Environmental and Experimental Biology (2024) 22: 185–200
http://doi.org/10.22364/eeb.22.18

Original Paper

Characterization of seminal fluid peptides/proteins of male *Helicoverpa armigera* and their plausible role in post-copulatory modulation of female reproductive behaviour

ISSN 2255-9582

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Abstract

In many insect species, female reproductive output is determined by male ejaculate components such as seminal fluid, plasma, and male accessory gland (MAG) peptides. These elements have additional roles in affecting insect receptivity and mating physiology. Studies indicate that the peptides/proteins of the male insects' seminal fluid are responsible for these behavioural changes in female *Helicoverpa armigera* insects. In particular, the virgin male's MAG peptide induces mating receptivity inhibition approximately 12 h after eclosion (emergence), which reaches peak concentration approximately at 24 h. This time coincides with the male's mating time, and is transferred to female moths during copulation. The aim of the study was to purify and characterize the virgin male's MAG-duplex peptides/proteins by biochemical and bioinformatics analysis. Analysis yielded a peptide of 5 kDa molecular weight. In insects several conserved proteins belonging to members of multigene families control a wide range of reproduction and related physiological processes. Data mining of the MAG-duplex peptides utilizing the public protein repository identified six proteins (heat shock proteins, diazepam-binding inhibitor, elongation factor 1 alpha, odorant-binding protein, serpin, and thioredoxin) with diverse functions. The diazepam-binding inhibitor protein, which binds coenzyme A and thiol esters of long fatty acids, was unique to *H. armigera*. The molecular evolutionary analysis of the proteins independently supports the widely accepted theory that genes in gene families with roles in reproduction have evolved over a generational time scale.

Key words: accessory reproductive glands, acyl-CoA-binding proteins, elongation factor, fecundity, *Helicoverpa armigera*, juvenile hormone, longevity, odorant binding protein, pheromone, post mating receptivity.
Abbreviations: ACBPs, acyl-CoA-binding proteins; AL, antennal lobe; DBI, diazepam-binding inhibitor; FAR, fatty acyl reductases; HSPs, heat shock proteins; JH, juvenile hormone; MAG, male accessory gland; OBPs, odorant-binding proteins; PBS, phosphate-buffered saline; PG, pheromone gland; PMR, post mating receptivity; RP-UFLC, reversed-phase ultra-fast liquid chromatography; RT, reproductive tract; SF, seminal fluid; SFPs, seminal fluid peptides/proteins; SP, sex peptide.

Introduction

Heliothines, such as *Helicoverpa armigera* (Hübner), are insects belonging to the order Lepidopterans and are major agricultural pests in many parts of the world (CABI 2018). In many insect species, mating results in an increase in fecundity and a transient decrease in receptivity along with a parallel increase in the rate of egg laying. The physiological mechanisms involved in receptivity appear to be initiated and coordinated by male seminal fluid (SF) and its constituents (Druart, Graaf 2018). Throughout their life cycle, certain species of insects exhibit patterns of ontogenetic receptivity. Additionally, in species that exhibit post-mating receptivity (PMR), cyclic receptivity, sperm retaining in spermathecae, presence of egg, or seminal chemicals (often peptides produced by the male accessory glands) may prevent re-mating. Insect SF proteins (SFPs) are produced by the testes, ejaculatory bulb, ejaculatory duct, and male reproductive tract accessory glands (Druart, Graaf 2018). The fluid provides a favorable environment for the sperm's well-being during transfer to the female insect. It is composed of lipids, carbohydrates, nucleic acids, peptides, also enzymes, chaperones, structural proteins, and antioxidants (Evans et al. 2019; Scolari et

al. 2021). Given their presence in SF across diverse taxa, including mammals and arthropods, these protein classes seem to be conserved. Post-mating the proteins alters the biochemistry, physiology, and transcriptome of female insects both short-term and long-term (Gillott 2003).

Female insects belonging to all genera show a reduction in mating receptivity, such as in *H. armigera* (Kiran et al. 2021), *Heliothis virescens* (Hosseini 2016), *Bombyx mori* (Zhang et al. 2014), and several other species. The relative molecular weights of the various SFPs vary (Avila et al. 2011). Prohormone-like polypeptides and large glycoproteins have 200 to 400 amino acids, whereas sex peptide (SP) comprises 36 amino acids. PMR research suggests that gene expression may also be influenced by the number of mating events (Apger-McGlaughon, Wolfner 2013). The metabolism of glucose, amino acids (including dopamine precursors), carboxylic acids, and the juvenile hormone (JH) pathway are all regulated by metabolism regulatory genes that are frequently downregulated during the second mating event (Newell et al. 2020). After the second mating event, there is an increase in the expression of genes linked to cell growth processes and reproduction (Blankers et al. 2022).

Proteomic methods that combine liquid chromatography and mass spectrometry may identify hundreds of low-concentration proteins present in complex biological materials like SFPs (Steen, Mann 2004; Karr 2008). The approach can be effectively used even in the absence of a whole genomic sequence by comparing spectra with protein predictions from expressed sequence database and protein databases (Andres 2008; Bräutigam 2008). This finding has been supported by a number of previous studies that used biochemical, proteomic, and transcriptomic techniques followed by in silico techniques. Thus, proteomic studies are effective in determining the protein components of the ejaculate from insects belonging to different species (Walters, Harrison 2010). Mature, sexually receptive female noctuid moths release a volatile mixture of sex hormones during the scotophase to signal that they are available for mating (Jurenka 2004). Because they encourage reproductive isolation within species as well as across species, these PMR mechanisms are crucial to the evolutionary process. The mix is generated by the pheromone gland (PG) in the majority of moths, using by-products of the fatty acid metabolism (Tillman 1999). Lepidopteran female sex pheromones are also derived from fatty acids. Saturated C10 – C18 fatty acyl moieties are the substrates for moth pheromone compounds, which are altered by the addition of alcohol, acetate ester, or aldehyde functional groups. (Matsumoto 2010).

Certain enzymes involved in the manufacture of fatty acids – encoded by the fatty acyl reductases (FAR) gene – perform the biosynthesis of pheromone bouquets. Pheromone production in female *H. armigera* during the later phases of the scotophase is regulated by the timely release of pheromone biosynthesis-activating neuropeptide (Hanin 2012). This peptide is present in many insects, and its pheromonotropic activity has been well-described in several lepidopteran insects (Rafaeli 2009). When pheromone biosynthesis-activating neuropeptide interacts with its G-protein-coupled receptor in pheromone gland cells, a calcium influx that triggers the synthesis of cyclic AMP is produced, activating calcium and calmodulin sensitive adenylate cyclase (Choi et al. 2003). The variables responsible for controlling the behavioural changes that the female moth experiences after mating remain unclear. One of the physiological changes is the termination of pheromone biosynthesis and calling behaviour (Jurenka 2017). *B. mori* PG cells accumulate a large number of lipid droplets within the cytoplasm just before eclosion (Fónagy et al. 2005). Additionally, the PG cells express two distinct acyl-CoA-binding proteins (ACBPs) during pheromonogenesis: pheromone gland ACBP and midgut ACBP. The lipid droplets include unsaturated C16 and C18 fatty acid triglycerides in addition to the pheromone. Research indicates that pheromone antagonist-mediated mating time optimization ensures maximum reproduction (Chang et al. 2017). Female *H. armigera* emits pheromone compounds, comprised of a 98:2 ratio of (Z)-11-hexadecenal to (Z)-9-hexadecenal, to attract males (Hughes, Carde 2020). In addition, females prevent less-than-ideal mating by blocking males with cis-11-hexadecenol (Z11-16:OH), a pheromone antagonist. In summary, these findings emphasize the significance of pheromone-mediated mating and the associated neuro-physiological alterations that occur in insects during reproduction.

The present investigation aims to correlate between the duration of peptide synthesis and the JH in male *H. armigera* moths, as well as its function in impeding pheromone-mediated behaviour in females. Biochemical and bioinformatics analyses were used to purify and characterize the virgin male's peptides and proteins. In addition, protein 3D homology modeling and molecular evolution analysis were conducted to determine the likely function of the proteins in PMR.

Materials and methods

Insect rearing and preparation of MAG-duplex crude extract

Neonates of *H. armigera* (NBAII-MP-NOC-01) were procured from the National Bureau of Agriculturally Important Insect Research, Bengaluru, India. Rearing of insects and preparation of proteins from virgin and mated male-MAG-duplex were carried out according to the protocols described by Rama et al. (2021). The tissue extracts in Bennett's buffer were subjected to chromatography techniques for purification. All reagents were prepared under aseptic conditions with high quality analytical grade chemicals. GLP and ISO-certified laboratories conducted

the experiments under stringent conditions. Care was taken to minimize human error, and all samples were processed in the same batch to avoid errors. The experiments were carried out by a single investigator.

Partial purification using cation-exchange chromatography

Standard laboratory procedure with specified protocol (WATERSTM) was adhered to with minor modifications. In the present study, Sep-Pak Accell plus CM short cartridges (pore size 300 Å) were used for the extraction of cationic analyses. Samples of each batch were lyophilized and reconstituted with phosphate-buffered saline (PBS) to carry out mating receptivity bioassays.

Purification of behaviour-modifying peptides from active fractions of the CM Sep-Pak cartridge by RP-UFLC

The active fractions from the cation-exchange chromatography were subjected to a reversed-phase ultra-fast liquid chromatography (RP-UFLC) gradient solvent system (Schmidt et al. 1993). The peaks eluted at 29.59, 39.26, 42.82, 45.62, 47.5, and 50.60 min were collected several times manually and lyophilized. The lyophilized samples were reconstituted with PBS to carry out the mating receptivity bioassays.

Bioassays

Lyophilized protein extracts were used for bioassay experiments to study their effect on the reproductive behaviour of female moths (Jinn, Gong 2001). Details are described in Rama et al. (2021).

MAG-duplex protein/peptides analysis by MALDI-TOF

The lyophilized active fractions of RP-UFLC were reconstituted with Milli-Q water and subjected to MALDI-TOF (UltrafleXtreme, Bruker Daltonics, Germany) analysis, which was carried out at the Molecular Biophysics Department, IISc, Bengaluru.

Preparation of a sample for de novo *sequencing*

Tris-glycine-SDS-PAGE analysis of the peptides fractionated by RP-UFLC was carried out using 15% polyacrylamide gel (Laemmli 1970) to ensure purity before submission of the samples for *de novo* sequencing. Low-range protein markers (SRL, India) were used to determine the relative molecular weights of the bands.

De novo *sequencing by in-gel digestion and nano-LC-MS/MS analysis*

The Coomassie blue-stained band from Tris-glycine-SDS-PAGE showed activity subjected to de novo sequencing at C-Camp, NCBS, Bengaluru. Initially, in-gel digestion was carried out with the trypsin enzyme; later, all the fragments generated were subjected to *de novo* sequencing by nano-LC-MS/MS. Generated data was acquired using Peak (version 7) software, and the data was searched for similar annotations in various databases such as NCBI, SWISS-PROT, and TrEMBL using the MASCOT search engine.

Proteome analysis of virgin male MAG-duplex crude extract by in-solution digestion and nano LC-MS/MS analysis – a shotgun/bottom-up approach

The proteome analysis (C-Camp, NCBS, Bengaluru) of virgin MAG-duplex was carried out with a crude sample reconstituted with Milli-Q water. The sample was initially subjected to acetone precipitation, and its quantity was estimated using a BCA assay. Further, the sample was subjected to an in-solution digestion protocol with additional alkylation and reduction (Wiese et al. 2007). Digested peptides were reconstituted for nano-LC-MS/MS analysis. Generated data were acquired using Protein Discover (version 1.4) software, and the data were searched for similar annotations in the NCBI repository using MASCOT as a search engine.

Protein bioinformatics

Since *H. armigera* is not a common model organism and there is no complete genome sequence available to date, proteomics is a novel and direct method to identify unannotated peptides/proteins. A database search of the putative protein in the NCBI protein database was carried out (https://www.ncbi.nlm.nih.gov/protein/, accessed on December 2023). Several internet servers were used to retrieve cellular and structural information about the protein to elucidate the structure-function relationship: Uniprot (https://www.uniprot.org/), GO-Annotation (https://geneontology.org/), SMART (http://smart.embl-heidelberg.de/), Alpha Fold (https://alphafold.ebi.ac.uk/), Phyre (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index), and Ka/Ks analysis (https://ngdc.cncb.ac.cn/tools/kaks).

Time of secretion of behaviour-modifying peptide

The extracts of MAG-duplex tissue were collected from virgin male moths at every six-hour interval from the time of emergence (0 to 36 h) and analyzed on Tricine-SDS-PAGE, as explained earlier, to assess the time of secretion of the behaviour-modifying peptide in the accessory glands.

Fecundity and longevity study of female moths

As reported in our previous papers the experiment was set up with two groups of females: Group 1 – virgin females (n = 30) and Group 2 – mated females (n = 30). Group 2 females were obtained when virgin females mated with naïve males of age 2 to 6 day old in the ratio 1:2 (female:male).

Statistics

The chi-square (χ^2) test was used to study the unreceptivity of female moths at the 1% ($\alpha < 0.01$) significance level. All experimental analyses were carried out using R program software (version 3.0.2).

Results

Effect of cation exchange fractions on mating receptivity behaviour

The cation-exchanged fractions F2 and F3 elicited noticeable unreceptive behaviour upon injection into virgin females (Fig. 1). On injection of fraction-2, unreceptivity was approximately 82% ($\chi^2 = 11.240$, df = 1, $p < 0.01$) on the day of treatment, and 73% ($\chi^2 = 7.812$, df = 1, $p < 0.01$) on the second day in virgin females. Similarly, when fraction-3 was injected, 85% of virgin females ($\chi^2 = 14.333$, df = 1, $p < 0.01$) were unreceptive on the day of treatment, and 78% were unreceptive ($\chi^2 = 8.56$, df = 1, $p < 0.01$) on the second day. Mated female moths were 96% unreceptive ($\chi^2 = 23.860$, df = 1, $p < 0.01$) on the day of treatment, and the percentage of unreceptive mated female moths was reduced to 83% ($\chi^2 = 13.830$, df = 1, $p < 0.01$) on the second day. After injection, both the virgin and mated females resumed receptivity ($p > 0.01$), with 74, 75, and 72% on the first, second, and third day, respectively.

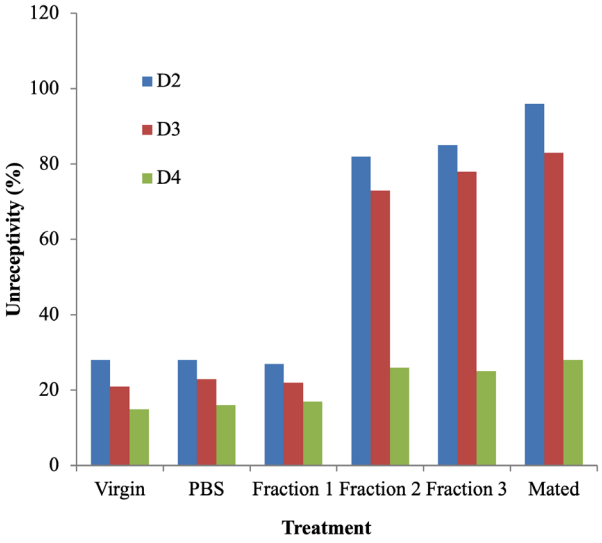

Fig. 1. Effect of CM Sep-Pak fractions on receptivity of *Helicoverpa armigera*. Mating inhibition exhibited by virgin females, PBS (control), CM Sep-Pak fractions injected females and mated females ($n = 25$). The percentage unreceptivity was recorded from the 2nd day after emergence. D2, D3, D4 indicate days.

Purification of behaviour-modifying peptides from active fractions by RP-UFLC

The pooled cation exchange fractions (2 and 3), eliciting unreceptivity behaviour, were further fractionated by RP-UFLC using a Phenomenex C18 column. Proteins/peptides with different hydrophobicities were eluted into six peak fractions at different retention times from the 29.59th min (P1) to the 50.60th min (P6). The peak fractions eluted at 29.59 (P1), 39.26 (P2), 42.82 (P3), 45.62 (P4), 47.5 (P5), and 50.60 min (P6) were collected several times manually and lyophilized (Fig. 2). The reconstituted samples with PBS were subjected to bioassays for mating receptivity.

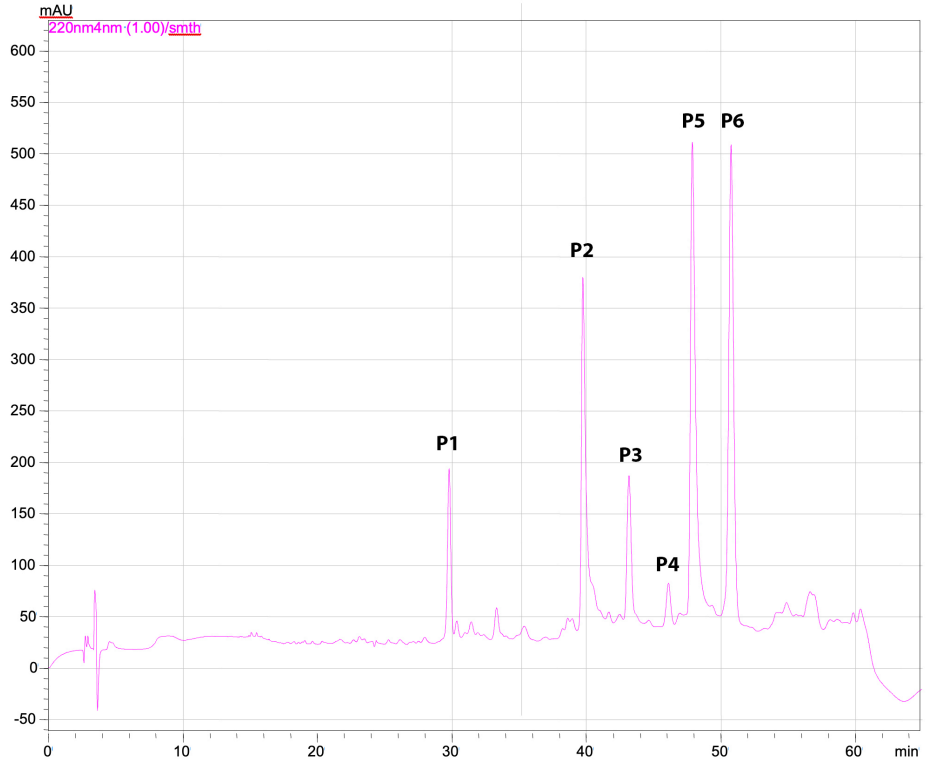

Fig. 2. RP-UFLC chromatogram of cation-exchanged pooled fractions (2&3). The peak fractions eluted at time 29.59 (P1), 39.26 (P2), 42.82 (P3), 45.62 (P4), 47.5 (P5) and 50.60 (P6) min.

Effect of RP-UFLC fractions on the mating receptivity behaviour

Injection of fraction P4 to virgin females resulted in 80% unreceptivity on the mated day (χ^2 = 10.800, df = 1, p < 0.01), and on the second day it was 73% (χ^2 = 7.812, df = 1, p < 0.01). Mated female moths showed approximately 93% (χ^2 = 22.071, df = 1, p < 0.01) unreceptivity on the day they mated and 73% (χ^2 = 7.812, df = 1, p < 0.01) on the second day. On the third day, receptivity was resumed by both virgin females (73%, p > 0.01) and mated (72%) P4 fraction-injected (Fig. 3).

Intact mass analysis of RP-UFLC fractions by MALDI-TOF

The active fraction was subjected to MALDI-TOF analysis for intact mass identification on an ultrafleXtreme instrument. The peptide profile suggested that the peptide was intact and had not undergone any cleavage during the fractionation process. The peptide that showed positive results in the bioassay had a molecular weight 4962.3 (P4) Daltons (Fig. 4).

De novo *sequencing of a female behavior-modifying peptide*

As shown in Fig. 5, a prominent single band was observed in fraction P4, which was subjected to de novo sequencing. The de novo sequencing results revealed 10 partial sequences with ≥ 90% average local confidence. The database searches performed using NCBI BLAST showed less significant sequence similarity, with a score < 40 for any of the partial sequences generated from the peak P4 peptide, suggesting that this peptide is novel and not been previously reported.

Bioassays for the fecundity and longevity study of female moths

The results of bioassays have been described in detail in our previous publications. A brief description of the results is provided in the following paragraphs. The fecundity analysis showed a significant difference in fecundity between the virgin and mated female groups (W = 176, p < 0.01) with the Wilcoxon rank-sum test, which is based on null hypothesis rejection. Mated females laid more eggs (1656.96 ± 542.80) compared to virgin moths (824.86 ± 641.64). Then oviposition rate analysis showed the mated females laid 12.23 and 22.24% of their total eggs (1656.96 ± 542.80) on the second and third scotophases respectively, whereas in virgin females the percentage of eggs laid was 1190.69 and 1112.1% on respective days. The egg-laying peaked on the seventh day in virgin females, which accounted for 14.36% of their total eggs, with a similar

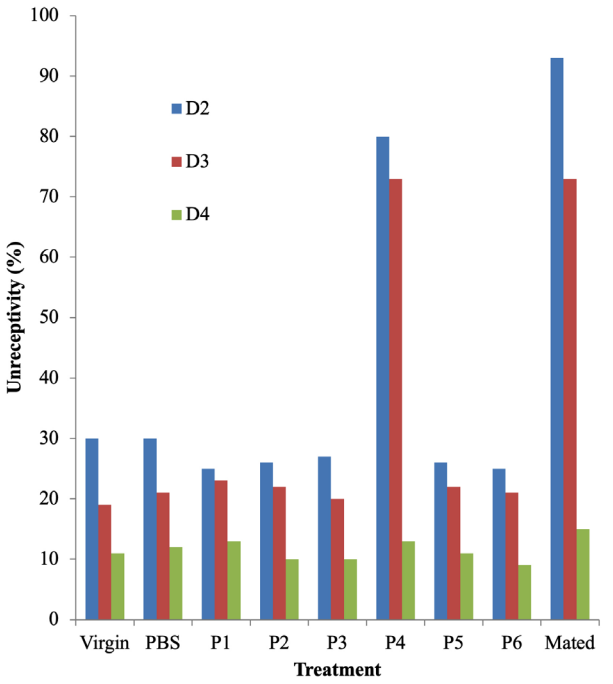

Fig. 3. Effect of RP-UFLC peak fractions on receptivity of *Helicoverpa armigera*. Mating inhibition exhibited by virgin females, PBS (control) and mated females, RP-UFLC peak fractions injected females (n = 20). The percentage unreceptivity was recorded from the 2nd day after emergence. D2, D3, D4 indicates days.

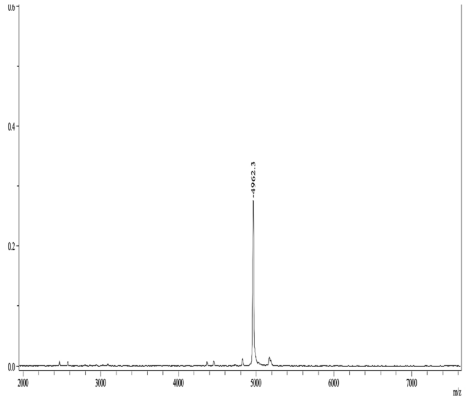

Fig. 4. The MALDI-TOF spectrum of peak P4 depicting a molecular mass of 4962.3 Da.

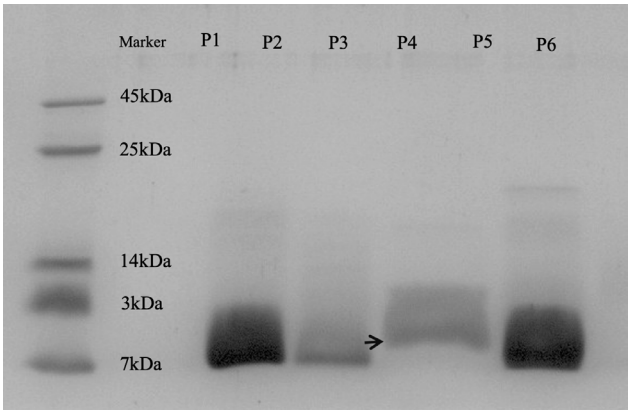

Fig. 5. Tris-glycine-SDS-PAGE (15%) of RP-UFLC peak fractions showing a prominent single band in lane 4.

percentage of eggs laid by mated female moths. Finally, by the 9th day, very few mated females lay eggs, and they terminated before the virgin females.

Longevity studies showed a significant difference between the virgin and mated groups ($W = 771.5$, $p < 0.01$). The mated moths had a shorter lifespan (10.68 ± 2.47 days) than virgin moths (17.06 ± 3.81 days), which lived longer by approximately one week. Regression analysis showed that the behaviours (fecundity and longevity) of mated females are coupled and are negatively related.

Time of secretion of behaviour-modifying peptide

Freshly emerged females did not show either mating receptivity or egg-laying behaviour during the first scotophase. From the second scotophase, the females started mating as well as egg- laying irrespective of mating status. In total, 71% of virgin females ($\chi^2 = 4.459$, df = 1, $p > 0.01$) showed mating receptivity, and 29% showed unreceptive. Conversely, none of the mated females ($\chi^2 = 25$, df = 1, $p < 0.01$) were ready for second mating. However, the mated females started exhibiting receptivity to approached males in the third scotophase, and the percentage of such females was 9% ($\chi^2 = 21.324$, df = 1, $p < 0.01$). Mating receptivity increased to 68% ($\chi^2 = 5.899$, df = 1, $p > 0.01$) on the fourth scotophase. Finally, the percentage of mating receptivity or unreceptivity was similar to that of virgin females on the fifth scotophase (~ 98% in both groups). The virgin as well as mated female moths started oviposition on the second scotophase.

MS-based proteome analysis of virgin MAG-duplex crude extract

The direct inference of the MAG-duplex revealed a range of proteins from the shotgun proteome analysis (Table 1). These include the heat shock proteins, diazepam-binding inhibitor, elongation factor 1 alpha, odorant-binding protein, serpin, and thioredoxin from the protein database (https://www.ncbi.nlm.nih.gov/protein/, accessed on December 2023). Table 2 summarizes the biophysical features of the proteins from proteome analysis of virgin males.

Protein bioinformatics

Using the crystal structure of the insect *Maduca sexta*

Table 1. Proteome analysis of MAG-duplex of *H. armigera* virgin males

No.	Accession	Description	Length	Unique peptides	Peptides
1	328670877	Heat shock protein	659 aa	8	11
2	219671577	Heat shock protein 70	654 aa	6	9
3	40022264	Diazepam-binding inhibitor	90 aa	2	2
4	767847	Elongation factor 1-alpha, partial	413 aa	0	2
5	268527749	Elongation factor 1 alpha, partial	319 aa	0	2
6	767845	Elongation factor 1-alpha, partial	413 aa	0	2
7	441481897	Thioredoxin	106 aa	2	2
8	314912147	Serpin	391 aa	2	2
9	365919066	Odorant-binding protein, partial	121 aa	0	2
10	385275513	Odorant-binding protein 18	137 aa	0	2

Table 2. Cellular and structural properties of protein classes from the proteome analysis of MAG-duplex of *H. armigera* virgin males

No.	Description	Function (GO-ontology)	Cellular location	Cysteine residues	Unique feature of protein domain
1	Odorant-binding protein, partial	Odorant-binding	Secreted	Present	Chain
2	Heat shock protein 70	ATP-dependent protein folding chaperone	Endoplasmic reticulum lumen	Absent	Coiled coil
3	Diazepam-binding inhibitor	Fatty-acyl-CoA binding	Mitochondria	Absent	Ligand acyl-CoA-binding
4	Elongation factor 1-alpha, partial	GTP binding	Cytoplasm	Absent	Tr-type G
5	Thioredoxin	Thioredoxin-disulfide reductase (NADP) activity	Mitochondria	Present	FAD/NAD(P)-binding. Pyridine nucleotide-disulphide oxidoreductase dimerisation
6	Serpin	Serine-type endopeptidase inhibitor activity	Extracellular space	Absent	Serpin domain-containing protein

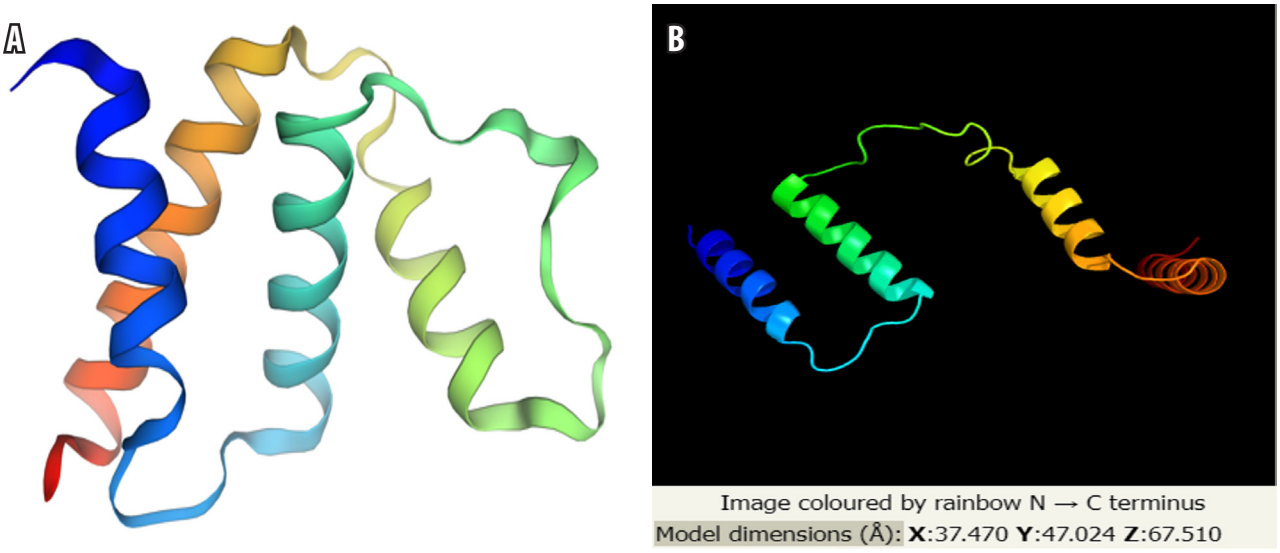

Fig. 6. A, alpha fold-3D model of *H. armigera* DBI modelled using *Maduca sexta* acyl-CoA-binding protein depicting the four α helices (ribbon representation). B, 3D model (ribbon representation) of *Helicoverpa armigera* DBI modelled using crystal structure of human acyl- CoA binding domain7 protein depicting the four α helices.

acyl-CoA-binding protein as a template, 3D modeling was utilized to propose the four α helices of the protein (Fig. 6 and 7). In total, 67% of amino acids fold into α helices, according to the crystal structure of proteins containing the human acyl-CoA binding domain 7. The protein was then examined further, and active areas that might be representative of the ligand binding properties of the protein were found (Fig. 8). A Ramachandran plot analysis anticipated a 100% favored secondary structure growth with no steric hindrances (Fig. 9).

The NCBI protein blast data of DBI shows indicates several insects belonging to butterflies, moths, beetles,

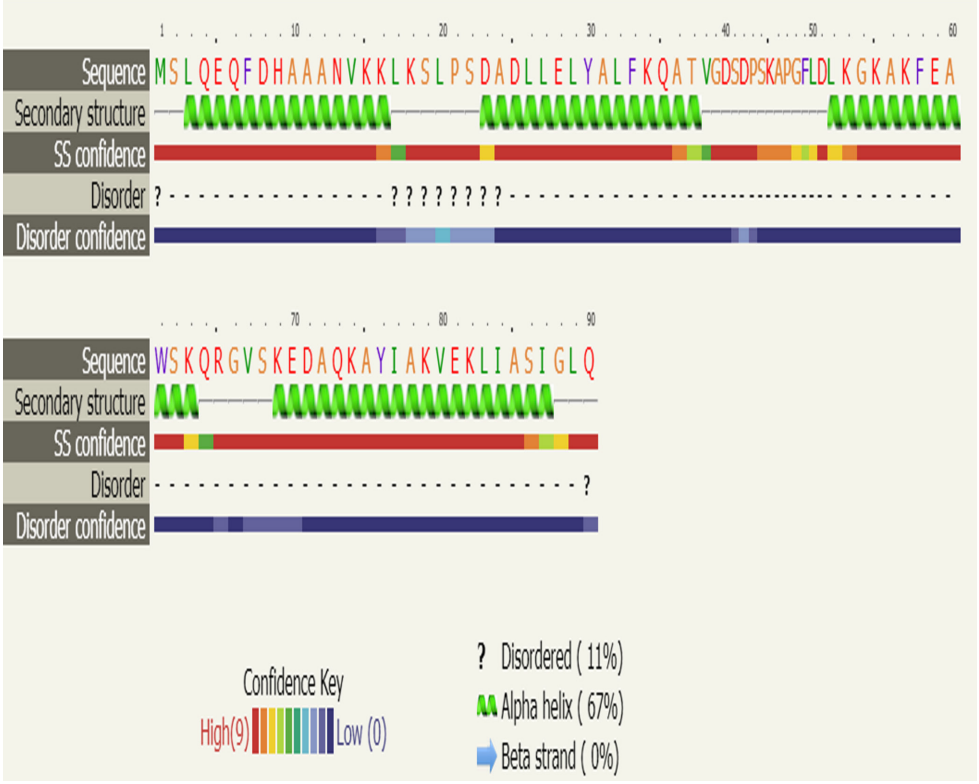

Fig. 7. Predicted secondary structure of *Helicoverpa armigera* DBI modelled using *Maduca sexta* acyl-CoA-binding protein depicting various primary structures (alpha, beta and disordered regions).

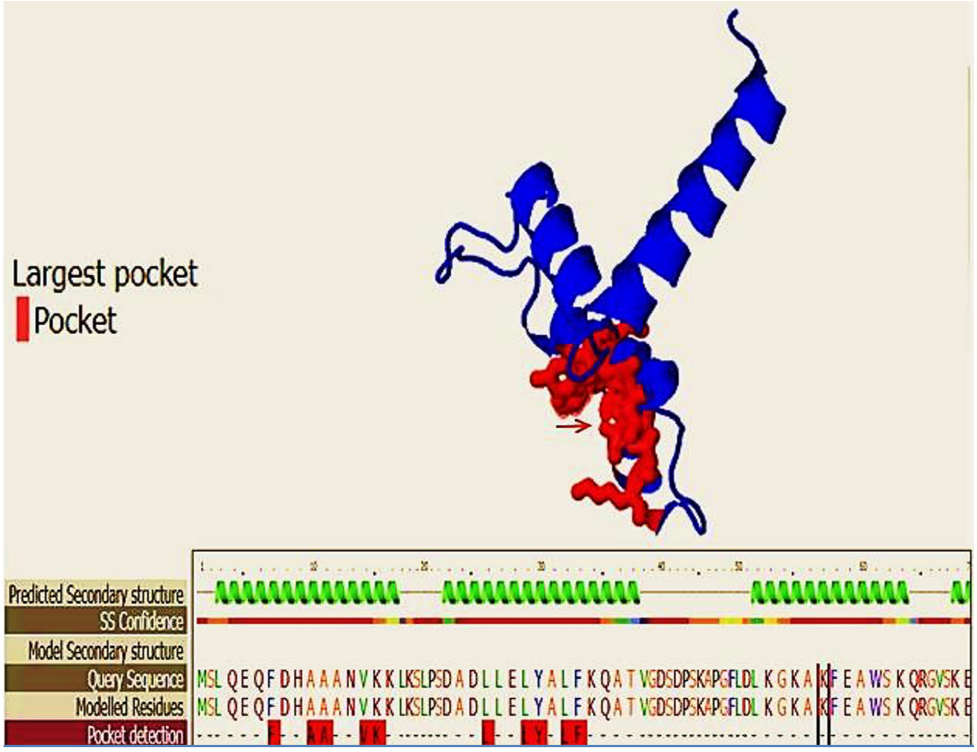

Fig. 8. Model of DBI *Helicoverpa armigera* illustrating the pocket detection. Arrow indicates location of active sites.

wasps, and flies, have hits surpassing 100 (above 75% homology). This pattern suggests that this protein is important for traits related to reproduction. The multiple sequence alignment and phylogenetic tree of the ACB protein are displayed in Fig. S1 and 10.

Discussion

Examining the constituents and functions of SFPs is necessary to comprehend the role of insect reproductive compounds. When their SFPs are engaged, females go

Fig. 9. Ramachandran plot suggesting the secondary structure contour map of *Helicoverpa armigera* DBI protein.

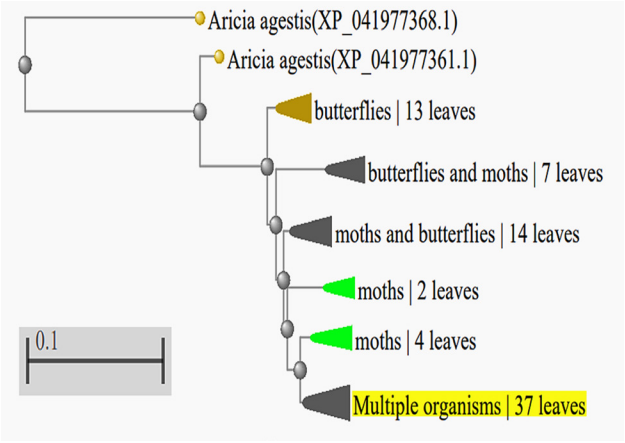

Fig. 10. Rooted phylogenetic tree of DBI protein in various insects constructed using https://www.ncbi.nlm.nih.gov/tools/treeviewer/(Rectangle Cladogram sorted by distance).

through a complex transition into a post-mating state. This complex process entails the identification and disintegration of SFPs, which is followed by the firing of brain circuits to control modifications in the female reproductive organs (Rubinstein, Wolfner 2013). As stated in the procedures, bioassays were performed to investigate the physiological alterations brought about by SFP. The PSP derived from the heliothine *H. zea* is cationic and causes unreceptivity by blocking pheromone production. Hence, ion exchange chromatography was used to purify the bioactive components of the MAG-duplex. In many species of moths (Lepidoptera), sex related endpoints usually depend on the female emitting a unique blend of sex pheromones to attract conspecifics. Further research suggests that in order to control mating behaviours particular to their species, female moths may produce a multi-component pheromone mix with specially regulated blends (Blankers et al. 2022). Finally, several findings imply that the PG (pheromonostatic action) and CNS (allatotropic activity) of the female moth may include target receptors for the SFPs.

According to the SDS-PAGE analysis, the peptide appears approximately 12 h after the moth ecloses and reaches critical concentrations at 24 h. These results are in line with previous research (Hou 1998). A male *H. armigera* reaches sexual maturity 24 h after emergence, and it is fully developed at 48 hours following emergence, according to the study. Nonetheless, the stages of reproductive development and activity during adult emergence vary throughout insect species (Wolfner, Applebaum 2005). In gypsy moth *Lymantria dispar* and *B. mori* gametogenesis at the time of eclosion is complex. The adult moths of these species are ready for mating, do not feed, and oviposition occurs immediately after eclosion without the requirement for hormone control. This age-dependent behavioural plasticity is determined by the biosynthetic activity of JH, which controls the sensitivity of neurons in the primary olfactory centre, the antennal lobe (AL) (Jarriault et al. 2009). It also observed that small number of insect species have been shown to exhibit age- and hormone-dependent olfaction-guided behavioural modifications. Males of migratory species, such as *H. armigera* moths, change their reaction to sex pheromones with age and endocrine circumstances, but males in non-migratory species respond to the female sex pheromone immediately after hatching (Barrozo et al. 2010). The peptide that was found in the accessory glands of virgin males but disappears in mated males may be the same peptide that was eluted at 45.62 min in HPLC and reduced mating receptivity (Rama et al. 2021). SFPs helps in sperm preservation and release in the oviducts, which enhances reproductive efficiency. After mating, female sexual receptivity is lost in the majority of insect species, either permanently or intermittently. This loss of receptivity to mating often occurs in conjunction with sex pheromone depletion in moths. There is a direct correlation between calling behaviour and sex pheromone production. Calling activity, which might be intermittent or continuous, is not done by female mates (Wedell 2005; Rama et al. 2021). It is possible that male *H. armigera* moths have an SP-like component that is transferred to females during copulation or at least one that functions similarly to the *D. melanogaster* SP (Nagalakshmi et al. 2004). It was demonstrated that a partial sequence of produced pheromonostatic peptide from (HezPSP) in *H. zea* could inhibit pheromone synthesis (Eliyahu et al. 2003), whereas the *Aedes aegypti* mosquito uses Head Protein 1 peptide (Duvall et al. 2017). These data suggest that the accessory glands of male *H. armigera* may include both PSP-like and SP-like components.

This trend indicates that there is a high level of evolutionary turnover among the regulators of female post-mating alterations in insects, with new regulators emerging and older regulators disappearing from populations over a generational timescale. Six proteins with distinct cellular and structural functions including heat shock proteins (chaperone), diazepam-binding inhibitor, elongation factor 1 alpha (eEF1A), thioredoxin, serpin, and odorant-binding proteins were identified from the protein database by the proteome analysis of virgin MAG-duplex crude extract, as shown in Tables 1 and 2. Insects release HSPs in response to external stimuli. Heat shock proteins (HSPs) are chaperone proteins that have important functions in spermatogenesis, mitosis, and fertilization during reproduction. They function as signaling molecules that facilitate sperm-oocyte interaction (Asquith et al. 2005). HSPs have been found in the SF protein of *Tribolium castaneum* (Xu et al. 2013) and the mature testis proteome of *B. mori* (Zhang et al. 2014). Few HSPs use differential phosphorylation and protein interactions to influence hormone-dependent signaling pathways. HSP 70 and HSP 90 have been found in *H. armigera*, indicating their participation in the immunological response, while HSP 60 has been reported from the mated females of *D. melanogaster* (Mack et al.

2006; Rafaeli, Hanin 2013). In collaboration with co-chaperones and accessory proteins, HSPs mediate essential functions such folding, assembly, intracellular localization, secretion, regulation, and destruction of other proteins (Hendrick, Hartl 1993). These proteins exhibit distinct and characteristic expression patterns in insects at various developmental stages (Michaud et al. 1997). In the current investigation, heat shock protein and heat shock protein 70 were discovered.

ACB proteins are big, multipurpose, completely independent proteins conserved in mammals and yeast (Burton et al. 2005). Their activity clearly indicates that they bind both coenzyme A and the thiol esters of long fatty acids. Essential intermediaries in lipid metabolism, acyl-CoAs also affect gene regulation and intermediary metabolism control. It has been demonstrated that many insect species have acyl-CoA-binding and diazepam-binding inhibitor proteins, which are essential for the growth, transformation, and oogenesis of insects (Snyder, Antwerpen 1998; Pagán 2021). Additionally, FAR functions as a semi-selective funnel in the pheromone gland of heliothine moths, producing a fatty-acyl precursor ratio unique to a particular species (Hagström et al. 2012). The hemolymph can respond to specific humoral cues to start the transcription of ACBPs. Support for this observation comes from Takahashi et al. (2007), who discovered that titres of D-glucosyl-O-L-tyrosine grew significantly before eclosion and reached a maximum on the day before eclosion. Based on the biological and proteomic data, it is feasible to conclude that the DBI proteins are crucial for female fecundity through the pheromone-mediated ligand-signaling pathway. Elongation factor 1-alpha has been identified in the current work. Elongation factors are highly conserved proteins required for both protein production and the elongation of peptides during translation. eEF1A is expressed in germ cells (Zhao et al. 2012). Further, EF-1alpha was found in the accessory glands of *Cimex lectularius* bed bugs. (Reinhardt et al. 2009).

During early reproductive development in the female adult, EF-1 expression is upregulated in the fat body of *Locusta migratoria*, and this expression varies in tandem with changes in the hemolymph juvenile JH titer in the fifth instar. In addition, JH stimulates the accumulation of LmEF-1 alpha, assisting in the massive synthesis of proteins required for egg formation (Zhou et al. 2002). *Anopheles gambiae* male accessory glands contain thioredoxin, which is expressed and identified in the mating plug (Baldini et al. 2012). In mated females, thioredoxin in MAG is essential for preventing oxidative damage to the reproductive system and/or sperm (Baer et al. 2009; Azevedo et al. 2012). Metazoans use intermolecular disulfide bonds to oligomerize proteins during sperm chromatin compaction. It has been shown that during fertilization, protamines' intermolecular disulfide bonds are broken down and expelled from sperm via the thioredoxin deadhead factor in *D. melanogaster* (Emilyanov, Fyodorov 2016).

Serine proteases are a class of enzymes found in bacteria, viruses, and eukaryotes. They account for more than one-third of all proteases. These proteases are largely β-protein structures, but they also contain three amino acid residues: Ser, His, and Asp, in their active sites, whose sequences have differed greatly over evolution (Betzel et al. 2001). For apoptosis, cell differentiation, embryonic development, reproduction, and immunity in insects, serine proteases are essential (Yang et al. 2017; Lee et al. 2018).

In the SF of *Drosophila simulans*, serine proteins control proteolysis (Mueller et al. 2004). Odorant-binding proteins (OBPs) are small soluble proteins which bind odorants and pheromones, serving as carrier molecules to transport them to insect receptors (Dong et al. 2016; Cai et al. 2021). They are found in the pheromone glands (Pelosi et al. 2014). Numerous investigations have demonstrated that OBPs are present in male accessory glands (MAGs) of diverse insect orders (Mamtha et al. 2019; Saraswathi et al. 2021).

The rate of female re-mating determines the level of sperm competition in a population. How sperm competition results in selection depending on the distribution of male ejaculates has been extensively studied. A multitude of factors such as fertility, mortality and the mating process itself impact female fitness. When re-mating simultaneously, females are more fit because they are selected to mate more frequently, which increases sperm competition and favours traits that provide them an edge over competitors. Genes involved in reproduction have a tendency to evolve quickly and exhibit positive selection because they enable detection of food or predators in the environment. Also, the genes are linked to chemoreception (olfactory), this information precisely regulates social and sexual interactions between members of the same species. Olfactory information includes the detection of odours and pheromones essential for survival and reproduction. These tasks are performed by the DBI and OBP proteins in *H. armigera*. We assessed this theory of fast evolution of genes by computing the ratio of neutral mutations and purifying selection to beneficial mutations acting on these two proteins using Ka/Ks substitution. The results for the OBP and DBI proteins (Ka/Ks values of 1.72 and 0.87, respectively) suggested that positive selection could be altering the genes.

According to the phylogenetic tree, the paralogs of the FAR gene have undergone a variety of evolutionary events, such as duplication, in order to aid in certain reproductive functions within their respective species. Knowledge of the genes and enzymes involved in pheromone synthesis is necessary to comprehend the mechanisms underlying the specificity of pheromone signals. In an examination of ACB in closely similar heliothine species, different pheromone mix ratios were dependent on the proportion of the precursor (Hagström et al. 2012). Furthermore, as there are no known orthologues of heliothine or in other arthropods or mammals, the study demonstrated that

moths have evolved and recruited a single, distinct group of FARs for the exclusive purpose of producing pheromones. Collectively, these results underscore the importance of ACB gene in the pheromone synthesis range of insects, as evidenced by its high degree of amino acid residue conservation.

Unique properties, biological qualities, and functions of a protein are determined by its structure. It also assigns unique features related to variety of physiological processes and molecular interactions. Most of the proteins identified in this study have unique, specialized domains that allow them to perform specific reproduction related functions. Structural analysis of a typical SFP protein suggested that the hydroxyproline-rich mid-section of SP elicits the innate immune response (Domanitskaya et al. 2007), the disulfide bridge-containing C terminus stimulates the core post-mating responses of increased oviposition and decreased sexual receptivity (Schmidt et al. 1993; Ding et al. 2003; Moehle et al. 2011), and the tryptophan-rich N terminus binds to sperm and stimulates JH synthesis (Moshitzky et al. 1996; Fan et al. 2000; Peng et al. 2005). The thioredoxin protein and the odorant-binding protein have cysteine residues which facilitates sperm-oocyte interaction, pheromone-mediated signaling, oxidative damage, and protease activity. However, other proteins possessed distinct and specialized domains. Fig. 11 depicts the phylogenetic relationship of the MAG proteins based on their functions: OBP and EF1 protein are grouped in a clade with serpin as outgroup, thioredoxin and DBI as a group and heat-shock protein as a single clade. Cumulatively, the appearance of these classes of proteins in *H. armigera* highlights their role in insect PMR.

The significance of the non-sperm components of male SF is becoming increasingly recognized by several researchers in PMR and insect reproduction. Understanding the roles of these proteins requires an understanding of their structure-function relationship, which could determine their physiological functions. Distinct protein structures were observed in each of the proteins, which may account

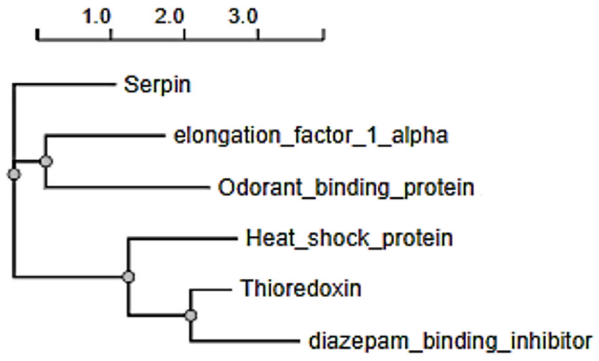

Fig. 11. Phylogenetic tree (PhyML) of SFP proteins identified in the study drawn using https://ngphylogeny.fr/. The proteins are grouped based on their protein structure and functions.

for their biochemical and physiological roles in insect reproduction. A coiled-coil domain is made up of two to seven alpha helices, known as trimers and dimers that are wound together like a rope strands (Martinez-Goikoetxea, Lupas 2023). Large macromolecular complexes can be nucleated and scaffolded by these proteins. Proteases and serpin proteases combine to generate covalent complexes. Serpins are made up of many beta and alpha strands as well as an exterior reactive centre loop that houses the active site that the target enzyme recognizes (Stawiski et al. 2000). The intact ACB domains of the DBI protein are its defining feature. These domains been found in several big, multipurpose proteins found in several eukaryotic species (Islinger et al. 2020). The four alpha helices that make up the acyl-CoA-binding site of the ACB domain are recognizable and positioned prominently. The ligand is bound via specific interactions with residues in proteins. The FAD-binding domain was present in the thioredoxin protein (Fraaije et al. 1998). The oxidoreductase FAD-binding domain is an evolutionary conserved protein domain. Numerous biological processes are regulated by these proteins (Chakraborty et al. 2022). Odorant proteins contain the chain GTPases domain. These proteins function as molecular switches regulating the switch between an inactive GDP-bound state and an active GTP-bound one. Elongation factor 1-alpha partially contained the tr-type G domain. The basic structure is made up of five α-helices encircling a core β-sheet with six strands.

Chemosensory mechanisms and pheromone-based communication are essential to the richness and diversity of Lepidoptera, the second largest order of insects. Virgin male sperm and auxiliary reproductive glands produce SFP peptides/proteins when they are activated or released at appropriate time intervals. These proteins, many of which are evolving quickly, are essential for male fertility. Male *D. melanogaster* flies have been shown to intentionally alter their SFP levels in response to different forms of competition (Fedorka et al. 2011), also in response to changing environmental factors like age or mating frequency (Koppik, Fricke 2017). Finally, males reduce SFP release and instead utilize SFPs from previous mates to minimize reproductive costs (Gioti et al. 2012). Of the several proteins identified, the DBI protein was unique to *H. armigera*, whereas other proteins have been reported in other insects. In the metabolism of fatty acids and the control of genes, acyl-CoA enzymes act as regulators of intermediates. Biochemical and structural studies of these ACP proteins suggest extensive conservation at various residues in the protein, suggesting that they have important reproduction-related functions and perturbations could lead to reduced reproductive fitness. Extrapolating findings from the current investigation of female unreceptivity on the second day and subsequent receptivity on the fifth day, since this period involves increased lipid metabolism, it could be proposed that the DBI protein could facilitate

this crucial process during mating pauses and egg-laying. Recent studies on humans and mice support the roles of ACBP as a unique "hunger reflex" protein that efficiently increases lipogenic processes and appetite (Pedro et al. 2019). Furthermore, due to its neuropeptide nature, it may influence the pheromone pathway and subsequent signaling cascades mediated by pheromones, including unreceptivity and calling cessation.

Male and female moths' ACBP expression is up-regulated the day before eclosion and happens concurrently in the PG (Matsumoto et al. 2001). The information suggests that ACBP proteins play a major role in the physiological changes brought on by pheromones in female moths. In summary, these several lines of data indicate a significant involvement of ACBP proteins in pheromone-mediated physiological modifications in female *H. armigera* moths. Heat shock proteins, which aid in the folding and stability of proteins, are typically produced in response to stress. HSP 70 is positively connected with age and reproductive diapause (Kankare et al. 2010) and prolongs the life span of *D. melanogaster* (Le Bourg et al. 2001). Lastly, it has been proposed that HSP 90 expression controls JH-inducible gene expression in *H. armigera*, indicating yet another possible pathway for HSP family-mediated reproductive regulation. EF-1 alpha is essential for controlling apoptosis and protein synthesis. It interacts with the actin and ubiquitin-dependent proteolysis pathway. Increased expression of EF-1 alpha during stressful events has been linked to a prolonged lifespan (Talapatra et al. 2002; Wang et al. 2004). Thioredoxin are a class of antioxidant protein that protects against oxidative damage. Furthermore, the oocyte-to-embryo transition, which occurs in the absence of transcription, requires post-transcriptional and post-translational regulation.

Redox proteins offer an additional extremely dynamic layer of regulation that modifies this crucial stage (Petrova et al. 2018). The involvement of thioredoxin system in modifying sperm chromatin appears to have been conserved across evolution. Serine proteases are extensively distributed enzymes that are necessary for the catalysis of both extracellular and intracellular hydrolytic activities (Yang et al. 2017). Serpins are involved in hormone storage, transport, and insect immunity, among other physiological functions. Serpins are structurally conserved proteins with metastable characteristics that act as suicide substrates (Huntington 2011). In the silkworms *B. mori* and *Plutella xylostella*, serine protease 2 is a component of SF, and reductions in its expression cause male sterility (Xu et al. 2020).

OBPs are essential parts of the insect olfactory system that are required for host recognition, mating, and oviposition (Zhang et al. 2018). In conclusion, the data presented above points to a potential involvement of these proteins in gametogenesis, insect fertility, and associated reproductive phenomena. Some limitations of the study include differences in the life cycles of various animals. Furthermore, genetic pleotropic effects (autosomal dominant, additive, and recessive) resulting from intra-species variability may influence female re-mating and receptivity. Further characterization of the N and C terminals, biophysical structure, examining mutation(s), and identifying homologous receptors will enable elucidation of their roles in female reproduction.

The evolution of the female re-mating interval has a direct effect on sperm competition and could affect the selection of male and female traits associated with mating, sperm competition, and conception. Thus, it is imperative to imagine how male SFP affects female mating choices and other reproduction-related aspects. The findings of the present investigation imply that these traits are significantly influenced by the SFP of male moths.

In conclusion, through biochemical analysis we identify a 5 kDa molecular weight peptide. Our database search identifies six class of reproduction related proteins conserved proteins belonging to members of multigene families. The diazepam-binding inhibitor protein was unique to *H. armigera*. Genes from gene families linked to insect reproduction could have evolved under various selection pressures according to the prevailing views in evolutionary biology. In the current study, almost all of the proteins were included in this group. Notable is the evolution of the FAR and OBP multigene families. Due to gene duplication events, the FAR and OBP genes in insects have experienced significant expansions in comparison to those in plants and mammals and have evolved through birth-death evolution (Antony et al. 2016).

Acknowledgements

We thank the NBAIR, Bengaluru, for providing insect culture, Department of Crop Physiology, University of Agricultural Sciences, Bengaluru, for providing RP-UFLC facility, and IISc and C-Camp, NCBS, Bengaluru, India, for extending mass spectroscopy facilities. Rama Thyloor wishes to express her gratitude towards Department of Microbiology and Biotechnology, Bangalore University, and UGC, Department of Collegiate Education, Department of Higher Education, Government of Karnataka for permitting me to carry out research as Teacher Fellow under FDP program. Our sincere thanks to Vice Chancellor, Nrupathunga University for the encouragement and for providing logistic space for manuscript drafting and associated work.

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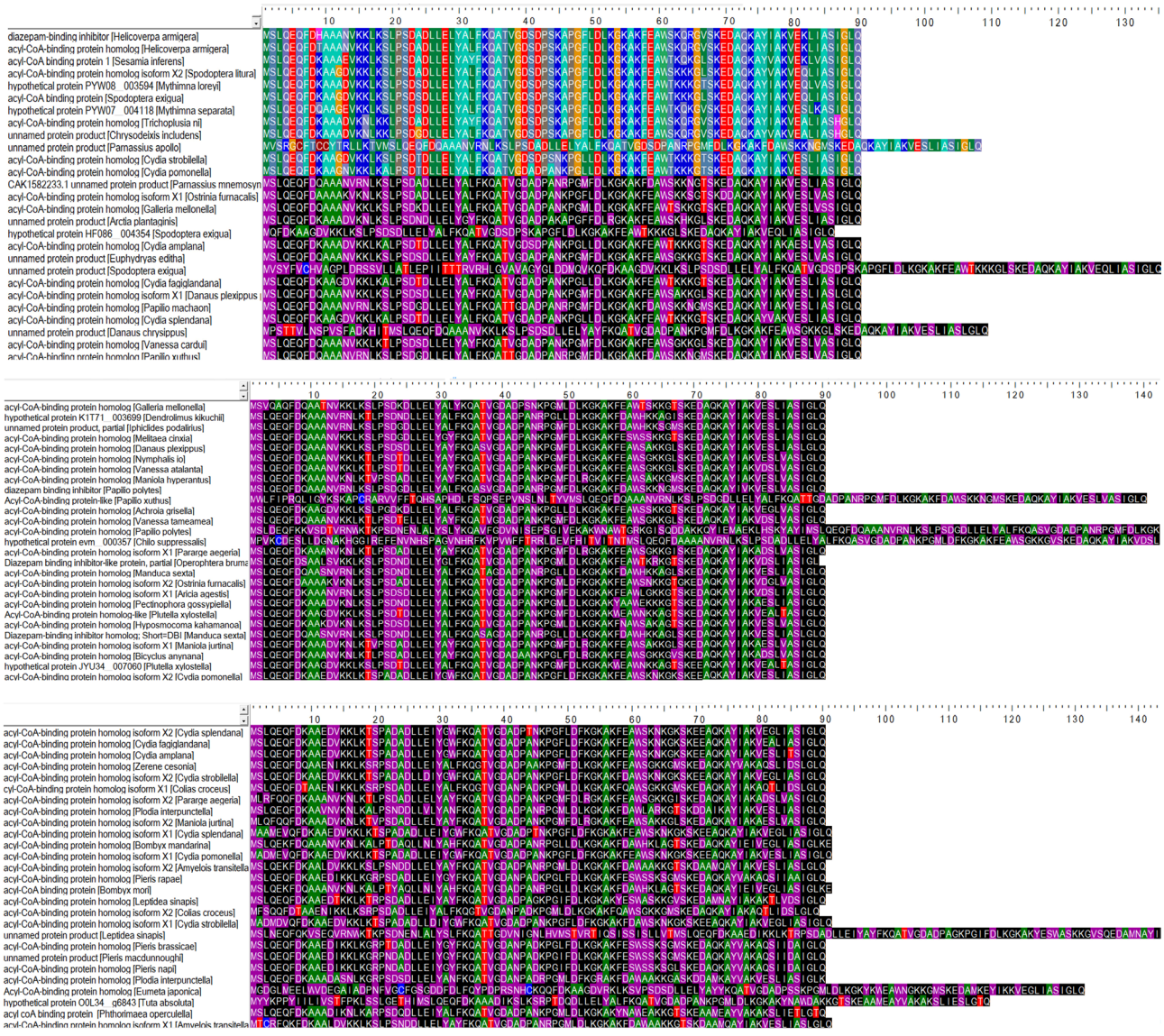

Fig. S1. ClustalW of DBI protein across insect taxa showing conserved amino acids.

Received 29 September 2024; received in revised form 28 October 2024; accepted 30 October 2024