Mating receptivity peptides in *Helicoverpaarmigera*: Molecular and evolutionary perspectives

Abstract

In many insect species, female reproductive output is promoted by mating and components of the male ejaculate. Beyond the impact of receiving sperm, male ejaculate components can result in increased rates of oocyte development, ovulation, and oviposition, as well as other changes, such as reduced mating receptivity. Previous studies have implicated the role of peptides/proteins in male accessory glands (MAGs), which are responsible for the induction of behavioral changes in *Helicoverpaarmigera* females. ~~Our and~~In several studies, we have ~~shown~~seen the MAG showed post-mating behaviors such as accelerated unreceptivity to re-mating and other post-mating traits. We have reported several peptides responsible for reduced mating receptivity, including a putative 4.9 kDa, a novel peptide with various functions in female post-copulatory ~~behaviour~~behavior. In this study, we reporta detailed analysis of the proteins from a molecular and evolutionary perspective. Major classes of multigene family proteins belonging to heat shock proteins, porphobilinogen synthetases, calmodulin, and elongation factor 1 were detected after database searches. All proteins were found to bind nucleic acids, possess signal peptides, and undergo posttranslational modification by seminal fluid proteins (SFPs).The Porphobilinogen synthetase protein is unique to the lepidopteran order. Our molecular studies support the observation that the molecular weight of MAG proteins varies significantly across species, suggesting the existence of different target sites or faster evolution of SFP. Our results independently support the hypotheses that the variation in the molecular weight of female reproductive behavior-modifying peptides/proteins between closely related species is due to rapid evolutionary pressure. MAG proteins not only ensure male reproductive fitness by constantly evolving under postcopulatory sexual selection but also maintain species-specific barriers to fertilization, mediate reproductive conflicts between the sexes, and potentially contribute to the formation of new species.

Keywords:*Helicoverpaarmigera*, Male accessory glands (MAGs), Gene families, ~~Postcopulatory~~post-copulatory sexual selection (PCSS).

Introduction

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At present, more than one million insect species have been described, and it is estimated that an equivalent number of species remain to be identified. Insects, the species-richest animal class, can change the economics of a country. On one hand, ~~as~~ pollinators ~~aids~~ aidin more than one hundred crop species that provide food for mankind; on the other hand, as pest species, pollination affects many crop species. Among insect pests, the order Lepidoptera represents a diverse and important group of agricultural pests. Lepidopteran, the world's major crop pest, is a Heliothine, which includes*H. armigera* and is responsible for major agricultural losses in several parts of the world (CABI, 2018).

In the overall life cycle of insects, mating plays a crucial role, as it is essential for reproduction and evolution. Successful reproduction involves a range of behaviors, from finding an appropriate mate, courting, and copulating to successful production and (in oviparous animals) deposition of eggs following mating. Furthermore, factors such as copulation itself, the presence of eggs, the presence of sperm stored in spermathecae, or seminal factors, usually peptides secreted by the MAGs, may arrest remating in a species. These peptides/proteins are synthesized, activated, or released at appropriate periods from the male accessory glands (MAGs), and elicit short- and long-term ~~post-mating~~post-mating responses through proteins and neural networks. The network spans from the female reproductive organs and abdominal ganglion to the brain and operates by activating receptors for seminal fluid proteins and octopamine. This activation leads to the modulation of various signals influencing a female's physiological behavior, organ structure, and hormone secretion, it facilitates enhanced nutrient intake,upregulation of reproduction-related genes, ovulation, and sperm storage. Furthermore, the actions of seminal proteins are prime targets for sexual conflict that impacts male and female fitness and have been proposed to evolve under postcopulatory sexual selection (PCSS). Profound remodeling of behavioral, physiological, and transcriptomic pathways in females of many insect taxa is induced by male seminal fluid proteins (SFPs) (LaFlamme ~~BA~~ and Wolfner ~~MF~~, 2013).

SFPs are made by males and are provided to females during mating. These classes of proteins are found in seminal fluids across taxa ranging from arthropods to mammals, suggesting their conservation and importance in gametogenesis and reproduction. SFPs identified to date encompass a variety of protein categories, such as proteases/protease inhibitors, lectins, prohormone precursors, peptides, and protective proteins (e.g., antioxidants). The

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relativemolecular weights of different SFPs also vary significantly, ranging from 36 amino acids in sex peptide (SP) to 200–400 amino acids in prohormone-like polypeptides and large glycoproteins (Avila,~~FW~~ 2011).Several studies have shown that male accessory gland proteins inhibit mating receptivity in different female insects, viz., *Helicoverpa* (Kiran, 2021), *Heliothis virescens* (Seyed~~ Ali Hosseini~~, 2016), *Lymantria dispar* (Wedell~~ N~~, 2005), *Bombyx mori* (Songdou~~ Zhang~~, 2014) and several other species. More than one mechanism has been proposed to demonstrate the effects of male-derived substances on female mating receptivity. The physiologically active SP-like factor directly inhibits pheromone biosynthesis, which is released duringscotophase by PBAN (Fan et al., 2000) and subsequently, it also stimulates the production of JH by the CA (Astrid~~ T Groo~~t, 2005). Since major differences in reproductive physiology exist between species of insects, seminal protein information from multiple insects could shed light on the evolutionary patterns of reproductive traits in insects. These proteins, many of which are rapidly evolving, are essential for male fertility and represent formidable modulators of female post-mating behavior.

Sexual reproduction requires coordinated contributions from both sexes to proceed efficiently. However, the reproductive strategies that the sexes adopt often have the potential to give rise to sexual conflict because they can result in divergent, sex-specific costs and benefits. Sexual conflict arises through actions mediated by seminal fluid proteins. Several lines of experimental evidence suggest that mating systems can impose selection that influences the evolution of nonsexual phenotypes, such as development, activity, metabolism, and nutrient homeostasis. These conflicts can occur at many levels, from molecular to behavioral. These proteins provide many excellent examples to trace the operation of sexual conflict from molecules through behavior. Since these seminal fluid proteins often rapidly evolve under positive selection between species, because of this divergence, these proteins may play a role in speciation by contributing to reproductive isolation between populations.

The molecular characterization of peptides/proteins of MAGs that modulate female reproductive behavior has been reported for several insects (Saraswathi et al., 20~~0~~20; Mamatha et al., 2023), similar information on *H. armigera*, is ~~emerging~~ emergedin the last decade using genomic and proteomic methods (Dario Trujillo, 2024;Jian-Yu Meng, 2010). Our previous study demonstrated various physiological changes such as (Rama et al., 2021) in female insects. In the

present study, we mine data ~~with respect to~~concerning the biochemical, molecular, physiological, and evolutionary roles of the MAG peptides which could shed light on the mechanisms operational on these ~~protein~~ proteinsin *H. armigera* reproduction and ~~in~~ insects.

Materials and Methods

Insect rearing and MAG-duplex crude extract, preparations,and bioassays are described in Rama et.al. (2024).Further, Proteomics analysis (nano-LC–MS/MS analysis) is described in Rama et.al. (2024) unpublished ~~data under~~data under review.

Bioinformatics analysis

The database searches were performed using nonredundant databases from the SWISS-PROT and TrEMBL with the automated database-searching program using the MASCOT search engine version 2.4 (Matrix Science, Boston, MA, USA). Proteome analysis of the MAG-duplex tissue from virgin male moths was carried out by nano-LC–MS/MS to identify peptides/proteins involved in female behavior.

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Phylogenetic tree construction

The peptide sequences of the retrieved peptides from the database were used to construct a phylogenetic tree to determine the possible evolutionary trajectory of these classes of proteins across the tree of life using the ClustalX program. The phylogenetic tree was constructed by the maximum likelihood (ML) method using the program Mega 5.05 (http://www.megasoftware.net/). Homologous relationships were determined using bootstrap analysis with 1000 replications. Multiple protein sequences were aligned using the ClustalW method (http://www.clustal.org/).

Protein interaction network and 3D structure deduction

The ~~protein protein~~protein-proteininteractions were determined using STRING (https://cn.string-db.org/, accessed on March 2024), and3D protein structures were generated using https://swissmodel.ExPASy.org/servers.

Results

Protein Bioinformatics

Since *H. armigera*is not a model organism and there is no complete genome sequence available to date, proteomics is a novel and direct method for identifying unannotated peptides/proteins. Our proteomic study of male MAGs by the shotgun method validated the presence of 5 classes of peptides/proteins (Tables 1 and 2)~~,~~ when searched in the ~~SWISSPROT~~ SWISS-PROTand TrEMBL databases.

As shown in Figure 1, in the phylogenetic tree of the MAG proteins, the proteins were grouped into clades a, b, c, and d, with clade a containing the heat shock protein (HSP) cognate 3 ofAsian swallowtail, speckled wood butterfly, heat shock protein of cotton bollworm (belonging to the order Lepidoptera), delta-~~aminolevulinic~~aminolaevulinic acid dehydratase protein of monarch butterfly(Lepidoptera), tsetse fly, and oriental fruit fly (Diptera). Clade b- uncharacterized proteins of mountain pine beetle (Diptera), small parasitoid wasps (Hymenoptera), heat shock 70 kDa protein cognate 3 of Indian jumping ant (Hymenoptera), and uncharacterized proteins ofhoney bees (Hymenoptera).

A phylogenetic tree of the MAG proteins across Phyla grouped the calmodulin proteins into clades a, b, c, d, e, and f (Figure 2). Clade-a, b, and c of marine invertebrates (Ascidians) consisted of ~~Sea sponge~~sponges,sea~~urchin~~urchins,sea peaches,sea~~squirt~~squirts, and Amphioxus. Clade-d of free-living nematodes and annelids (epi-endogenic earthworm), clade-e of single-celled alga, Human/Animal infectious protozoan causing African sleeping sickness and ~~chagas~~ Chagasdisease in humans. Clade-f, of Plant pathogenic fungi—potato late blight fungus—and two species—algae—giant kelp and brown algae and finally clade- g, h of Insects-fruit fly, Carolina sphinx moth, owlet moths, and Fall armyworm. ClustalW alignment of Calmodulin proteinfrom corn to zebrafish to protozoans demonstrated high conservation at various amino acid residues (Figure 3) suggestive of its importance in Ca^{2+} mediated cellular processes.

UniProt functional annotation of the proteins revealed that the HSP and uncharacterized proteins were nucleic ~~acid-binding~~acid-binding proteins to possess a signal peptide and undergo posttranslational modification. Finally, the proteins were present in the three orders of insects (Table 3). ~~Several criteria are essential for classifying a protein as an SFP, and~~ our preliminary

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analysis indicated that the peptides in our study satisfied a few of these criteria, necessitating future studies.

Proteins are vital components of a ~~cells~~ cell'scomplex biological network of macromolecules, acting as molecular machines, sensors, transporters, and structural elements. ~~Protein-protein~~Protein-proteininteractions are dynamic~~ in nature~~, adjusting in response to different endogenous and exogenous stimuli. Reproduction and related physiological mechanisms are subjected to several cues in insects. This provides considerable flexibility in function and allows cells to adapt in a measured way to changing stimuli. The protein interaction map of porphobilinogen synthase (PBGS) suggested a PPI enrichment p value of < 1.0e-16 (Figure 4).Go-annotation suggested 11 proteins in the hub with 11 nodes converging on Heme biosynthesis. Further, 3D structure delineation of PBGS with *Drosophila* PBGS suggested a homology model with 8 ligands (2 active sites) with 60.19% identity and an alpha-fold model (high pLDDT-90) (Figure 5A & 5B). In summary, these observations highlight the importance of these proteins in *H. armigera* reproduction and related physiological ~~phenomenon~~phenomena.

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Discussion

Mating in most species of insects leads to a transient or permanent loss of sexual receptivity in females. Our previous study (Rama et. al 2021)supports the findings of~~ previous~~ studies in related *Helicoverpazea* species in which MAG secretions were able to induce unreceptivity. These data suggest that both SP-like and PSP-like factors may be present in the male accessory glands of *H. armigera*.~~ males.~~ The relative molecular weights of different SFPs also variedy significantly, ranging from 36 amino acids in sex peptide (SP) to 200–400 amino acids in prohormone-like polypeptides and large glycoproteins (Sebastian Sturm et al., 2021). This indicates two possible reasons for different molecules having similar functions: one is that their targets are different, and the other is the rapid evolution of proteins involved in reproduction and related traits.

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The MAGs of many insect species produce and secrete several reproductive proteins collectively named accessory proteins (Acps). These proteins, many of which are rapidly evolving, are essential for male fertility and represent formidable modulators of female post-mating behavior. Sexual reproduction requires coordinated contributions from both sexes to proceed efficiently.

However, the reproductive strategies that the sexes adopt often have the potential to give rise to sexual conflict because they can result in divergent, sex-specific costs and benefits.

In silico proteomics study of the MAG revealed the presence of a cocktail of peptides/proteins, ~~includes~~ including a. chaperone proteins or heat shock proteins, b. delta-aminolevulinic acid dehydratase, c. calcium ~~calmodulin-binding~~calmodulin-binding proteins, and d. uncharacterized proteins (Tables 1 & 2). Many of these proteins have been identified as key housekeeping proteins that are involved in various cellular pathways and signaling mechanisms and have important functions in reproduction in both sexes. Multiple Sequence alignment of Insect MAGs retrieved from database using MUSCLE (3.8) is depicted in (the supplement notepad). Several amino acids were conserved in the C terminal of the proteins suggesting their role in post-copulatory reproductive behavior.

Heat shock proteins are typically produced in response to stress, aiding in protein folding and stability. Researchers have detected heat shock proteins in the male accessory glands of various insects, including *H. armigera*(Rafaeli et al., 2013), *Anopheles gambiae* (Dottorini et al., 2007), and *Apis mellifera* (Gorshkov et al., 2015). Chaperone proteins function as intercellular signaling molecules and facilitate sperm-oocyte interactions (Tutuncu et al., 2004; Nixon et al., 2005). Heat shock cognate 70 (HSC 70) proteins have high sequence homology to Hsp70 family proteins, which are expressed at relatively constant levels under normal conditions and can be induced, to some extent, by heat shock (Rubin et al., 1993). These proteins are highly expressed in both the testes and ovaries of Drosophila, suggesting their roles in gametogenesis (Fly Base, http://flybase.org/). Orthologous of HSP ~~geneshave~~ genes have been identified in Lepidoptera, Diptera and Hymenoptera.

Porphobilinogen synthases (PBGSs) are evolutionarily conserved~~metal-binding~~metal-binding proteins (zinc and magnesium or potassium at the active site) that catalyze an early step in the biosynthesis of tetrapyrroles (Jaffe,~~e EK~~ 2000). These enzymes form the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) enabling the transfer of electrons from NADH to the respiratory chain. The current sequence database has a repository of 130 different organisms spanning ~~Archea~~Archaea, bacteria, eukaryotes, and metazoans. The proteins exhibit differences in ~~the~~ kinetic, physiological, inhibitory and immunological activities. The PBGS of *Drosophila melanogaster* (DmPBGS) shows sequence

homology to the two ~~zinc-binding~~zinc-binding sites found in yeast and mammalian PBGS (Lenka Kundrat 2003). In *Pieris brassicae* this protein has been reportedas a bilin-binding protein (BBP) in the hemolymph, fat body, epidermis and wings of *Pieris brassicae*. Increased heme biosynthesis in fat body (16%) and ovaries (36%) of animal tick insects has been reported implicating their role in reproduction-related physiological phenomena in insects.

As evident from the phylogenetic tree in Fig. 4, paralogs of the calmodulin protein were found to exist in Unicellular euglena, fungi, certain species of Brown Algae, *C.elegans*, in human parasites and insects. Ca2+ signaling proteins bind to Ca2+ and maintain intracellular Ca2+ levels (Tang, ~~S~~ 2020). ~~Calcium-dependent~~Calcium-dependent, calmodulin-stimulated protein phosphatase, which plays an essential role in the transduction of the intracellular Ca2+-mediated signal calcium (Ca2+), regulates many cellular and physiological processes from development to reproduction. cAMP-dependent phosphorylation of the sperm axonemal component is the most important intracellular trigger causing the initiation, activation, and hyperactivation of sperm motility in diverse organisms, such as tunicates, sea urchins, mussels, salmonid fish and mammals (Mamoru Nomura 2004; Marin-Briggiler et al., 2005).

In the free-swimming stage, Euglena ~~reproduce~~ reproducesrapidly via a type of asexual reproduction method known as binary fission. The euglenoid cell reproduces its organelles by mitosis and then splits longitudinally into two daughter cells. The unicellular flagellate *Euglena gracilis* shows negative gravitactic behavior.CaM.2 is an element in the signal transduction chain of gravitaxis in *E. gracilis*. Actin and myosin were detected by immunofluorescence in *Euglena* flagella, suggesting that an actomyosin contractile system controlled by calmodulin, similar to that in human sperm, enables various reproduction-related biological processes (Viktor Daiker 2010).

CaM is a multifunctional Ca2+-sensing protein that is ubiquitous in fungi and has important functions in growth, development, fertility, stress tolerance, and virulence in several fungi, such as*N. crassa* (Laxmi and Tamuli 2017), *S. pombe* (Itadani et al. 2010) and *C. neoformans* (Kraus et al. 2005). These proteins mediate ~~plant-pathogen~~plant-pathogeninteractions (Cheval et al., 2013; Xiangzi Zheng 2018). *Phytophthora infestans* an oomycete pathogen, including downy mildews ~~and~~ are the causal agents of tomato and potato late blight disease. The fungi secrete

hundreds of candidate effectors that contain the RXLR motif, which have been shown tosuppress plant immunity and effect Ca2+/CaMsignaling (Whisson et al., 2016).

Brown algae belong to the stramenopilesgroup in the eukaryotic lineage, and the eukaryotic branch is highly divergent from the animal, fungal, and green plant lineages. In the early stages of development, fucoid zygotes are apolar and possess no inherent cytoplasmic order; during a period termed “axis formation”, a potential polar axis is generated within the zygote. This polarity may be facilitated by environmental stimuli (Kropf DL 1992) or by Calmodulin (Pu and Robinson 1998). Studies related to calcium and calcium-binding proteins have suggested their roles in gametophyte and embryonic development, fertilization, zygotic photopolarization, and physiology including stress response in *S. japonica*.

In*C. elegans.* Ca2+ signaling is coupled with sperm activation, although the underlying mechanism is inhibition rather than activation. Calcium orchestrates various events that prepare and facilitate gamete maturation, interaction, and, ultimately, fertilization in *C. elegans* (Singaravelu G, Singson A 2013). The *C.elegans* mutant KCNL-2 null strain kcnl-2(tm1885) exhibited a mild egglayingegg-layingdefect phenotype. Sequence analysis of the KCNL-2 protein revealed six transmembrane domains, a potassium-selective pore, and a calmodulin-binding domain, suggesting the plausible role of these proteins in reproduction (Chotoo CK 2013).

CaMproteins have been reported in *Trypanosoma brucei* (Ruben et al., 1983) and *Trypanosoma cruzi* (Benaim et al., 1991).They are associated with CaM-dependent protein kinase function *Trypanosoma brucei*(Ogueta et al., 1940). In the flagellum CaM, an elaborate extra-axonemal structure, is found within the paraflagellar rod (PFR), and the axoneme and Ca2+-dependent signaling cascades contribute to PFR function (Portman and Gull, 2010). Calmodulin functions in both developmental stages of the *Trypanosoma brucei* life cycle. Manipulating intracellular Ca (2+) homeostasis of these parasites is proposed as a drug treatment strategy (B Gustavo Benaimand Célia R S Garcia 2011). The CaM protein profile of *T. cruzi* is complex, and several of these are differentially expressed at specific stages of development (Garcia-Marchan Y, 2009).

Lumbricusrubellus (Annelida, Oligochaeta) is a species oflower invertebrate earthworm that naturally lives in soils high in organic matter and is related to *Lumbricusterrestris*.

Immunohistochemically study provided evidence for the presence of calcium-binding proteins (CaBPs) in the epidermis of the earthworm- *L.terrestris* (A Licata 2000). Furthermore, evidence suggests that the worm absorbs a considerable proportion of ingested calcium, most of which is subsequently eliminated from the body via the calciferous glands. Taken together, these data suggest that CaM plays a role in the physiology of*Lumbricus* species.

In lower marine invertebrates, Ca2 enables hyperpolarization of the plasma membrane through the efflux of K+ and subsequent cAMP synthesis, which contributes to the initiation of sperm motility and sperm activation through the calmodulin/calmodulin kinase system(Lambert 2011). In sea urchin eggs, calmodulin transiently stimulates a membrane guanylyl cyclase in a Ca2+-independent manner and activates a K (+)-selective channel that hyperpolarizes the sperm. Ca (2+) regulates microtubule sliding in flagella (H Bannai 2000). In *C. intestinalis*, a plasma membrane Ca2+-ATPase (PMCA), referred to as a sperm-activating and attracting factor (SAAF)-binding protein, functions as a CaM protein.SAAF activates the Ca2+/CaM pathway and subsequently mediates the activation of sperm motility through an increase in cAMP (Kaoru Yoshida, 2018). Immunolocalization indicates the abundance of these proteins in spinal cord and the gonads tissues.

CaMfunctions as a high-fidelity signal transducer for more than 300 target proteins, but diversity among its four Ca2+-binding sites, called EF-hands, may contribute to the functional versatility of CaM. Each of the four EF-hand motifs has distinct biophysical and structural properties that span*archea* to present mammals (D Brent Halling 2016). ClustalW analysis of the calmodulin protein across phyla revealed that 80% of the residues were conserved, highlighting its importance in Ca (2+)-mediated mechanisms.

The endoplasmic reticulum chaperone (HSPs) plays a key role in protein folding and quality control in the endoplasmic reticulum lumen. These proteins facilitate the correct folding of proteins and the degradation of misfolded proteins. The chaperone activity is regulated by ATP-induced allosteric coupling of the nucleotide-binding (NBD) and substrate-binding (SBD) domains (UniProt). In the present study, HSP homologs were identified in two lepidopteran species. Genes encoding the Hsc70 group of proteins show high levels of expression in both testes and ovaries in *Drosophila*.Maho Azuma 2021 indicated the involvement of HSP70

proteins in the activation of various steps in the insulin signaling pathway, essential for spermatocyte growth. Finally, in *Manduca sexta*,the brain neuropeptide prothoracicotropic hormone (PTTH) acts in the ecdysteroid hormone synthesis accompanied by specific increases in synthesis of heat shock protein (HSC 70) cognate proteins.

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The eukaryotic elongation factor-1 (eEF1) complex plays central roles in peptide elongation during eukaryotic protein synthesis (Riis et al., 1990; Le Sourd et al., 2006). The eEF1 complex consists of two functional parts, the eEF1A and eEF1B complexes. eEF1A, in its GTP-bound form, binds and delivers aminoacyl-tRNAs and eEF1B ensures proper scaffolding of the eEFcomplex (Le Sourd et al., 2006). In the present study, homologs of eEF-1were identified in two species of Noctuidae moths. The elongation factor eEF1A is expressed in insect germ cells, indicating its role in gametogenesis (Zhao, 2012), bed bug *Cimex lectularius* (Reinhardt et al. 2009).S Zhou(2002) suggested that the production of eEF-1 is increased during JH production, contributing to the excess protein synthesis required for egg production.

Behaviors associated with reproduction are major contributors to the evolutionary success of organisms and are subject to many evolutionary forces, including natural and sexual selection and sexual conflict. As a consequence, behaviors and genes associated with reproduction are often under strong selection and evolve rapidly. Several hypotheses of adaptive evolution, such as sexual selection and sexual conflict, have been proposed to explain this phenomenon. Sexual conflict is involved in many aspects of reproduction where evolutionary interests between males and females diverge (Tim Clutton-Brock, 2007). A potential evolutionary outcome of sexual conflict is a continual co-evolutionary chase between the sexes where adaptations in one sex led to counter adaptations in the other sex (Rice WR 2006; Claudia Fricke, 2009). According to Arnqvist& Rowe (2002) and Gavrilets (2014), sexual conflict and sexually antagonistic coevolution are potent and ubiquitous drivers of evolutionary change. Proteins/peptides of male accessory glands have wide-ranging evolutionary implications for the fitness of many insects. Observations from diverse taxa indicate that seminal fluid proteins transferred at mating affect the reproductive success of both sexes and are also found to evolve rapidly. Sequence data from diverse taxonomic groups reveal that reproductive genes are rapidly changing due to adaptive evolution (Swanson and Vacquier, 2002). For example, comparisons of sequence data between *Drosophila* species have shown that male reproductive genes are evolving faster than non-

reproductive genes are (Civetta and Singh, 1995; Swanson et al., 2001). The presence of different molecules with similar functions and sometimes in closely related insects probably indicates the rapid evolution of these peptides in insect reproduction.For example, Acp26Aa (Li L, 2015) and OSP (Yi and Gillott, 1999) may act on ovaries, while SP is suggested to act directly on CA, hence, it becomes necessary to characterize the female behavior-modifying proteins/peptides of the individual insect species.

Reproductive genes typically evolve more rapidly than non-reproductive genes, and seminal fluid protein (SFP)-encoding genes are considered among those that evolve the fastest. With respect to molecular and population genetics approaches, studies focused on a single or few SFP genes have shown evidence of rapid adaptive evolution by positive selection (Holloway and Begun 2004; Haerty et al. 2007). Eleven to 15% of all SFPs evolving under positive selection (Rowe et al., 2020) are compatible with a variety of selective constraints acting on male reproductive proteins and on each protein and its functions (Carnahan-Craig and Jensen-Seaman, 2014; Sirot, 2019). Selection based on variations in pre, peri and postmating behaviors can lead to reproductive isolation and incipient speciation. The response to selection at the genetic level can include the expansion of gene families.Since many SFPs are multigene families, gene duplication is proposed to play an important role during evolution, contributing to their high divergence between species (Sirot, 2019). Our MAG duplex data revealed that several peptides belong to the multigene family of proteins. Nevertheless, the biochemical and functional characteristics of several peptides from the ~~MAG-cocktail~~MAG cocktail implicate their roles in reproduction and related physiological ~~phenomenon~~ phenomenawarranting future detailed analysis.

Conclusion

Dissection of the nature and function of insect SFPs is relevant for understanding insect reproductive molecules and their actions. SFPs have target receptors that are retainedor metabolized by the female reproductive system. Thisputative protein is retained and has various biochemical and physiological ~~on~~ postcopulatory traits in females. It is tempting to speculate its direct role in unreceptivity after drawing inferences from the present study and several studies. Since the putative peptide is involved in mating or receptivity inhibition in females, delivery of this peptide to a virgin female will result in the laying of unfertilized eggs, as mating is not an

absolute necessity for egg laying. The extreme polyphagy of the two heliothines*H. armigera*and *H. zea* is associated with extensive amplification and neofunctionalization of genes in response to host range, resistance, and adaptation coupled with versatile proteomic/transcriptional changes. Pearce et al., 2017 suggested that the two genomes remain very similar in terms of gene content and order; however, *H. armigera* is more polymorphic, and *H. zea* has lost several genes. Nonsynonymous/synonymous sites in the expanded gene families have rapidly diverged both between orthologs in the two species and between paralogs of other insect orders.Phylogenetic analysis revealed that the HSP protein and an uncharacterized protein were present in three orders of insects and calmodulin across six phyla in the tree of life.

PBGSs are proposed to have species-specific sensitivity to certain active site-directed inhibitors. This class of proteins plays important roles in insect flight and in basal and reproduction reproductive physiological processes, which require aerobic respiration.Enhanced antioxidant activity in mitochondria preserves mating performance following oxidative stress during and after mating. Furthermore, fly models have demonstrated the possible involvement of the oxidative stress-mediated ovotoxicity of VCH 4-vinylcyclohexene (VCH) (Amos O Abolaji 2015). Taken together, these data highlight the role of this protein in receptivity-related phenomena. Identification of this porphobilinogen synthetase unique to lepidopteran species-specific proteins in the MAG of *H.armigera* could aid in the development of novel methods for controlling this pest, such as design of inhibitors of tetrapyrrole biosynthesis.

Calmodulin is a small, acidic protein that enables Ca^{2+} to mediate numerous intracellular Ca^{2+}-dependent events in diverse kingdoms. The protein is conserved from lower to higher eukaryotes (Chin and Means 2000). The spectrum of evolutionary trajectories displayed by calmodulin family members may mirror a variable spectrum of selective restraints related to the unique physiological role of the protein. The results of the present study corroborate the above statement since homologs of the CaM protein were observed in unicellular eukaryotes, fungi, Nematodes, Ascidians and insects. The marked evolutionary conservation of calmodulin may be explained by its extraordinary biochemical and functional versatility. Unlike higher plants, flagellated cells are involved in the reproduction of algae as well as in early diverging land plants. The coordination of cell polarization and cell cycle progression is crucial for proper zygotic development. Ca2+ signals coordinate zygotic polarization and cell cycle progression in brown algae.The inhibition

of photopolarization by calmodulin antagonists and the effects of artificially increasing the cytoplasmic calmodulin concentration in developing *F. serratus* zygotes are compelling examples of calmodulin involvement during polar axis formation. The CaMs and CMLs of brown algae have been shown to have evolved independently of the well-studied green plant lineage, indicating a different ancestor.

The roles of increased intracellular Ca2+ in the motility, activation and chemotaxis of sperm in marine invertebrates, such as sea urchins and ascidians, and in vertebrates, such as fish and mammals,have been reported. CaM exists as a multigene family in amphioxus, which may have evolved independently from the multigene family in vertebrates. As described by several researchers, truncated variants of CaM are found almost exclusively in parasitic protists, such as apicomplexans, kinetoplastides or animal-pathogenic oomycetes, and in green algae, suggesting that its loss could be related to certain adaptations in these species. Trypanosomatids possess relatively large amounts of calmodulin. There is 89% amino acid sequence similarity between T. cruzi and vertebrate calmodulin, suggesting that these variants may be associated with their pathogenicity. Thus, the CaM protein has undergone various structural modifications, enabling its function/s in the respective phyla. Interactions between gene duplication and natural selection are suggested to occur during the molecular evolution of CaM proteins, a class of proteins with multiple interactions(Baba et al., 1984). The gene has evolved rapidly in ancestral species in the evolutionary tree of life and more slowly in recent species. A striking pattern in molecular evolution is that genes encoding proteins involved in reproduction tend to evolve rapidly. CaMhas evolved slowly, but its evolutionary rate is substantially faster in fungi.

EF-1a nuclear genes have been characterized in other animals, including brine shrimp (Lenstra et al. 1986), mice (Roth et al. 1987), humans (Brands et al. 1986), and honeybees (Walldorf and Hovemann 1990), with 70% nucleotide and 80% protein conservation. EF-1alpha is essential for protein synthesis and apoptosis regulation and interacts with actin and ubiquitin-dependent proteolysis. While normally viewed as a single-copy gene in insects (Friedlander, Regier, and Mitter 1996), the presence of two copies of EF-1a in bees raises the possibility that two copies are widespread, which could result from an ancient gene duplication event. Furthermore, the ~~intron less~~intronless feature of the gene serves as a substrate for synonymous substitutions. Nonsynonymous sites in the expanded gene families are rapidly diverging, both between

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orthologs and between paralogs. This is true in our study, as several proteins unique to *H.armigera* were observed in other insect orders. Homologs of the HSP protein (protein1, 2) varied between lepidopteran *Drosophila* and tobacco hornworm andascidian calmodulin(1, 2, b fragment) varied among theamphioxus,sea peach and sea urchin strains.

The *D. melanogaster* sex peptide (SP) has been a focal point for research on male–female conflict and sexually antagonistic coevolution. A single trait mapped to a single locus has evolved to promote male reproductive success at the expense of females. Our fecundity and longevity studies on mated and MAG-duplex extract-injected females (Rama et al., 2016 &2021) support the findings of SP's sexual conflict studies supporting the antagonistic coevolution model. Future studies aimed at identifying cognate receptors/s, developing antagonists, and studying the evolutionary patterns of reproductive traits in *H. armigera*will help determine structural-function relationships.

Since major differences in reproductive physiology exist between species of insects, MAG-duplex peptide information could shed light on identifying the target sites of peptides in *H. armigera* female moth. Further, this could aid in the development of antagonists of target sites and eco-friendly pest control regimes.

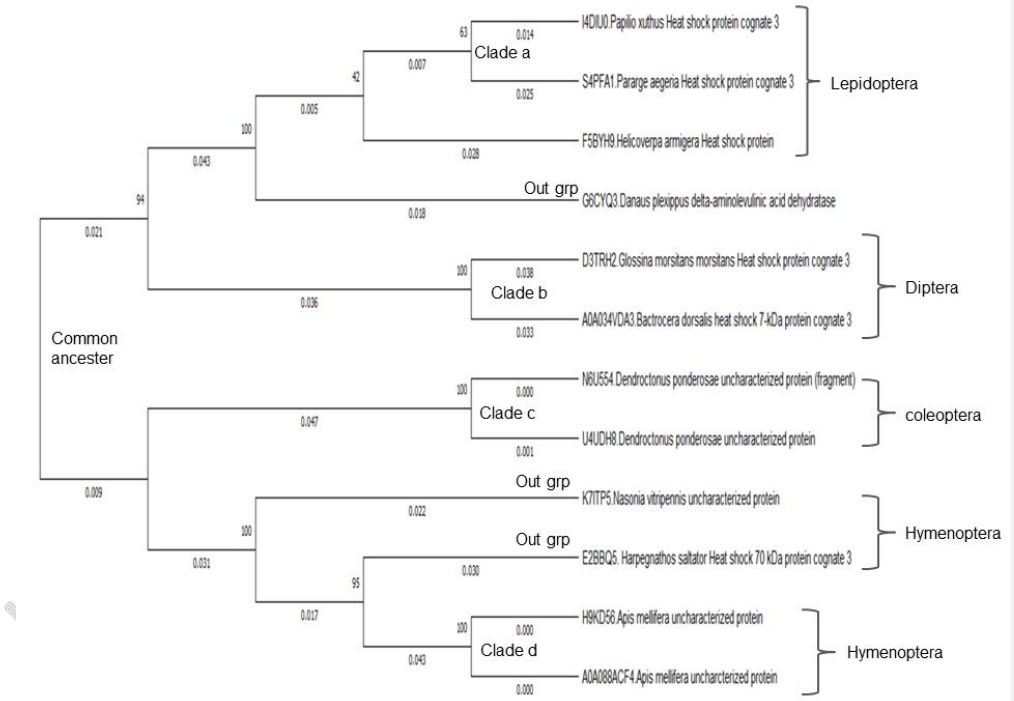

Figure 1:Phylogenetic tree of peptides across insect ordersusing the ClustalX program

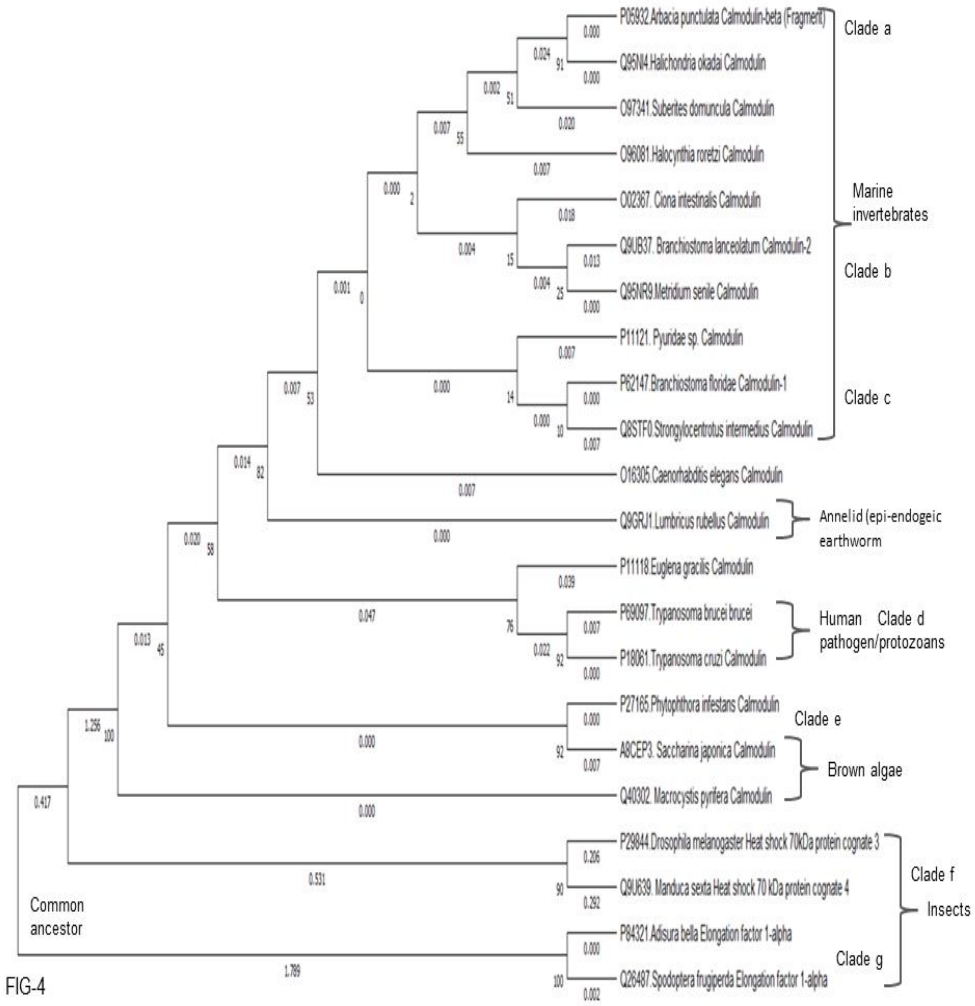
P05932.Arbacia punctulata Calmodulin-beta (Fragment)
Q85N4.Halichondria okadai Calmodulin
O97341.Suberites domuncula Calmodulin
O96081.Halocynthia roretzi Calmodulin
O02367. Ciona intestinalis Calmodulin
Q9UB37. Branchiostoma lanceolatum Calmodulin-2
Q6VNR9.Metridium senile Calmodulin
P11121. Pyuridae sp. Calmodulin
P62147.Branchiostoma floridae Calmodulin-1
Q8STF0.Strongylocentrotus intermedius Calmodulin
O16305.Caenorhabditis elegans Calmodulin
Q9GRJ1.Lumbricus rubellus Calmodulin
P11118.Euglena gracilis Calmodulin
P69097.Trypanosoma brucei brucei
P18061.Trypanosoma cruzi Calmodulin
P27165.Phytophthora infestans Calmodulin
A8CEP3. Saccharina japonica Calmodulin
Q40302. Macrocystis pyrifera Calmodulin
P29844.Drosophila melanogaster Heat shock 70kDa protein cognate 3
Q9U639. Manduca sexta Heat shock 70 kDa protein cognate 4
P84321.Adisura bella Elongation factor 1-alpha
Q26487.Spodoptera frugiperda Elongation factor 1-alpha
Clade a
Marine invertebrates
Clade b
Clade c
Annelid (epi-endogeic earthworm
Human pathogen/protozoans
Clade d
Clade e
Brown algae
Clade f
Insects
Clade g
Common ancestor

FIG-4

Figure 2:Phylogenetic tree of the calmodulin protein across various organisms using the ClustalX program

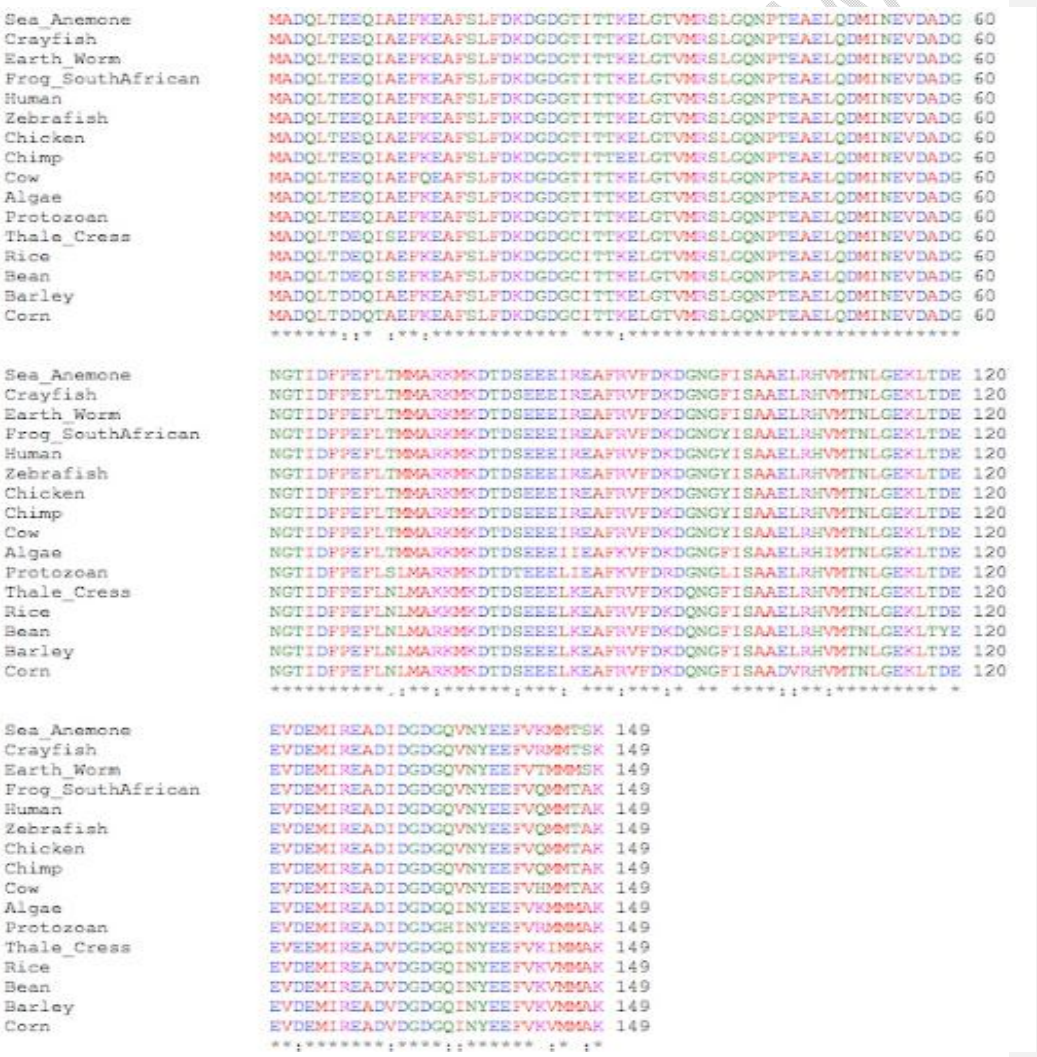

Figure 3: ClustalW of calmodulin protein sequences from different eukaryotic species from corn to zebrafish to protazoans highlighting the conservation between various and extremely diverse species.

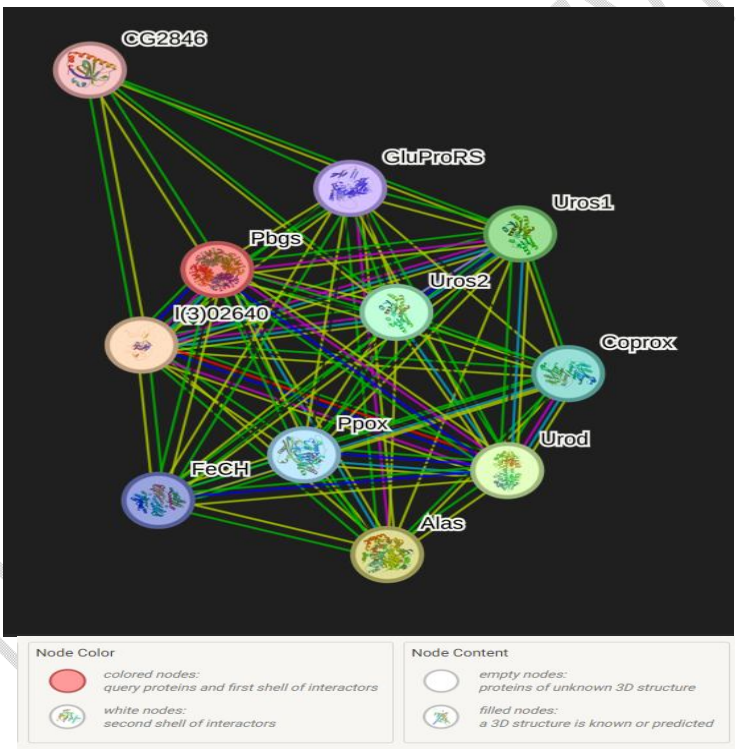

Figure 4:Protein interaction map of the PBGS protein using STRING.

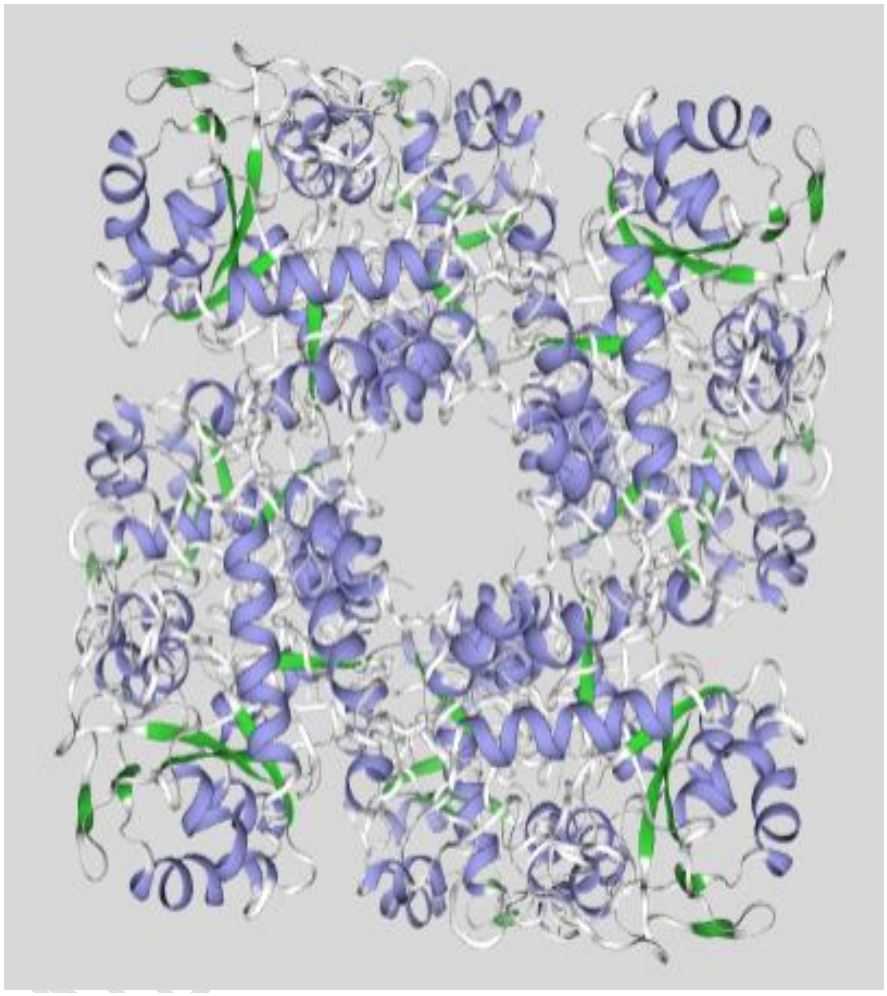

Figure 5A: Homology model with 8 ligands(2 active sites),with 60.19% identity.

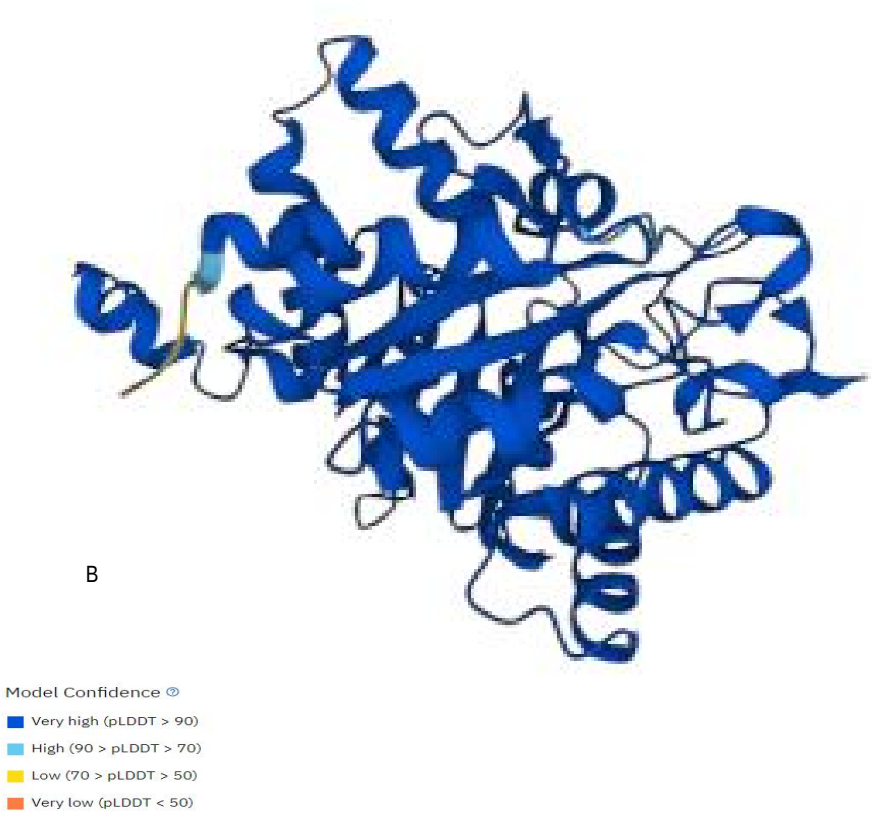

Figure 5B:Alpha fold model(High pLDDT-90)

Figure -5A &B:3D protein model of diazepam binding protein using the SWISS-MODEL homology-modeling pipeline.

Table 1: Proteomic analysis of MAG duplexes of *H. armigera* virgin males using the SWISS-PROT database across phyla

Sl.no	Accession	Description	Proteins	Unique Peptides
1	P29844	*Drosophila melanogaster* Heat shock 70 kDa protein cognate 3	1	3
2	Q9U639	*Manduca sexta* Heat shock 70 kDa protein cognate 4	1	3
3	P62147	*Branchiostomafloridae* Calmodulin-1	1	0
4	Q9UB37	*Branchiostomalanceolatum* Calmodulin-1	1	0
5	O96081	*Halocynthiaroretzi* Calmodulin	1	0
6	O16305	*Caenorhabditis elegans* Calmodulin	1	0
7	O02367	*Ciona intestinalis* Calmodulin	1	0
8	Q9GRJ1	*Lumbricusrubellus* Calmodulin	1	0
9	Q40302	*Macrocystis pyrifera* Calmodulin	1	0
10	Q95NR9	*Metridium senile* Calmodulin	1	0
11	P27165	*Phytophthora infestans* Calmodulin	1	0
12	P11121	*Pyuridae sp.* Calmodulin	1	0
13	A8CEP3	*Saccharina japonica* Calmodulin	1	0
14	O97341	*Suberitesdomuncula* Calmodulin	1	0
15	P05932	*Arbacia punctulata* Calmodulin-beta (Fragment)	1	0
16	P11118	*Euglena gracilis* Calmodulin	1	0
17	Q95NI4	*Halichondriaokadai* Calmodulin	1	0
18	Q8STF0	*Strongylocentrotus intermedius* Calmodulin	1	0
19	P69097	*Trypanosoma brucei brucei*	1	0
20	P18061	*Trypanosoma cruzi* Calmodulin	1	0
21	P84321	*Adisurabella* Elongation factor 1-alpha	1	0
22	Q26487	*Spodoptera frugiperda* Elongation factor 1-alpha	1	0

Table 2: Proteome analysis of MAG duplexes of *H. armigera* virgin males using the TrEMBLE database in insects

Sl. No	Accession	Description	Proteins	Unique Peptides
1	D3TRH2	*Glossina morsitansmorsitans* Heat shock protein cognate 3	1	0
2	E2BBQ5	*Harpegnathos saltator* Heat shock 70 kDa protein cognate 3	1	0
3	F5BYH9	*Helicoverpaarmigera* Heat shock protein	1	0
4	G6CYQ3	*Danaus plexippus*Delta-aminolevulinic acid dehydratase	1	0
5	I4DIU0	*Papilio xuthus* Heat shock protein cognate 3	1	0
6	K7ITP5	*Nasoniavitripennis* Uncharacterized protein	1	0
7	N6U554	*Dendroctonusponderosae* Uncharacterized protein (Fragment)	1	0
8	H9KD56	*Apis mellifera* Uncharacterized protein	1	0
9	S4PFA1	*Parargeaegeria* Heat shock protein cognate 3	1	0
10	U4UDH8	*Dendroctonusponderosae*Uncharacterized protein	1	0
11	A0A088ACF4	*Apis mellifera* Uncharacterized protein	1	0
12	A0A034VDA3	*Bactrocera dorsalis* Heat shock 70 kDa protein cognate 3	1	0

Table 3: Summary of MAG peptides identified in the study.

Sl. no	Peptide/protein	Category of protein based on GO ontology	Present in Lepidoptera (a), Diptera (b) Hymenoptera (c)	Present in other insects	Post transcriptional modification (Yes/no)	Signal peptide (Yes/no)
1	*Glossina morsitans*morsitans Heat shock protein cognate 3	Nucleotide binding	a,b,c	Data not available	Yes	Yes
2	*Harpegnathos saltator* Heat shock 70 kDa protein cognate 3	Nucleotide binding	a,b,c	Hymenoptera	Yes	Yes
3	*Helicoverpaarmigera* Heat shock protein	Nucleotide binding	a	Data not available	Yes	Yes
4	*Danaus plexippus*Delta-aminolevulinic acid dehydratase	Nucleotide binding	a,b,c	Data not available	Yes	Yes
5	*Papilio xuthus* Heat shock protein cognate 3	Nucleotide binding	a,b,c	Data not available	Yes	Yes
6	*Nasoniavitripennis* Uncharacterized protein	Nucleotide binding	c	Data not available	Yes	Yes
7	*Dendroctonusponderosae* Uncharacterized protein (Fragment)	Nucleotide binding	Data not available	Coleoptera	Yes	Yes
8	*Apis mellifera* Uncharacterized protein	Nucleotide binding	Data not available	Coleoptera	Yes	Yes
9	*Parargeaegeria* Heat shock protein cognate 3	Nucleotide binding	a,b,c	Data not available	Yes	Yes
10	*Dendroctonusponderosae*Uncharacterized protein	Nucleotide binding	b	Coleoptera	Yes	Yes
11	*Apis mellifera* Uncharacterized protein	Nucleotide binding	c	Data not available	Yes	Yes

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