRLGGSCMLHGSSEVVGGGLAGVSVEEEILSPVPDPPPGFSHSACP
                                                             : *******:**

CpomnAChRα4     PEVHRTCFCVRFIAEHTRMLEDSTKVKEDWKYVAMVLDRLFLWIFTLAVLVGTAGIILQA
BmornAChRα4     PEVHRTCFCVRFIAEHTKMLEDSTKVKEDWKYVAMVLDRLFLWIFTLAVLVGTAGIILQA
                *****************:******************************************

CpomnAChRα4     PTLYDDRVPIDKAFNQIATSTLVRCPPPA
BmornAChRα4     PTLYDDRVPIDKHFNQYATSTLVRCPPPV
                ************ *** ***********.
```

```
CpomnAChRα_5    MKILMCHVSDMLCSSITKVLIWVAISFLIKGAVCCDEEYRLVRHLMQNYDASVRPVENSS
BmornAChRα_5    -------------MSLRKLAVILVILCIRDSLCCDEEYRLVRHLMQKYDASVRPVENSS
                            : *  : : : *:.:;**************:************

CpomnAChRα_5    HPLLVTFGVSLHHIIDVEEKDQLLTTNCWITQVWMDYHLRWNTSDFDGIGVIRIPFERVW
BmornAChRα_5    HPLLVTFGVSLHHIIDVEGKDQLLTTNCWITQIWTDFHLRWNTSDFDGISVIRIPYERVW
                ****************** *************:* *:************.*****:****

CpomnAChRα_5    KPDIILYNNADPNYRSAVINTNVIVRHTGEVTWLSHGIYVSVCDINVEQFPFDVQLCTMK
BmornAChRα_5    RPDIILYNNADPNYRSAVINTNVIVKYTGEVTWLSHGIYVSVCDINVEQFPFDAQLCTMK
                :************************;:*************************.******

CpomnAChRα_5    WASWTYDGFQLDLIKQFDEGDTTNNQPNGEHDLVSFEANKHMKYYSCCVEPYPDITYVIK
BmornAChRα_5    WASWTYDGFQLDLKKQFDEGDTTNYQTNGEFDLVSFEAIRHDQYYSCCVEPYPDITYVIK
                ************* ********** *.***.******* :* :*****************

CpomnAChRα_5    LRRRPMFYVFNLILPCLLINGIALLVFYVPSESGEKVTLGISALLSMTVFLMTIRDTLPP
BmornAChRα_5    LRRRPMFYVFNLILPCLLINGIALLVFYVPSESGEKVTLGISALLSMTVFLMTIRDTLPP
                ************************************************************

CpomnAChRα_5    TEKTPLISLYYGVS----------------------TGQPVPAALRELVLQRLARLLCIN
BmornAChRα_5    TEKTPLISLYYGVSTCLVSFSASLSVVTLNISYRGVRGRPVPAVVRDVVLHRLARLLFIN
                **************                      *:****.:*:;**;****** **

CpomnAChRα_5    FDMDAKGDSTPVAVGVQVNPRTGSRLKTELSCDGAPLPPPSPRFARHNHNHMAGAMAAQS
BmornAChRα_5    FDIDAKGDSRAQSS-VPVNPRTGSRLKAEVRCDGVPLTPASPRFVRHNQNHLAGAAATVG
                **;****** . :  * **********:*: ***.**.*.****.***:**:*** *: .

CpomnAChRα_5    DVSTRPVR-------QRASAAHAALREAAARHEQRVAANERLDRANLEWKQVAVVADRAL
BmornAChRα_5    GASTTSSEPVCVASCARCVCCGCTLREAAARHEQRVAANERLERANLEWKQVAVVADRAL
                ..** . .        *. .. .:******************:*****************

CpomnAChRα_5    LAVFVLVTAISTAAILLPQLTNLHVGNTPLKPPI
BmornAChRα_5    LAVFVLVTTIATAAILLPQLNNLHVGNTPLKPPI
                ********:*:*********.**************
```

```
CpomnAChRα6     MAPLLVALALMTLLPVSEQGPHEKRLLNALLASYNTLERPVANESEPLEVKFGLTLQQII
BmornAChRα6     MVPILAAFALLALLPVSEQGPHEKRLLNALLASYNTLERPVANESEPLEVRFGLTLQQII
                *.*|*.*:**:;**************************************:*********

CpomnAChRα6     DVDEKNQILTTNVWLNLEWNDYNLRWNDSEYGGVKDLRITPNKLWKPDVLMYNSADEGFD
BmornAChRα6     DVDEKNQLLITNIWLSLEWNDYNLRWNESEYGGVKDLRITPNKLWKPDVLMYNSADEGFD
                *******;* **;**.**********;*********************************

CpomnAChRα6     GTYQTNVVVRSSGSCLYVPPGIFKSTCKMDITWFPFDDQHCDMKFGSWTYDGNQLDLVLK
BmornAChRα6     GTYQTNVVVRSGGSCLYVPPGIFKSTCKMDIAWFPFDDQHCDMKFGSWTYDGNQLDLILK
                ***********.******************;************************;**

CpomnAChRα6     DENGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYVDVTFTIMIRRRTLYYFFNLIVP
BmornAChRα6     DEAGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYVDVTFTIMIRRRTLYYFFNLIVP
                ** ************************************************************

CpomnAChRα6     CVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNLVAETLPQVSDAIPLLGTYFNCI
BmornAChRα6     CVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNLVAETLPQVSDAIPLLGTYFNCI
                ************************************************************

CpomnAChRα6     MFMVASSVVLTVVVLNYHHRTADIHEMPQWIKSVLLQWLPWILRMSRPGKKITRKTIMLS
BmornAChRα6     MFMVASSVVLTVVVLNYHHRTADIHEMPQWIKTVFLQWLPWILRMSRPGKKITRKTIMMS
                *********************************:*:**********************:*

CpomnAChRα6     NRMRELELKERSSKSLLANVLDIDDDFRHGPPAPNSTASTGNLGPGCSIFRTDFRRSFVR
BmornAChRα6     NRMRELELKERSSKSLLANVLDIDDDFRHAPPPPNSTASTGNLGPGCSIFRTDFRRSFVR
                *****************************.**.**************************

CpomnAChRα6     PSTMEDVG---VGHHRELHLILRELQFITARMKKADEEAEVISDWKFAAMVVDRFCLFVF
BmornAChRα6     PSTMEDVGGGLSSHHRELHLILRELQFITARMKKADEEAELISDWKFAAMVVDRFCLFVF
                ********    .*************************:******************

CpomnAChRα6     SLFTIIATVAVLLSAPHIIVQ
BmornAChRα6     TLFTIIATVAVLLSAPHIIVQ
                :*******************
```

```
CpomnAChRα7     MGGRARRALLAAPAGLLLLLG-LLWPRGVRGGYHEKRLLHHLLDHYNVLERPVVNESDPL
BmornAChRα7     MGGRARCSLLAAPAGLLLLLLGLLWPRGVCGGHHEKRLLHHLLDHYNVLERPVVNESDPL
                ****** :************  ******* **:**************************

CpomnAChRα7     QLSFGLTLMQIIDVDEKNQLLITNIWLKLEWNDMNLRWNTSDFGGVKDLRVPPHRLWKPD
BmornAChRα7     QLSFGLTLMQIIDVDEKNQLLITNIWLKLEWNDMNLRWNTSDFGGVKDLRVPPHRLWKPD
                ************************************************************

CpomnAChRα7     VLMYNSADEGFDSTYPTNVVVRNNGSCLYVPPGIFKSTCKIDITWFPFDDQRCEMKFGSW
BmornAChRα7     VLMYNSADEGFDSTYPTNVVVRNNGSCLYVPPGIFKSTCKIDITWFPFDDQRCEMKFGSW
                ************************************************************

CpomnAChRα7     TYDGYQLDLQLQDEAGGDISSFVTNGEWELIGVPGKRNEIYYNCCPEPYIDITFAVVIRR
BmornAChRα7     TYDGYQLDLQLQDEAGGDISSFVTNGEWELIGVPGKRNEIYYNCCPEPYIDITFAVVIRR
                ************************************************************

CpomnAChRα7     KTLYYFFNLIVPCVLIASMALLGFTLPPDSGEKLSLGVTILLSLTVFLNMVAETMPATSD
BmornAChRα7     KTLYYFFNLIVPCVLIASMALLGFTLPPDSGEKLSLGVTILLSLTVFLNMVAETMPATSD
                ************************************************************

CpomnAChRα7     AVPLLGTYFNCIMFMVASSVVSTILILNYHHRHADTHEMSDWIRCVFLYWLPWILRMSRP
BmornAChRα7     AVPLLGTYFNCIMFMVASSVVSTILILNYHHRHADTHEMSDWIRCVFLYWLPWILRMSRP
                ************************************************************

CpomnAChRα7     GSAATPPPARAPPPPDLELRERSSKSLLANVLDIDDDFRHGHAQQPPCCRYYRSLDDLHE
BmornAChRα7     GSATTPPPARAPPPPDLELRERSSKSLLANVLDIDDDFRHAQSQQPPCCRYYR-------
                ***:**********************************.:::**********

CpomnAChRα7     HYSPPGGGEENGAGIAAHSCFGVDYELSLILKEIRVITDQMRKDDEDADISRDWKFAAMV
BmornAChRα7     ------GGEENGAGLAAHSCFGVDYELSLILKEIRVITDQMRKDDEDADISRDWKFAAMV
                      ********:**********************************************

CpomnAChRα7     VDRLCLIIFTLFTIIATLAVLLSAPHIMVS
BmornAChRα7     VDRLCLIIFTLFTIIATLAVLLSAPHIMVS
                ******************************
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```
CpomnAChRα8     MKLQVVWFFIAFTRTCMGLKLLEANPNVKRLYDDLLSNYNRLIRPVTNVSDILTVRLGLK
BmornAChRα8     MNFKLCLLILIVYKGTEAVKLLEANPDVKRLYDDLLSNYNRLIRPVTNVSDILTVRLGLK
                *:::: ::: . :  .:*******:**********************************

CpomnAChRα8     LSQLMEVNLKNQVMTTNLWVEQKWFDYKLQWNPDEYGGVEMLYVPSEHIWLPDIVLYNNW
BmornAChRα8     LSQLMEVNLKNQVMTTNLWVEQKWFDYKLQWNPDDYGGVEMLYVPSEHIWLPDIVLYNNW
                **********************************:************************

CpomnAChRα8     DGNYEVTLMTKATIKYTGEVNWKPPAIYKSSCEINVEYFPFDEQTCFMKFGSWTYSGFQV
BmornAChRα8     DGNYEVTLMTKATLKYTGEVNWKPPAIYKSSCEINVEYFPFDEQTCFMKFGSWTYNGAQV
                *************:*******************************************.* **

CpomnAChRα8     DLKHMDQPAGSSLVHVGIDLSEFYLSVEWDILEVPATRNEEYYPCCPNEPFSDITFKLTM
BmornAChRα8     DLKHMDQSSGSSLVHVGIDLSEFYLSVEWDILEVPATRNEEYYPCCP-EPFSDITFKLTM
                *******.:************************************* ************

CpomnAChRα8     RRKTLFYTVNLIIPCVGLTFLTVLVFYLPSDSGEKISLCISILVSLTVFFLGLAEIIPPT
BmornAChRα8     RRKTLFYTVNLIIPCVGLTFLTVLVFYLPSDSGEKISLSISILVSLTVFFLLLAEIIPST
                ************************************.************ ******.*

CpomnAChRα8     SLAIPLLGKYLLFTMILVSLSVWVTVCILNVHFRSPSTHTMSPWMKKLFLQLMPKLLMMR
BmornAChRα8     SLAIPLLGKYLLFTMILVSLSVWMTVCVLNVHFRSPSTHTMSPWMKKLFLQLMPKLLMMR
                ***********************:***:**********************************

CpomnAChRα8     RTKYSLPEYDDTFVSNGYTNELEMSRDSLTDAFGDSKNGDNGDYRKSPNPEDDMMAAGGH
BmornAChRα8     RTKYSLPDYDDTFVSNGYTNELEMSRDSLTDAFGDSK-CDNGDYRKSPAPEDDMMGASAH
                *******:******************************  ********* ******.*..*

CpomnAChRα8     QRPSVTESENMLPRHLSPEVAAALQSVRFIAQHIKDADKDNEVVEDWKFMSMVLDRFFLW
BmornAChRα8     QRPSVTESENMLPRHLSPEVAAALKSVRFIAQHIKDADKDNEVIEDWKFMSMVLDRFFLW
                ************************:*****************:*****************

CpomnAChRα8     LFTIACFVGTFGIIFQSPSLYDTRVPVDQQLSSIPMRKSNFFYPKGIETIGIIS
BmornAChRα8     LFTIACFVGTFGIIFQSPSLYDTRVPVDQQISSIPMKKNNFFYPKDIETIGIIS
                *****************************:*****:*.******.********
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```
CpomnAChRα9     MWPRRFLRACPVLTLVLWACVLLARAETDDCPDKKEQ-LTEDARLRKDLKCAYNSNYRPV
BmornAChRα9     ---------MKLYVGFLIILTIIFNVNCNECPTEREQPFHDEARLHQDLLCAYNVDFRPV
                         : . .*   .:: ..: ::** ::** : ::***::** **** ::***

CpomnAChRα9     LHHQDTVNVEVGFLLKYISFDYLEETFTVHSWVTMTWKDEFLKWKPSDYGGLNMTLLESH
BmornAChRα9     KDHKTSVTVKVRFAMKYLSFDSLEETFTLHSWVALRWKDEFLTWTPSDYGNIKEIQIESH
                 .*: :*.*:* * :**:*** ******:****:: ******.*.*****.::   :***

CpomnAChRα9     EIWTPRLALFNADAS--RYQSDSFYTTCKVHNNGSVTCVPHMAHSGICRTTLRRWPYDAQ
BmornAChRα9     LIWTPSMSLFNADANTAMFQSDQFYTTCLLSSDGTVTCVPHLGHTGICSTSLRRWPYDTQ
                 **** ::******.   :***.***** : .:*:******:.*:*** *:*******:*

CpomnAChRα9     NCTLYFGSWMHTGEQINFTFDPVQAVNTDEYQDGPGWRLLQVVKKRYSGKYACCPNDTYP
BmornAChRα9     NCTLYFGSWMHTGEQVNFTFYKNDPVVLTDFHDGPGWKLLKVVNERLSETYTCCPNSTYP
                ****************:****   :.*   :::*****:**:**::* * .*:****.***

CpomnAChRα9     MLKYTFVMQREAAGPAAIVVVPSIAIVMLTLISLMLDIKDNTRLIVACFSLFCHFIFLTE
BmornAChRα9     MLKYRFVLKRAAAGPAAIVVVPSIVIVILTLTSFLLDVKDSTRLFLVCFSLFGHFIFLTE
                **** **::* **************.**:*** *::**:**.***::.***** *******

CpomnAChRα9     IGYDIPKESADTPIILLFIRDSMVVSLFAVLLTLGLMSLRTRATPPPVWLLRVTRFVAAG
BmornAChRα9     IGYDIPKHSSDTPIILLFLRDSMIVTLVGIIETLVLMDLRERKLPPPTWIISMNRLVSNS
                *******.*:********:****:*:*.::: ** **.** *  ***.*:: :.*:*: .

CpomnAChRα9     PVKYAVFTEFDPDRAPDEKVTLSEDDAGTSAVEDPKQVSSWLQLSNIMNSAVFIISVITY
BmornAChRα9     PGKYVVFTEFDPSNSAEEKVLTEDVTEERSRSSG----SDWIQFANILNSVLFCIAFLIY
                * **.*******..:.:***  .:     *  ..    *.*:*::**:**.:* *:.: *

CpomnAChRα9     AVLIGIYIPRDPY
BmornAChRα9     FILICVYIPYD--
                 :** :*** *
```

```
CpomnAChRβ1     MSGGTRACLLLAALLAVLYSGWCSEDEERLVRDLFRGYNKLIRPVQNMTQKVDVRFGLAF
BmornAChRβ1     -MSCVSRAFLATTLLAILYSGWCSEDEERLVRDLFRGYNKLIRPVQNMTQKVDVRFGLAF
                  . .  .:* ::***:*********************************************

CpomnAChRβ1     VQLINVNEKNQIMKSNVWLRLVWMDYQLMWDEADYGGIGVLRLPPDKVWKPDIVLFNNAD
BmornAChRβ1     VQLINVNEKNQIMKSNVWLRLVWMDYQLMWDEADYGGIGVLRLPPDKVWKPDIVLFNNAD
                ************************************************************

CpomnAChRβ1     GNYEVRYKSNVLIYPNGEVLWVPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQVS
BmornAChRβ1     GNYEVRYKSNVLIYPNGEVLWVPPAIYQSSCTIDVTYFPFDQQTCIMKFGSWTFNGDQVS
                ************************************************************

CpomnAChRβ1     LALYNNKNFVDLSDYWKSGTWDIIEVPAYLNIYEGNHPTETDITFYIIIRRKTLFYTVNL
BmornAChRβ1     LALYNNKNFVDLSDYWKSGTWDIIEVPAYLNIYEGNHPTETDITFYIIIRRKTLFYTVNL
                ************************************************************

CpomnAChRβ1     ILPTVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLVLPLIAKYL
BmornAChRβ1     ILPTVLISFLCVLVFYLPAEAGEKVTLGISILLSLVVFLLLVSKILPPTSLVLPLIAKYL
                ************************************************************

CpomnAChRβ1     LFTFIMNTVSILVTVIIINWNFRGPRTHRMPLWIRSVFLHYLPAALLMRRPRKTRLRWMM
BmornAChRβ1     LFTFIMNTVSILVTVIIINWNFRGPRTHRMPLWIRSVFLHYLPAMLLMRRPRKTRLRWMM
                ************************************************ ***************

CpomnAChRβ1     EMPGMGAPPHAAAPHDLPKHIR----MELSDLHHPNCKINRAAGGGGEVGALGGLGALGG
BmornAChRβ1     EMPGMGAPPHAAAPHDLPKHISKMEAMELSDLHHPNCKINRAAGGG-EVGALGDLGALGG
                ********************     ********************* ******.******

CpomnAChRβ1     LGLGGERRESESSDSLLLSPEAAKATEAVEFIAEHLRNEDLYIQTREDWKYVAMVIDRLQ
BmornAChRβ1     LGLGGERRESESSDSLLLSPEAAKATEAVEFIAEHLRNEDLYIQTREDWKYVAMVIDRLQ
                ************************************************************

CpomnAChRβ1     LYIFFIVTTAGTVGILMDAPHIFEYVDQDRIIEIYRGK
BmornAChRβ1     LYIFFIVTTAGTVGILMDAPHIFEYVDQDRIIEIYRGK
                ***************************************
```

```
CpomnAChRβ2     MFSQIVLLIFLCILYPSYSQDCVMLVTKEDAWDKKLHMDKMASYGDRQPPRNQSCVPVKV
BmornAChRβ2     --MIRTVYISSLLVVLCRCECVIDHSSDENNWEQKLQKDLKANYDISQPPFVNRTFEVMS
                     .: *   ::  . .:  :   :.*: *::**: *  *.*.  ***  :  . *

CpomnAChRβ2     HFQLKTFAFDEYADRFTAKLWTRMIWRDERLTWSPEEYGGVSKMVVLPADIWTPSLKLLN
BmornAChRβ2     FIVIDSYALDSGNDRFEVTGSLILNWMDDRLRWNRSEYAGIADTVMRSAILWLPGFRQVN
                .: :.::*:*.  *** ..    : * *:** *. .**.*::. *: .* :* *.:: :*

CpomnAChRβ2     SVDP-DYFNMDIEECLVHNNGEVMCVPEIIYESFCNAKLRDWPFDVQNCTLQFGDGDRLS
BmornAChRβ2     AVGAGDDDVPFYTLCRVSSSGKVECKLKSVFTSRCRVDLRNWPLDVQQCDLEFGAWKGDK
                :*.. *        * * ..*:* *  : :: * *..**;**;***:* *:**  .  .

CpomnAChRβ2     SSRFRFKGRGLTLGLVEYGNGWNIMLKDLKEDPDSDVQLSFTLIMERQASGLVAVLVGPC
BmornAChRβ2     VLVRVKFPNQLYISEVTLETHWRLTDWEMKHSNESEVQTVWTYTLERQTRTLAAILVLPA
                       . * :. *   . *.:   ::*.. :*:**  :*  :***:  *.*:** *.

CpomnAChRβ2     SILCILTTTSILMNVNYYIRLGFLCFSLISHFVFLFFIDDFLPKHSGDTPVILFFVRDSI
BmornAChRβ2     VVLSLLSASCFLVDVKRTIRLLLCCFSHGCHYRFLSQLQQCIPKHAEDPPALLLLYRGSL
                 :*.:*:::.:*::*:  *** : ***  .*: **  ::: :***: *.*.:*:: *.*:

CpomnAChRβ2     ILTITSVLFTFVLSKIVKRKTVPFEWVSLVSNKVFFSFGQYLIFPRWRVDPDSKDTNVKN
BmornAChRβ2     VVSVLLIMLSIVLRWTATKDTVVPQPLSTMNKAVMCSRWKMLIWPQWHSSSESTCN----
                ::::  ::::;**   ..:.**  : :* :.: *: *  : **:*:*: ..:*. .

CpomnAChRβ2     KEIWTNMANILNSAYLVVDIIVYIILFRSYMPKQPPAQY
BmornAChRβ2     -KEWTHLANTMNSIALGVTFVVYAILFILFLPR------
                 : **::** :**  * * ::** ***  ::*:
```

```
CpomnAChRβ3     ------------------------------------------------------------
BmornAChRβ3     MSLLPGLLLLLVRGSAQDCVIDHRIPEDAWEQKLHTDLINTGSLEPLCNKTEPLDVYIRF


CpomnAChRβ3     ----------------------------LTWKPEDYNNIMSTQVSS-FDIWSPGLKLINT
BmornAChRβ3     TLRYFEYLSEESTFNIYTRVHISWTHDRLTWDPKDYGGIEETVLVSGTEMWYQTFRVLNS
                                            ***.*:**.,* .* : *  ::*   ::::*:

CpomnAChRβ3     ADADDFDFFY-TKCRLNSTGHVRCVIRAEHKVFCTSDLTNWPYDTKSCTLKFDQWRPQE-
BmornAChRβ3     PETDDTVHHYNVPCQVAHTGRVVCVPRFNDPAICVPDLTDWPYDRQTCSLLIAPPDHNTG
                .::**  ..* . *::  **:* ** * :. .:*..***:**** ::*:* :     :

CpomnAChRβ3     --TKVFFSLSHIKFVMQAYQNAGWMVAREKVFKNLTTGIQSAVKFEFERLSEGLEAIIVV
BmornAChRβ3     GANKIKLSFGGRAITMFGAEYGAEWMIIDYLQTNGTDTLN--MTFVMERHGEGLAAVVVF
                  .*: :*:.   :.* . : ..  :  : : .* *  ::  :.* :** .*** *:.*.

CpomnAChRβ3     PAFVISVLTVTALLLDYRNINRLGILFMTVISHFLFSNVVADNVPKHSANTPVILSFISC
BmornAChRβ3     PGVMLSALTLTALALDPRQSTRASLLGFSVAAHVYFINQLRAMAPLHQLASVPSLVYYYR
                *..::*.**:*** ** *: .* .:* ::* :*. * * :   .* *.  :   * :

CpomnAChRβ3     STVVTVICFVLSFYFRYLGKKKTQPSPWIISYNDFVLDGYGKYGVFTKWEVVEQDLKVKP
BmornAChRβ3     GSLLATLVALVSSLCLGALCRKSSPAPRWLHELQAAVGSHWTRLLIPRWECGETNNTERD
                .:::::.:  ::*        :*:.*:*  :   : .:..: .  ::.:**  * : . :

CpomnAChRβ3     NVEWVNFISIVNSVCLFLVIITYIYLFSAFIPKKPVFTFVFDIA
BmornAChRβ3     TERWTNVANIANAACLLIFPVVYVSMYFSLVPQPYPITN-----
                . .*.*. .*.*:.**::. :.*: :: :::*:   :*
```

Supplementary Data 3. Alignment of nAChRα5 sequences from honey bee *A. mellifera* (Amel) with that of codling moth *C. pomonella* (Cpom). Accession numbers for *A. mellifera* sequence is listed in the legend of Figure 2.

Alignment of nAChRα5 of codling moth *C. pomonella* (Cpom) and honey bee *A. mellifera* (Amel).

```
CpomnAChRalpha5     MKILMCHVSDMLCSSITKVLIWVAISFLIKGAVCCDEEYRLVRHLMQNYDASVRPVENSS
AmelnAChRalpha5     ------------------------------FGDEHEYRLTKYLLDGYDAGVRPAENSS
                                                 .  :.****.::*::.***.***.****

CpomnAChRalpha5     HPLLVTFGVSLHHIIDVEEKDQLLTTNCWITQVWMDYHLRWNTSDFDGIGVIRIPFERVW
AmelnAChRalpha5     QPLAVVFGLSLHHIIDVDEKNQILTTNCWVTQIWTDHHLKWNASEFAGIRVIRVPYNRVW
                    :** *.**:********:**:*:******:**:* *:**:**:*:* ** ***:*::***

CpomnAChRalpha5     KPDIILYNNADPNYRSAVINTNVIVRHTGEVTWLSHGIYVSVCDINVEQFPFDVQLCTMK
AmelnAChRalpha5     RPDTILYNNADPQYSSAVINTNVIVSHTGEVVWLSHGIFRSSCDIDVEFFPFDEQRCVLK
                    :** ********:* ********** *****.******: * ***:** **** * *.:*

CpomnAChRalpha5     WASWTYDGFQLDLIKQFDEGDTTNNQPNGEHDLVSFEANKHMKYYSCCVEPYPDITYVIK
AmelnAChRalpha5     WASWTYDGYQLELEKQSEQGDVSNYQANGEFDLVNFSARRNVEYYSCCPEPYPDITYEIR
                    ********:**:* ** ::**.:* *.***.***.*.*.:::*****  ******** *:
                                 TM1                              TM2
CpomnAChRalpha5     LRRRPMFYVFNLILPCLLINGIALLVFYVPSESGEKVTLGISALLSMTVFLMTIRDTLPP
AmelnAChRalpha5     LRRRPMFYVFNLILPCILINSVALLVFYVPSESGEKVTLGISALLSMTVFLMTIRESLPP
                    ****************:***.:**********************************::***
                                    TM3
CpomnAChRalpha5     TEKTPLISLYYGVST----------------------GQPVPAALRELVLQRLARLLCIN
AmelnAChRalpha5     TEKTPLISLYYGVSICLVTFASALAVVTLNLHHRGVRGTRVPGIVRSLVLDKLARIVLLN
                    **************                        *  **. :*.***::***:: :*

CpomnAChRalpha5     FDMDAKGDSTPVAVGVQVNPRTGSRLKTELSCDGAPLPPPSPRFARHNHNHMAGAMAAQS
AmelnAChRalpha5     FQEERRSEPVEPPR---------RKNNCPLHCKPELGQSQSSPKFVARREESNSSSPSLD
                    *: : :.:..  .           : :  * *.     . *.     .::.  .: .: .
                                                                        TM4
CpomnAChRalpha5     DVSTRPVRQRASAAHAALREAAARHEQRVAANERLDRANLEWKQVAVVADRALLAVFVLV
AmelnAChRalpha5     LGKEGGLEAQWSRVLGRVHATIERNEKRLAEQDRRERMEFDWKQVALVSDRALLCVFFLT
                      .   :. : * . . :: :  *:*:*:* ::* :* :::*****:*:*****.**.*.

CpomnAChRalpha5     TAISTAAILLPQLTNLHVGNTPLKPPI
AmelnAChRalpha5     TIVSTTVILCGSPPSANIAKEG-----
                    * :**:.**  . .. ::.:
```

This alignment shows the first, second and fourth transmembrane regions (TM1, TM2, TM4) in Cpom and Amel aligning fairly closely with one another. Amel's third transmembrane region (TM3) aligns in the region identified above between GVST and GQPV in the Cpom sequence. This further supports the conjecture that once this region in CpomnAChRα5 has been cloned and sequenced, we would expect to find a predicted third transmembrane region in this area.

This alignment shows nearly identical alignment from the N-terminal end after the N-terminal signal peptide through the second transmembrane region (TM2). As Bmor enters its third transmembrane region (TM3), the sequences diverge sharply from one another. Bmor was identified as having only three transmembrane regions, and is apparently a truncated sequence (Shao et al., 2007). There were two head transcriptome sequences identified as nAChRα5 that had apparently unidentified sequence data between GVST and GQPV. We have not yet successfully cloned and sequenced this region. It is likely that once we clone and sequence this region, we will have additional sequence that will lead to a transmembrane region being predicted around this area.

Chapter 2: CRISPR/Cas9 Gene Editing of the Codling Moth Genome

Abstract

RNA interference (RNAi) has greatly enhanced the ability to explore gene and protein function within non-model organisms. However, RNAi has proven in many instances to be an unreliable tool in Lepidoptera. This has been disappointing for those seeking ways to control Lepidopteran insect pests, including codling moth, *Cydia pomonella* (L.) (Lepidoptera: Torticidae), a major pome fruit pest throughout the world. The recently discovered CRISPR/Cas9 gene editing system has introduced a new tool to the field that could possibly outperform RNAi for gene function studies. This study has two main goals: (i) determine whether codling moth eggs could withstand the stress of a fluid injection, and (ii) introduce mutations into a target gene sequence with the CRISPR/Cas9 system. Regarding the first goal, saline-injected eggs matured to healthy larvae in 26.1% of cases, compared to uninjected eggs reaching this stage in 43.4% of cases. Regarding the second goal, preliminary data indicate that intended genetic modifications were successfully introduced into the genomes of 85% of CRISPR/Cas9 injected eggs.

Introduction

The transcriptomic and temporal expression data on nicotinic acetylcholine receptor subunits in codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) discussed in Chapter 1 can also be used to select target subunits of interest for gene knockdown or knockout studies. This can help determine the relative importance of, and roles played by, various subunits in development and insecticidal susceptibility. Since the effectiveness of double stranded RNA (dsRNA) in RNA interference (RNAi)

experiments was established in nematode, *Caenorhabditis elegans* (Maupas) (Rhabditida: Rhabditidae) (Fire et al., 1998), RNAi has been used in many different organisms for gene knockdown and knockout studies. Instead of searching for phenotypically aberrant samples for genetic studies, the ability to specifically target a gene product for silencing has accelerated gene product characterization in both model and non-model organisms.

RNAi is initiated by the introduction into the cell or organism of a long exogenous dsRNA strand with one strand designed to base pair with the messenger RNA (mRNA) being targeted in the study. The dsRNA is cut into small RNA duplexes by a ribonuclease III enzyme called Dicer. These duplexes are unwound and one of the strands, referred to as the guide strand, is loaded into the RNA-induced silencing complex. This complex then targets mRNAs present in the cell that share complete or partial sequence homology with the guide strand. This association then triggers Slicer, a RISC-bound endonuclease, to cleave the target mRNA. Destroying the target mRNA present disallows the cell from producing the protein for which it encodes (Terenius et al., 2011). The efficiency of the destruction of the target mRNA determines whether a knockdown of a gene product (in which the protein produced is reduced in quantity) or a knockout of the gene product (in which the protein is not produced at all) is achieved.

Initial successes with the use of RNAi in Lepidoptera began to accumulate in 2002 with the effective silencing of the *slapn* gene in Oriental leafworm moth, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Rajagopa et al., 2002), the *Hemolin* gene in cecropia moth, *Hyalophora cecropia*, (L.) (Lepidoptera: Saturniidae) (Bettencourt et al., 2002), and the *white* gene in silkworm, *Bombyx mori* (L.)

(Lepidoptera: Bombycidae) (Quan et al., 2002). However, by the 8th International Workshop on Molecular Biology and Genetics of the Lepidoptera in 2009, it was becoming apparent among researchers that the effectiveness of RNAi in Lepidoptera was not as widespread as had been initially demonstrated in other organisms. This led to the construction of a database (http://insectacentral.org/RNAi) with the invitation to researchers working with RNAi in Lepidoptera to deposit details of their successful and unsuccessful experiments for the purposes of comparative analysis. This database facilitated the identification of many trends, including the observation that RNAi appeared to be completely ineffective at knocking out gene products in some insect families, highly effective in other insect families, and incredibly variable within still other insect families (Terenius et al., 2011).

Very few RNAi studies have been done on insects within the Tortricidae family, of which codling moth is a member, and therefore they are not very well represented in the aforementioned analysis. A recent study targeted five different gene products to examine the effectiveness of RNAi in codling moth (Wang et al., 2015). Of the five targets tested, four of them had no phenotypic or mRNA transcript-level impact on the organism. The one that did have an impact on the organism only caused a two-fold mRNA transcript reduction at the maximum dose tested (250 ng/µl) leading to a knockdown of the gene product, as opposed to a full knockout. Since RNAi has failed to produce effective results, there exists no reliable system for functional genomic studies for those researchers studying Lepidoptera.

A newly developed gene-editing system introduces the possibility of being able to advance such studies. The CRISPR/Cas (Clustered Regularly Interspaced Short

Palindromic Repeat/CRISPR-Associated Sequences) system, found in 40% of bacteria and 90% of archaea, was initially discovered as a microbial defense mechanism against viral infection. A Cas complex present in the microbe detects the foreign genetic material of the invader (virus or plasmid), creates a "spacer" identical to the sequence, and incorporates it into future Cas complexes. When the new Cas complex identifies the same foreign genetic material, this triggers Cas proteins in the complex to destroy the foreign genetic material specified by the "spacer" (Horvath and Barrangou, 2010).

After it was determined that the Cas9 protein could be utilized to introduce double-strand breaks into a piece of DNA with a single-guide RNA (sgRNA) to direct it to mutate a gene of choice (Jinek et al., 2012), the system was tested in many different organisms, including fruit fly, *Drosophila melanogaster* (Fabricius)(Diptera: Drosophilidae), a commonly used insect model. Initial studies in fruit fly have focused on knocking out gene products that translate to clear phenotypic indicators, such as eye and body color, to facilitate ease of recognition of genetic mutation (Gratz et al., 2013; Bassett et al., 2013; Yu et al., 2013; Kondo and Ueda, 2013; and Ren et al., 2013). One study in which Cas9 mRNA and sgRNA were injected into embryos induced desired mutagenesis in up to 88% of injected flies (Bassett et al., 2013). Such precise targeting at the embryonic stage allows for knockout of target gene products in an otherwise wild-type background (Bassett and Liu, 2014), which is ideal for the study of field populations. Maintaining this wild-type background also allows for more accurate study of subtle, behavioral phenotypes (Bassett and Liu, 2014), such as plant- and mate-seeking behaviors.

To test the CRISPR/Cas9 gene editing system in codling moth, the *maleless (mle)* gene was targeted. In fruit fly, in which the male has XY chromosomes and the female has XX chromosomes, dosage compensation is executed via hypertranscription of the single X chromosome in the male. This hypertranscription is mediated by a ribonucleoprotein complex made up of 5 autosomal genes, and 2 non-coding RNAs that originate from the X chromosome (Ruiz et al., 2000). Of the autosomal genes, one of them is the *maleless* gene that encodes a helicase. If this complex is unable to form, males do not have appropriate levels of X-linked transcripts and they are unable to develop (Lucchesi et al., 2005).

Homologues of all 5 of these autosomal genes have been identified in silkworm (Liu et al, 2008). This would indicate that a similar dosage compensation mechanism would exist within this species. Lepidoptera have a different sex chromosome system from fruit fly, with males containing ZZ chromosomes and females possessing ZW chromosomes. So if hypertranscription took place in the same way, it would do so in the female. Instead, studies have found that males possess twice as many transcripts of Z-linked genes as females suggesting that dosage compensation does not occur (Koike et al., 2003; Suzuki et al., 1998; Suzuki et al., 1999; and Zha et al., 2009).

As of this writing, the function of the *maleless* gene product has not been established in codling moth. Codling moth has the same sex chromosome structure as silkworm (Fukova et al., 2005). If the *maleless* gene product functions the way it would be expected to based on the results in fruit fly, then knocking out the *maleless* gene product in codling moth would result in all male progeny with a mutation in a gene that is not transcribed. This result would grant the CRISPR/Cas9 experiment a phenotypic

marker of successful mutation. If this is not the case, then the *maleless* gene product likely plays a different role in codling moth from that played in fruit fly.

There were two goals in the present study. First, it needed to be determined whether codling moth eggs could withstand the stress of injection of a fluid and still develop normally. Second, it needed to be determined whether gene mutations could be introduced in the codling moth genome with the CRISPR/Cas9 system.

The first goal was successfully executed, with 26.1% of saline-injected eggs resulting in healthy mature larvae, compared to 43.4% of uninjected eggs resulting in healthy mature larvae. For the second goal, preliminary data indicate 85% of injected insects had a form of the intended genetic modification within their genomic DNA.

Material and Methods

Insects

Codling moth were collected from field sites around the Yakima Valley in the summer of 2012 and mated at the Yakima Agricultural Research Laboratory, Wapato, WA, USA. Resulting larvae were reared individually on artificial diet (Southland Products, Lake Village, AR, USA). Healthy larvae were transferred to small cups to pupate and emerge as individual adults. Adults were then collected together into plastic bags to mate and lay eggs. To grow and maintain a laboratory colony, generations were reared at 25°C, 45% RH with a 16:8 light: dark cycle. To maintain a wild-type genetic profile, first and second generation codling moth were collected from the field every summer to make backcrosses into the laboratory colony.

Egg Collection

The timing of codling moth egg collection proved challenging as mating and oviposition generally occur within six hours of darkness (Riedl and Loher, 1980) and eggs are laid on a flat surface from which they cannot be removed without destruction of the egg. Codling moth eggs were collected for injection by placing 80-150 2-3 day-old adults together into a black felt-lined dark mating box with a 1/4" wire mesh screen bottom placed on top of a layer of glass slides (Fig. 1). Moths would lay eggs inside of the mesh squares directly onto the glass slides. This collection was done within 4-6 hours of the dark period established in the rearing rooms. The box was moved to a new layer of glass slides every 15 minutes to facilitate collection of eggs for injection and to allow for targeting of freshly laid eggs. Eggs were collected in this manner across 2-5 hours.

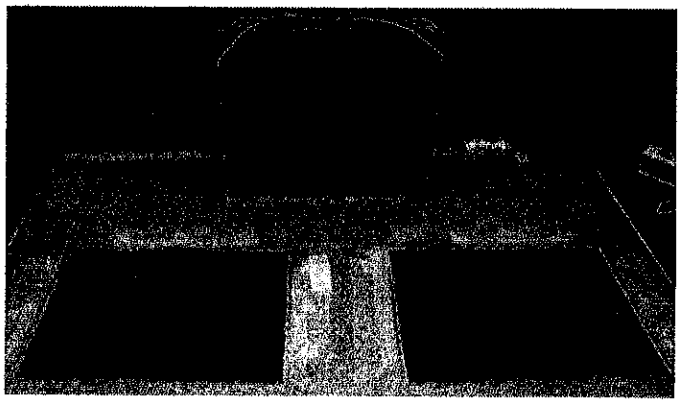

Fig. 1 Black felt-lined dark mating box with a ¼" wire mesh screen bottom with two sets of glass slides.

Egg Injection

Slides with eggs were placed under a Zeiss Stemi 2000 stereo microscope and injected using a FemtoJet 4i microinjection system controlled with a Transferman 4r micromanipulator (Eppendorf) with Femto II tips (Fig. 2).

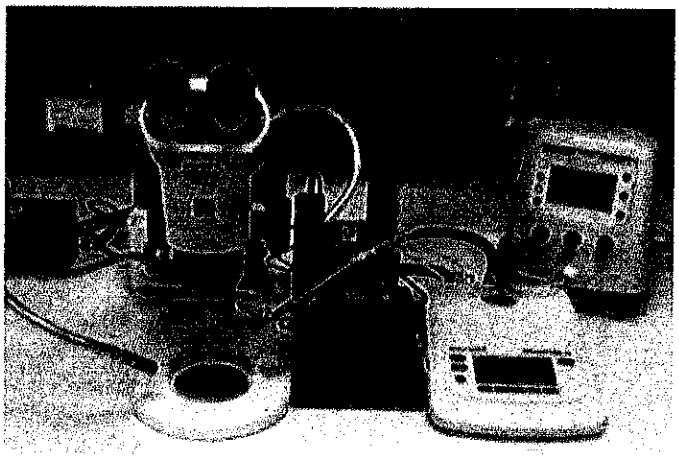

Fig. 2 FemtoJet 4i microinjection system with a Transferman 4r micromanipulator and a Zeiss Stemi 2000 stereo microscope.

Injection conditions were initially set at 1000 hPa injection pressure, 0.1 second injection time, and 55 hPa compensation pressure, and then adjusted as needed throughout the injection period. Injections were performed for 1-3 hours starting when at least 10 eggs were consistently collected, so as to prevent the injection needle from clogging by sitting idle.

Saline Injection

To compare survival of injected eggs to uninjected eggs, injected eggs were injected with a DPBS (Dulbecco's phosphate buffered saline) calcium and magnesium saline solution (HyClone). Slides with injected eggs were stored in a plastic slide storage box inside of a ziplock bag with a small slice of filter paper moistened with deionized water, and allowed to mature in the rearing room described above. A cohort of equal number of uninjected eggs was stored separately under the same conditions. Emerged neonates were collected from boxes and placed on artificial diet on the sixth and seventh days after egg collection, and reared to adulthood separately in the same conditions as the laboratory colony.

Gene Editing with CRISPR/Cas9

OVERVIEW

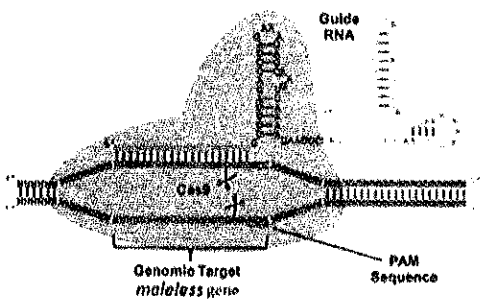

Fig. 3 Mechanism of CRISPR/Cas9 gene editing (Baron et al., 2013).

To utilize the CRISPR/Cas9 gene editing system, a single guide RNA (sgRNA) is designed to target ~20 nucleotide section of the gene of interest followed by the protospacer adjacent motif (PAM) sequence "NGG" (Fig. 3). The sgRNA also includes a sequence that binds the Cas9 protein enabling it

to perform efficient endonucleolytic cleavage in this area of the DNA strand. The sgRNA and the mRNA for the Cas9 protein are then co-injected into the embryo. This portion of the protocol was largely based on that published for fruit fly (Bassett and Liu, 2014).

THE *maleless* GENE

For this study, a homologue of the *maleless* gene was identified in codling moth

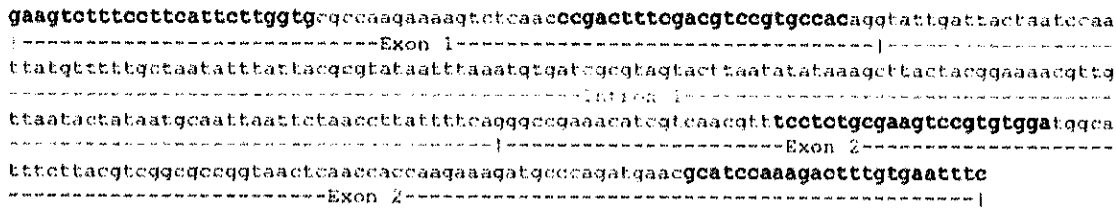

Fig. 4 *Maleless* (MLE) gene structure from partial exon 1 to partial exon 2. Sequence at which amplification primers lay down are in purple, canonical dinucleotide spice sites are boxed in red, sequence at which sgRNAs were designed are in bold, and PAM sequences are in boxes.

from transcriptome data generated in the laboratory of Dr. Amit Dhingra at Washington State University (Hendrickson *et al.*, manuscript in preparation). Oligonucleotide primers were designed using the Primer3 function contained within Geneious (version 6.8 created by Biomatters, available from http://www.geneious.com/) to obtain sequence data for intron 1 between exons 1 and 2 for use in CRISPR/Cas9 editing design (Fig. 4).

THE sgRNA

To create a 141, 144 or 145 base pair deletion in the gene (depending on allele), two sgRNAs each were designed to target two different portions of the *maleless* gene as indicated in Fig. 4. Sequence was chosen to delete splice sites between Exon 1 and Intron 1, and between Intron 1 and Exon 2 to disrupt the proper processing of the pre-mRNA that would result from transcription of the DNA sequence. The primers designed

for the PCR amplification of this region target a 338 base pair fragment for the wild type gene. The size of this deletion was expected to result in this fragment being reduced to about 200 base pairs. The difference in the size of these gene fragments was expected to be visually explicit on an agarose gel.

EGG INJECTION

A mixture consisting of 4 µg of Cas9 mRNA (Trilink Biotechnologies, Inc.) and 1 µg of each of the two sgRNAs was mixed together on ice, and 3 µl were injected into the microinjector tip. Eggs were collected, injected with an amount equal to ¼ the size of the egg and stored as described in the saline-injection experiment described above. All CRISPR-Cas9 injections were performed within an hour of sgRNA/Cas9 mRNA solution being taken off ice and added to a tip to limit possible RNA degradation at room temperature. An equal number of eggs were injected with saline to serve as an injection control. Additionally, an equal number of eggs were collected and maintained in the same conditions to serve as uninjected controls.

NEONATE COLLECTION

Eggs were checked on the sixth and seventh days after injection for emergence. Emerged neonates were placed individually into 1.5 mL tubes and stored in a -80°C freezer.

gDNA EXTRACTION

Genomic DNA (gDNA) extraction was performed on individual moths by homogenization in 600 µl of CTAB-ME buffer and heating to 65°C for 20 minutes. Cold chloroform in the amount of 600 µl was then added to separate the DNA layer that was then transferred to another tube. DNA was collected from this phase by adding 3 µl of

glycoblue and 500 µl of cold isopropyl alcohol and spinning down in a microcentrifuge for 10 minutes at 15,000 rpm. DNA was then washed with 70% ethanol and brought into solution with 50 µl of nanopure water.

PCR AMPLIFICATION

Primers detailed in Fig. 4 were used to amplify the gene fragment of interest in each of seven test samples and four uninjected control samples. Amplifications were done in 20 µl reactions containing 0.4 µl Titanium Taq (Clontech Mountain View, CA, USA), 1x PCR buffer (supplied with Taq), 0.25 µmol/L each primer, 200 µmol/L each dNTP and 3 µl of gDNA for each sample. PCR conditions were: initial denaturation for 5 min at 94°C, then amplification steps of 20 sec at 94°C, 10 sec at 60°C, and 1 min at 72°C for 40 cycles, followed by a final 5-min 72°C extension. Agarose gel electrophoresis was used to separate PCR products and notable bands were excised for cloning. DNA in the bands was extracted and purified with GenElute Minus EtBr Spin columns (Sigma, St. Louis, MO, USA), and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) with TOP 1 *Escherichia coli* chemically competent cells according to protocol provided by manufacturer. For each sample, 10 clones were selected to be grown overnight at 37°C. Plasmid DNA was extracted with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA). All cDNA clones were sent to MC Laboratories (MCLab, San Francisco, CA, USA) for sequencing.

Results and Discussion

Saline Injection Survival

A total of 696 eggs were injected with saline solution, and 694 eggs were retained as the uninjected control group (Table 1). Healthy larvae were collected 19-23

days after the injection day. In the uninjected control group, 43.4% of eggs collected made it to the healthy mature larval stage, and in the saline-

Saline-Injected Group				Uninjected Group			
Injection Day (Julian Date)	Eggs	Larvae Collection Day	Healthy Larvae	Injection Day (Julian Date)	Eggs	Larvae Collection Day	Healthy Larvae
162	52	183	4	162	52	183	16
163	17	183	0	163	18	183	4
169	34	190	12	169	37	190	7
170	75	190	22	170	83	190	28
174	29	197	10	174	23	197	5
175	42	197	5	175	45	197	27
176	15	197	4	176	15	197	5
177	5	197	4	177	5	197	2
181	26	204	5	181	53	204	24
182	57	204	19	182	56	204	32
183	14	204	4	183	16	204	14
223	50	244	19	223	37	244	20
224	50	246	19	224	40	246	23
225	50	246	6	225	50	246	28
230	0	0	0	230	19	251	9
231	30	253	9	231	28	253	12
232	50	253	20	232	49	253	23
233	50	254	6	233	31	254	12
239	50	258	14	239	37	258	10
TOTAL:	696		182	**TOTAL:**	694		301

Table 1 Egg-to-mature larva survival data per injection day for saline-injected and uninjected eggs.

Uninjected Group		**Saline-Injected Group**	
Total Average Survival	0.434	Total Average Survival	0.261
Daily Average Survival	0.432	Daily Average Survival	0.28
Standard Deviation of Daily Average Survival	0.168	Standard Deviation of Daily Average Survival	0.175

Table 2 Total average and daily average egg-to-mature larva survival calculated from data in Table 1.

injected group, 26.1% of eggs injected made it to the healthy mature larval stage (Table 2). The average survival of the uninjected control group per injection day was 43.2 +/- 16.8 %, and for the saline-injected group, it was 28.0 +/- 17.5 % (Fig. 5).

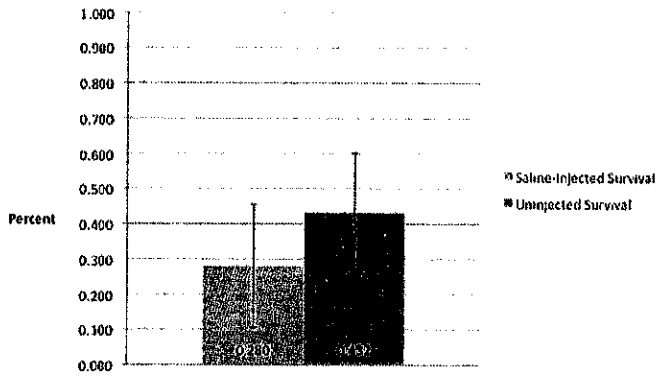

Fig. 5 Comparison of uninjected and saline-injected egg survival to mature larvae per injection day.

These data indicate that codling moth eggs are capable of withstanding needle injection of extra fluid reasonably well, allowing for the possibility of injecting them with experimentally useful substances. The similarity in value of the standard deviations for the uninjected control group and the saline-injected group indicate injector skill variability is minimal on average relative to the survival variability of uninjected eggs. These data also allow for estimation of injection targets. For example, in the worst-case scenario, 10.5% of injected eggs can be expected to develop to the mature larval stage. If the goal is to accumulate 100 healthy injected larvae, 953 eggs would need to be injected. It is important to note that this data only accounts for the stress of the injection and additional fluid being added to the egg. The potential toxicity introduced by an experimentally useful fluid would also have to be taken into account.

CRISPR/Cas9 Genetic Mutations

Initial PCR and gel electrophoresis showed that all sgRNA/Cas9 mRNA-injected larvae still contained a substantial band of the expected wild-type size between 300 and 400 base pairs. However, 2 out of 12 test larvae contained a smaller band relative to the wild-type fragment. The expected size of the smaller fragment was about 200 base

pairs, however, both smaller bands appeared to be too small to be the band with the full target deletion (Fig. 6). Analyses of sequences of clones from these two bands revealed that not only were they not the target, but they appeared to be a random artifact from the PCR reaction.

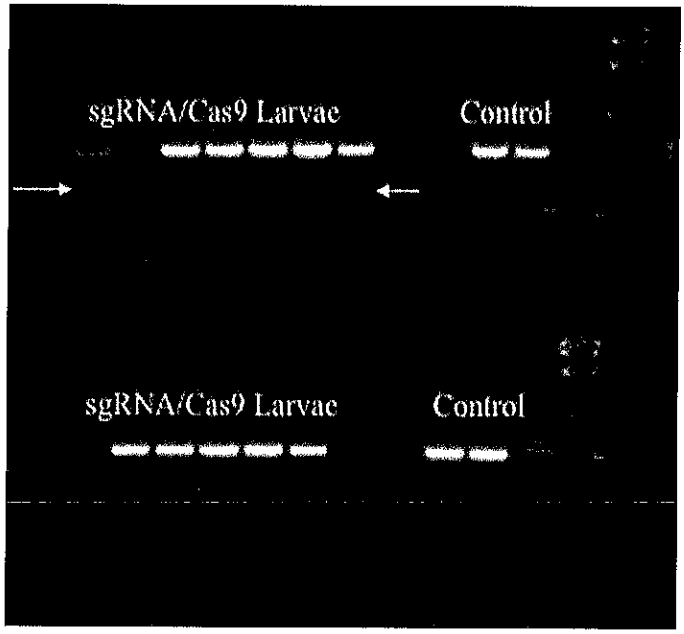

Fig. 6 Agarose gel of PCR results for 12 sgRNA/Cas9 mRNA-injected larvae and 4 saline-injected control larvae. Smaller bands of potential interest indicated by arrows.

Bands were cut and TA cloned for seven of the test larvae and four of the control larvae. Ten clones from each band were sequenced. No sequence modifications were found in the control larvae. Sequence modifications were identified in six of the seven (~85%) test larvae (Fig. 7, see next page). Of the 70 test clones, 26 contained some sort of sequence modification at one or both of the designed sgRNA targets. Two clones from one of the larvae contained the entire 145 bp deletion. These initial results indicate that a limited amount of genetic mutation was achieved at the intended targets in most insects tested. However, data analysis on a much larger population of injected insects will be required before any conclusions can be drawn on the success of the CRISPR/Cas9 gene editing system in codling moth.

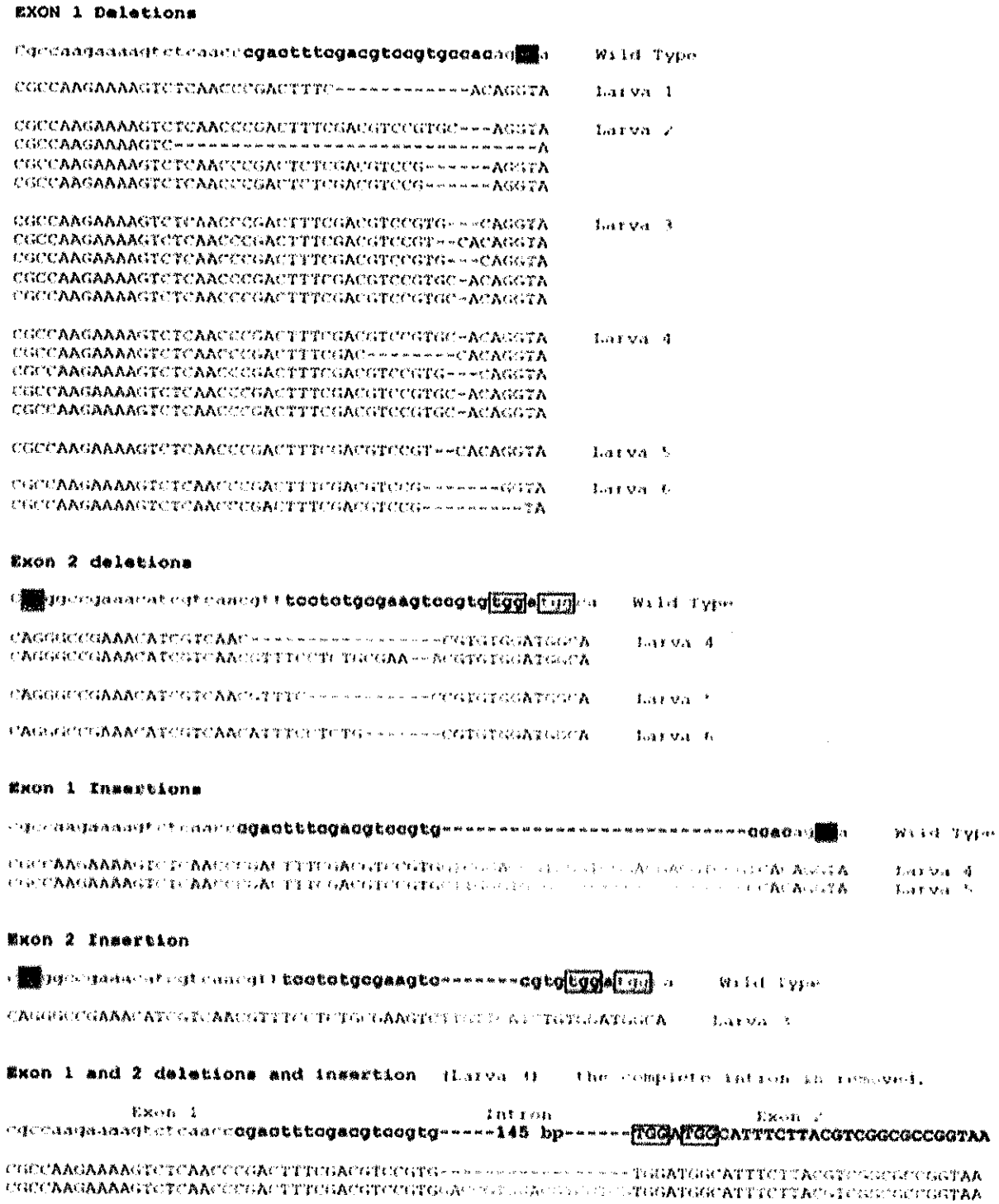

Fig. 7 Sequence modifications in the *MLE* gene identified in sgRNACas9 mRNA-injected larvae.

Future Directions

The ability to genetically modify specific targets within the codling moth genome opens up the possibility of exploring protein function through knockdown and knockout studies. Given the information now available for codling moth nAChR subunits, it will be possible to use the CRISPR/Cas9 gene editing system to knock down or knock out particular subunits. This would enable researchers to identify subunits necessary for survival and/or proper development. The technique could also be used in conjunction with toxicity studies of neonicotinoids and spinosads to determine which subunits are necessary and/or sufficient for insecticide sensitivity and agricultural insect pest control.

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