A Comparative Protein Profile of Virgin and Mated Male of Leucinodes Orbonalis Accessory Gland

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Abstract

Male accessory gland (MAG) proteins that are secretory in nature delivered to females, along with sperms affects female reproductive physiology and behavior. In our study, proteomic approaches were employed to identify the MAG proteins of *L. orbonalis*, a monophagous and most destructive pest on brinjal. A set of 117 spots in virgin MAG and 186 in mated MAG were obtained from 2-D gel electrophoresis. The differentially expressed MAG proteins after mating in comparison with the virgin male were 14 upregulated and 16 down-regulated. We have used MALDI- MS to identify the 13 unique proteins within the virgin MAG of the *L. orbonalis* and analyzed with the Swiss-Prot database using a Mascot search engine. The proteins were identified as proteolysis regulators, lipid transporter, olfactory protein, metabolism, DNA binding, and hexamerins. This is the first report on proteome analysis of MAG of the *Leucinodes orbonalis*.

Introduction

The reproductive proteins are the most evolutionarily divergent proteins among invertebrates and vertebrates ranging from gastropods (Metz et al., 1998; Riginos et al., 2006) to insects (Haerty et al., 2007) to mammals (Swanson et al., 2001; Clark & Swanson, 2005; Karn et al., 2008). These reproductive proteins generally, referred to as seminal fluid proteins (SFPs), in many insects, are secreted by the male reproductive system, including the testis, seminal vesicles, and the male accessory glands (MAG) (Xu, et al., 2013). Researchers have identified hundreds of SFPs in different orders of insects such as Diptera, Lepidoptera, Hymenoptera, Coleoptera, Orthoptera, and so on (Avila et al., 2011). By far in the best-studied system, *Drosophila melanogaster*, over 150 proteins have been identified, especially those produced in male accessory glands and transferred to females during mating (Avila et al., 2011, 2015a; Findlay et al., 2008, 2009; Yamamoto and Takemori, 2010). The accessory gland proteins (ACPs) transferred along with the sperm to females (Gillott, 2003; Ram and Wolfner, 2007) play an important role in sperm capacitation, storage, competition, and fertilization, thereby modulate the female post-mating behaviour and physiology (Mcgraw et al., 2015; Perry et al., 2013; Hopkins et al., 2017; Sirot et al., 2015; Rodríguez-Martínez et al., 2011; Avila et al., 2011; Sirot and Wolfner, 2015). For example, in *D. melanogaster*, the well-known ACPs ovulin (Acp26Aa) and sex peptide (Acp70A) both stimulate egg production. Ovulin induces the release of mature oocytes, whereas Acp70A stimulates egg production for several days after mating (Herndon et al., 1995; Heifetz et al., 2000; Chapman et al., 2003). The ACPs, which were studied by the evolutionary biologists for long, have generated interest among computational biologists recently owing to their significant role in reproduction. With the advancement in technology, the identification of these proteins is gaining momentum.

Currently, the most effective methods such as genomics, proteomics, custom microarray, RNA interference and a combination of two or more of these methods are useful and powerful tools for the identification and characterization of ACPs in various insects (Findlay et al., 2009; Wigby et al., 2009; Parthasarathy et al., 2009). Two-dimensional gel electrophoresis (2-DE) is routinely used for the analysis of complex protein mixtures, including whole-cell and tissue lysates (Gorg and Dunn, 2004). Several researchers have identified proteins from the accessory glands of males such as the proteases/protease inhibitors (LaFlamme & Wolfner, 2013), hexamerins (Burmester, 1999), olfactory proteins (Rafaeli & Hanin, 2013), defense/immunity proteins (Wei et al., 2015) and so on that were reported to be conserved across a wide taxonomic range of insect species. Using 2-D gel electrophoresis, in *Drosophila melanogaster*, a total of 440 reproductive organ proteins were identified, and among those, 129 were from the accessory glands (Takemori, & Yamamoto, 2009). In *Spodoptera litura*, Mamtha et al. (2019) reported a total of 566 distinct proteins and 91 differentially expressed in virgin and mated males. Another study in *Callosobruchus maculatus* MAG proteome showed that ≥ 127 proteins were found to be transferred to females at mating (Goenaga et al., 2015). However, proteins present in the reproductive system of several species are still uncharacterized as their encoding genes often rapidly diverge among different species. In the present study, we have used 2-DE along with Mass spectrometry (MS) for the comparative protein profile between the virgin and mated male MAG of *L. orbonalis.*

Leucinodes orbonalis Guenee (Lepidoptera: Crambidae) is a monophagous and most destructive pest of brinjal (Latif et al., 2010; Chakraborti, & Sarkar, 2011; Saimandir, & Gopal, 2012; Kariyanna et al., 2019), an essential annual vegetable in South and South-East Asia (Thapa, 2010). Recent reports displaying the production of brinjal cultivation in 2018 revealed that 1.87 million ha were cultivated in the world for a total output of 51.28 million tonnes, of which 62% and 24% of the world production were covered by China and India respectively (FAO, 2016). However, there is a downslide in the production due to the infestation by *L. orbonalis*, with yield loss up to 85 to 90% in India (Jagginavar et al., 2009). The pest starts damaging the crop soon after transplanting of the

seedlings and continues till the harvest of fruits (Nishad et al., 2019). As the egg hatches, the larvae mine young shoots and fruits of the brinjal, damaging the plant as well as the fruit (Son et al., 2016; Misra, 2008; Jagginavar et al., 2009). The pest is controlled by the use of heavy doses of insecticides such as organophosphates, carbamates and synthetic pyrethroids (Alam el al., 2003). The indiscriminate use of chemical pesticides in brinjal cultivation enhances the accumulation of residues in brinjal fruits, creates adverse effects on natural enemies, encourage pest resistance, secondary pest outbreak, health hazards and environmental pollution (Srinivasan, 2008; Bhadauria et al., 1999). Thus, the present trend of pest management moves towards the development of eco-friendly bio-pesticides wherein the employability of insect's own products that modify the behaviour of the insects to control the pest insects being investigated. Hence, the present study was conceptualized to identify the MAG proteins as they can be potent molecules employable to control the pests in the future.

Materials And Methods

Rearing Of Insects

Sample collection was achieved by collecting infested brinjals in and around Hesaraghatta, Bengaluru, Karnataka, to maintain a stock of wild population in the laboratory. *L. orbonalis* (Strain accession no. **MK422611**) was mass-reared at room temperature on potatoes washed with 4% sodium hypochlorite according to the protocol described by Mannan et al. (2015) until pupation. After adults' emergence, males and females were segregated and fed with 10% honey solution until dissection.

Sample Preparation

Approximately 50 MAG tissues were collected from virgin males dissected two hours after eclosion and from males mated for an hour. The tissues were homogenized in 100 µl sterile Milli-Q water and centrifuged at 10,000 rpm for 15 minutes at 4 ºC. The supernatants were stored at -80 ºC for further analysis.

Protein Separation By Two-dimensional Electrophoresis (2de)

Tissue samples containing 300 µg of total protein was diluted in rehydration buffer (8M Urea, 4% CHAPS, 40 mM Dithiothreitol (DTT), 2% Bio-lyte, pH 3–10; Bio-Rad) and incubated for 12 hrs at 25–28 ºC. After incubation prior to first dimension gel electrophoresis, the samples were centrifuged at 10,000 rpm for 15 minutes. The supernatants were loaded onto an 18 cm linear immobilized pH gradient strip (pH 3–10) and run at 250 V for 15 minutes and 10000 V for 3 hrs using an Ettan IPGphor3 system (GE Healthcare). Following the isoelectric focusing (IEF), strips were incubated twice for 10–15 minutes in equilibration solution (6M Urea, 75 mM Tris-HCl pH-8.8, 2% (w/v) SDS, 29.3% (v/v) Glycerol, 0.002% bromophenol blue) and for an additional 15 minutes in 1% DTT and 2.5% Iodoacetamine. Further, the IEF strips were transferred onto a polyacrylamide gel, and the second dimension (10% SDS-PAGE) was run according to Laemmli (1970) overnight at 70 volts using Ettan Daltsix Gel Electrophoresis system (GE Healthcare). The standard protein ladder (10–250 kDa, Bio-Rad) was also loaded on the gel to identify the molecular size of protein spots. After completion of electrophoresis, each gel was incubated overnight in fixing solution (50% methanol and 5% acetic acid in distilled water) and further rinsed with autoclaved distilled water several times for about 30 minutes. The gel was silver-stained according to the protocol described by Shevchenko et al. (1996).

Image Analysis

The silver-stained gels were documented using the Epson Expression 11000XL Scanner and subsequently analyzed with Image Master 2D Platinum 7 Software (GE Healthcare). The spot detection was automatically performed using the software algorithm, and the spots were verified. Each protein spots were carefully inspected in detail, based on peak height and 3D visualization. The matched spots of the mated and virgin gels were compared with each other to obtain differentially expressed proteins. Further, the selected unique protein spots from the virgin MAG gel were subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) for peptide mass fingerprinting (PMF) analysis.

Trypsin Digestion And Mass Spectrometry

For protein identification, selected spots were excised from the freshly silver-stained gel and rinsed twice with water (MS grade). The gel piece was minced into small pieces and transferred to a sterile micro-centrifuge tube followed by de-staining in a 1:1 ratio of 15 mM potassium ferricyanide and 50 mM sodium thiosulphate for 15 minutes with vortexing until the gels became translucent. The de-stained gel samples were washed with 25 mM ammonium bicarbonate solution and dehydrated by acetonitrile (HPLC grade) followed by drying using Speed Vac. The samples were incubated for an hour at 55 ºC in 100 mM dithiothreitol (DTT) and then 55 mM iodoacetamide (IAA) for 45 minutes to reduce and alkylate the proteins respectively followed by centrifugation at 10000 rpm. After discarding supernatant containing IAA, the gel was incubated with 25 mM ammonium bicarbonate solution for 10minutes. Further, the gel spots were rinsed with acetonitrile and dried in the Speed Vac. Proteins were digested with trypsin (12.5 ng/µl in 25 mM ammonium bicarbonate) by incubating overnight at 37 ºC (Shevchenko et al., 1996). After trypsinization, the peptides were extracted thrice with extraction buffer (50% acetonitrile, 1% trifluoroacetic acid v/v). The pooled extract was dried using the Speed Vac before MS. For protein identification, the dried samples were re-suspended in TA solvent solution (30:70 [v/v] acetonitrile: 0.1% TFA) and mixed with a saturated α-cyano-4-hydroxycinnamic acid solution (HCCA) in 1:1 ratio. In the MALDI target plate (MTP 384 ground steel, Bruker Daltonics, Germany) 2 µl sample was spotted. The samples and matrix were left to dry before peptide analysis and MALDI-TOF/TOF MS measurements were carried out using an Ultraflex III TOF/TOF device and its associated software (Bruker Daltonics, Germany). The spectra were acquired in positive reflector mode using the Auto Xecute option of the Flex Control software version 3.3. External calibration was done with standard peptide (Pepmix Mixture, Bruker Daltonics, Germany) with masses ranging from 1046 to 3147 Da. The MS or peptide mass fingerprint (PMF) spectra in the range of 500 to 5000 m/z with 500 laser shots for mass detection were recorded. Further, processing and peak picking of the spectra was carried out automatically using Flex analysis software (version 3.3. Bruker, Daltonics). The most intensive peaks were chosen for MS/MS product ion spectra (LIFT spectra) measurements in the laser-induced dissociation mode.

Protein Database Search

The generated data from MS was submitted to the MASCOT search engine (http://www.matrixscience.com) using the SwissProt database (2016_11 553231 sequences; 197953409 residues) against the Lepidoptera taxonomy (Moths_Butterflies, 665 sequences). The protein scores greater than 41 were considered as significant ($p < 0.05$) and the search parameters were set as follows: Trypsin digestion- up to two missed cleavage; fixed modifications-carbamidomethyl (C); variable modifications-oxidation(M); peptide tolerance: ±300 ppm; MS/MS tolerance: ±0.6 Da; peptide charge:1 + and mass values: monoisotopic.

Signal Peptide Prediction

The presence of signal peptide for the identified proteins was predicted using SignalP 4.1(http://cbs.dtu.dk/services/SignalP) (Petersen et al., 2011).

Results And Discussion

Two-Dimensional Gel electrophoresis involves the separation of complex protein mixtures based on pI and molecular weight, enabling the identification of different protein isoforms and post-translational modifications (Magdeldin et al., 2014; Oliveira et al., 2014; Pomastowski and Buszewski, 2014).

The 2DE profiles of virgin and mated males of *L. orbonalis* revealed (Fig. 1 and Fig. 2) 117 spots in virgin MAG and 186 in mated MAG with a molecular mass ranging from 15–84 kDa and 22–241 kDa respectively (Supplementary table. 1). The differentially expressed MAG proteins following mating were 14 upregulated and 16 down-regulated. More number of proteins in mated male MAG compared to virgin suggests the synthesis of new proteins during mating whose function or purpose is not reported in any of the insects. However, similar results were observed in the MAG proteome of *S.litura* (Mamtha et al., 2019).

The high intense 13 spots unique to virgins were excised, digested with trypsin subjected to MS, and identified using a Lepidoptera (Moths_Butterflies) taxonomy (Table 1). The identified proteins were grouped, according to their functions, as proteolysis regulators, proteins involved in lipid transporting, olfactory protein, metabolism-related, DNA binding, and hexamerins. As the spots were unique

to virgins, not found in mated moths, it was assumed that these proteins are probably transferred from male to female during mating. However, among the 13 identified proteins, eight proteins showed the presence of a secretory signal indicating the proteins encoded by these genes are destined for secretion predicting their possible transfer to females during mating (Table 2). Further, we discuss the possible functions of all 13 proteins based on the information available in other insects.

Table 1
The identification of proteins in the MAGs of *Leucinodes orbonalis* by 2D gel electrophoresis and mass spectrometry (MALDI-TOF MS) and Secretory signal prediction

SL. No.	Spots ID	pI	Molecular weight (kDa)	Spot intensity (% Vol.)	Accession No.	Protein	Organism	PMF Score	SignalP Prediction
1	1096	4.95737	15	0.45006	TRF_MANSE	Transferrin	*Manduca sexta*	69	YES
2	1091	7.91772	18	4.51543	VITM_MANSE	Microvitellogenin	*Manduca sexta*	112	YES
3	1092	7.5	18	4.90496	*PCNA_BOMMO*	Proliferating cell nuclear antigen	*Bombyx mori*	92	NO
4	1093	7.66265	18	0.82205	*PBP_BOMMO*	Pheromone-binding protein	*Bombyx mori*	64	YES
5	1125	7.10306	18	1.71525	*SSP2_BOMMO*	Sex-specific storage-protein 2	*Bombyx mori*	139	YES
6	1086	7.39584	21	1.78008	*SNMP1_HELAU*	Sensory neuron membrane protein 1	*Helicoverpa assulta*	48	NO
7	1129	6.7033	21	0.528896	*TPIS_HELVI*	Triosephosphate isomerase (Fragment)	*Heliothis virescens*	87	NO
8	1080	7.44088	23	0.127166	*SPI3_LONON*	Serine protease inhibitor 3/4 (Fragment)	*Lonomia obliqua*	99	YES
9	1075	7.19033	32	2.35006	*APLP_MANSE*	Apolipophorins	*Manduca sexta*	49	YES
10	1044	7.0918	67	2.03135	*C1TC_SPOFR*	C-1-tetrahydrofolate synthase, cytoplasmic	*Spodoptera furgiperda*	127	NO
11	1041	7.16781	74	2.3121	SPI3_LONON	Serine protease inhibitor 3/4 (Fragment)	*Lonomia obliqua*	127	YES
12	1019	7.04957	78	1.36198	*APLP_MANSE*	Apolipophorins	*Manduca sexta*	44	YES
13	1017	7.09743	79	2.72218	*BJSB1_TRINI*	Basic juvenile hormone-suppressible protein	*Trichoplusia ni*	50	YES

Table 2
Classification of identified MAG proteins based on the literature

Summary of predicted function	Protein name	Reference
Proteolysis regulators	Serine protease inhibitor 3/4 (Fragment)	Stephens et al., 2018
Olfactory proteins	Sensory neuron membrane protein 1	Shan et al., 2019
	Pheromone-binding protein	Findlay et al., 2008
Lipid transporting functions	Apolipophorins	Simmons et al., 2013
	Transferrin	Bonilla et al., 2015
	Microvitellogenin	Liu, et al., 2015
Metabolism	Triosephosphate isomerase (Fragment)	Rafaeli & Hanin, 2013
	C-1-tetrahydrofolate synthase, cytoplasmic	Chen et al., 2018
DNA binding	Proliferating cell nuclear antigen	Shikina et al., 2018
Hexamerins	Sex-specific storage-protein 2	Rafaeli & Hanin, 2013
	Basic juvenile hormone-suppressible protein	Rafaeli & Hanin, 2013

Proteolysis Regulators

Two proteins belonging to a family of conserved proteolysis regulatory group of proteins were identified as serine protease inhibitor 3/4 (Fragment) of different molecular weights and pI in the MAGs of *L. orbonalis.* Serine protease inhibitors are crucial in setting off several reproductive functions in insects and have been reported in the MAG of honeybees, mosquitoes, beetles, field crickets, and oriental fruit fly (Dottorini et al., 2007; Baer et al., 2009; Simmons et al., 2013; Xu et al., 2013; Wei et al., 2015; Bayram et al., 2017). These proteins have also been detected in the female sperm storage organs of *Crematogaster osakensis* (Gotoh et al., 2017b) and *D. melanogaster* (Allen & Spradling, 2008; Prokupek et al., 2008). In MAG of *Culex pipiens*, serine protease inhibitors were reported to play a role in downregulating protease activity before the transfer of sperm and semen to the female reproductive tract (Stephens et al., 2018). While others have also suggested their role in stimulating ovulation (Heifetz & Rivlin, 2010; LaFlamme et al., 2014), egg-laying (Marshall et al., 2009), decreasing receptivity to remating (Peng et al., 2005) and controlling the release of sperms (Avila et al., 2010). Likewise, we also speculate that these proteins identified in the MAGs may be transferred to females influencing their reproductive behavior as they were present in virgin males and absent in mated males moreover showed secretory signals.

Olfactory Proteins

Two proteins involved in olfaction, namely sensory neuron membrane protein1 (SNMP1) and pheromone binding protein (PBP)/odorant binding protein (OBP), were identified in *L. orbonalis*. SNMPs, localized in the peripheral olfactory system of insects, have been suggested to play a role in odorant perception (Rogers et al., 2001a, b; Vogt, 2003; Benton et al., 2007). Most of the insects rely on their sensitive antennae to perceive semiochemicals in the complex environment and contribute enormously to survival and reproduction (Ache & Young, 2005; Su et al., 2009; Sachse & Krieger, 2011; Leal, 2013; Shan et al., 2019). Several SNMPs that play an important role in male chemoreception have been identified in the lepidopteran and dipteran moths (Benton et al., 2007; Vogt et al., 2009; Gu et al., 2013; Liu et al., 2013; Liu et al., 2014; Zhang et al., 2015). For instance, the antennae of *Microplitis mediator* expressed SNMPs that are important for sex pheromone recognition (Shan et al., 2019). Similarly, in *Drosophila*, SNMP1 essential for sex-pheromone recognition (11-cis-vaccenyl acetate) was expressed mainly in the trichoid sensory cilia of the antennae (Benton et al., 2007; Jin et al., 2008).

The assumption that proteins present in virgins and missing in mated are possibly transferred to female does not hold good for SNMP1 identified in virgin and absent in mated male as several studies indicate its role in recognition of sex pheromone in male insects. Moreover, the protein has no signal peptide sequence. Possibly the protein disappears following mating, having performed its function of locating a mate.

Another identified protein is the Pheromone Binding Protein. PBPs are essential components of the insect olfactory system and are vital for the recognition of hosts, mating, and oviposition in insects (Zhang et al., 2012). They belong to a sub-class of odorant-binding proteins (OBPs) and are primarily meant to bind and transport the sex pheromones in insects (Liu et al., 2013; Pelosi et al., 2006; Senthilkumar, & Srinivasan, 2019). Evidences are showing the presence of PBPs/ OBPs in SFPs/MAGs of several insects, including *D. melanogaster* (Takemori &Yamamoto, 2009), (Findlay et al., 2008), *Tribolium castaneum* (South et al., 2011), *Helicoverpa armigera* and *Helicoverpa assulta* (Sun et al., 2012). These researchers have also suggested, the proteins are to be transferred to females during mating. In *H. armigera* and *H. assulta*, PBPs were identified on the surface of eggs that act as an oviposition deterrent and facilitates the scattering of eggs (Sun et al., 2012). In the MAGs of *Bactrocera dorsalis*, Wei et al. (2015) reported the transfer of PBPs to the females wherein it changes the reproductive physiology and behaviour by interacting with its specific receptors. Similarly, in *Bombyx mori*, Dong et al. (2016) identified four odorant-binding proteins from MAG and described their potential role as signaling molecules affecting female physiology and behaviour. In *L. orbonalis*, there is a possibility that it is transferred to females and involved in her post mated activities.

Lipid Transporting Function

The proteins with lipid transporting functions such as apolipophorins, transferrin, and micro-vitellogenin were identified in virgin MAG, and all are secretory proteins. Lipophorins are insect proteins, which are dispersed in the hemolymph and involved in lipid transport, acting as a shuttle of lipids between different tissues (Ryan & Horst, 2000). Sevala et al. 1997, (1999) demonstrated in *Blattella germanica* revealed that lipophorins also play a role as juvenile hormone (JH) binding protein and suggests that it may contribute to the regulation of JH titers in the hemolymph. It was also suggested that in case of two triatomine species, lipophorins play a role as yolk protein, being endocytosed by the oocytes, stored in yolk bodies and used to support embryonic development (Kawooya and Law, 1988; Ziegler and Van Antwerpen, 2006; Fruttero et al., 2011, 2017; Leyria et al., 2014).

In seminal fluid of *T. oceanicus*, apolipophorin has been shown to be the mediator of antibacterial immunity in the protection of sperm from bacterial attack or protect females from bacteria that may be introduced to their reproductive tracts during copulation (Simmons et al., 2013). The apolipophorin found in virgin MAG of *L. orbonalis* may also involve in the similar function of antimicrobial activity to protect either sperms or eggs in the female system.

Many reports have shown that transferrin is a multitask secretory glycoprotein that plays a vital role in iron transport, immunity, development, stress responses and juvenile hormone regulation in many insects (Geiser and Winzerling, 2012; Ampasala et al., 2004; do Nascimento et al., 2004; McCarthy et al., 2018). Sirot et al., (2008) and Hagai et al., (2007) reported the presence of transferrin in the male reproductive tract of blood-feeding insects and there are more evidences for their existence in the MAG (Simmons et al., 2013) including in *Teleogryllus oceanicus* and *Melanoplus sanguinipes* (Bonilla et al., 2015). In a study on *Bactrocera dorsalis* proteome of the testis, Wei et al. (2018) reported that transferrin is found to be abundant. It could play a role in testis development, spermatogenesis, and sperm formation. The properties such as sperm numbers, sperm motility, and male fertility are correlated to the quantitative variation in transferrin in the seminal plasma which suggest the possible role of transferrin in sperm capacitation (Ber et al., 1990; Ashrafzadeh et al., 2013; Frenette et al., 2004). One more example in fish, suggests that transferrin has a role to play in the nonspecific humoral defence of sperm against bacterial infection (Stafford & Belosevic, 2003; Jurecka et al., 2009), and it can offer protection of sperm from heavy metals toxicity (Dietrich et al., 2011). The transferrin chelates the iron and, in turn, controls lipid peroxidation in sperms, and thus the sperm is protected from oxidative damage (Sanocka & Kurpisz, 2004). Hence, the protective role of transferrin and its reproductive related functions might explain the possibility of its transfer to females along with sperm during mating.

Micro-vitellogenin is known as low molecular weight lipophorin protein (Maenaka, & Park 2018) is well documented in several insect species, such as *A*ntheraea *pernyi, Madoca. sexta, Helicoverpa zea, S. litura, Apis mellifera, Lymantria dispar, Amsacta albistriga* (Liu et al., 2015; Chen, & Yamashita, 1990; Raman, 2013). Similar to vitellogenin, micro-vitellin is also synthesized by the fat body virtually in females, secreted into the hemolymph, and then taken up by the maturing egg for egg development (Paes de Oliveira et al., 2012; Sato & Yamashita, 1991). The micro-vitellogenin protein from MAG of *L. orbonalis* may also possibly transferred to females while mating and facilitate egg development.

Metabolism

The triosephosphate isomerase (TPI), and C-1-tetrahydrofolate (C1-THF) synthase cytoplasmic identified in MAG of *L. orbonalis* belongs to the metabolism functional group. Both proteins are non-secretory in nature. Several reports on Triosephosphate isomerase are from higher animals. It is a glycolytic enzyme that plays a crucial role in sperm metabolism and leads to the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3- phosphate (Wierenga et al., 2010). Sun et al. (2018) profiled the sperm of mice and identified triosephosphate isomerase suggesting its role in sperm motility and being an important enzyme of glycolytic pathway that provides ATP required for motility of the sperm (Danshina et al., 2010; Vilagran et al., 2016). Previous studies demonstrated that TPI is located in the sperm head and is involved in sperm binding to zona pellucida (Auer et al., 2004; Petit et al., 2013). Another study in boar shows that triosephosphate isomerase 1 gets activated during capacitation and plays an important role in providing energy for sperm motility (Bailey et al., 2000).

Rafaeli & Hanin (2013) identified triosephosphate isomerase in the MAG of *H. armigera*, and assign the function as metabolism based on protein database. In the absence of direct evidence, we assign a similar task to this protein even in *L. orbonalis*.

One more protein belonging to the metabolism group is C1-THF synthase, a central enzyme for folate metabolism, protein synthesis, DNA repair, methylation, as well as the oxidation-reduction process (Cossins, 2000). Recently, Chen et al. (2018) showed that BmC1-THF synthase is highly expressed in the fat body of silkworm and plays a part in oxidative stress regulation. The C1-THF synthase has been extensively studied in some organisms such as *Saccharomyces cerevisiae* (Kirksey and Appling, 1996; Song and Rabinowitz, 1995), *Mus musculus* (Howard et al., 2003) and *Homo sapiens* (Prasannan et al., 2003; Walkup and Appling, 2005), while its function in insect reproduction is still not clear.

Dna Binding

Proliferating cell nuclear antigen (PCNA), a non-secretory protein reported to be involved in DNA binding activity, was identified in MAG of *L. orbonalis.* In a wide range of taxa, PCNA is an evolutionarily conserved protein (Bauer and Burgers 1990; Miura et al. 1999; Lin and Corstjens 2002; Orii et al. 2005; Ruike et al., 2006; Xie et al., 2008) and a member of the DNA sliding clamp for DNA polymerase activity (Maga and Hubscher 2003; Moldovan et al., 2007). Earlier studies show PCNA as an auxiliary protein for the mammalian DNA polymerase δ activity (Bravo et al., 1987; Bravo and Macdonald- Bravo 1987; Prelich et al., 1987), although some reports described that PCNA is involved in DNA repair, cell cycle control, DNA methylation, and apoptosis (Kelman 1997; Maga and Hubscher 2003; Moldovan et al., 2007).

PCNA is found to accumulate in the oocytes during early cleavage in Xenopus, (Leibovici et al., 1990). Recently, in the stony coral *Euphyllia ancora*, PCNA was identified in the testis and ovaries suggesting, its role in both the mitotic and meiotic process of the coral germ cells, gametogenesis, and early cleavage during embryonic development (Shikina et al., 2018).

In our study, we identified this protein in MAG of *L. orbonalis*. Since the presence of PCNA in MAG tissue is not been reported in any other insects, its function in this tissue is unknown. The absence of this protein in mated male MAG might indicate the possibility of this protein being utilized completely by the male itself during or after mating instead of its possible transfer to females as this protein doesn't contain a signal peptide.

Hexamerins

Sex-specific storage protein-2 (SP-2) and Basic juvenile hormone-suppressible protein 2 (BJHSP-2), identified in MAG of *L. obrbonalis* were classified as part of the hexamerin functional group. Hexamerins may simply be considered as 'storage proteins' (Fujii et al., 1989; Tojo and Yoshiga, 1994; Wang et al., 1993) or maybe repressed by juvenile hormone, and thus have been named 'juvenile- hormone suppressible proteins' (Jones et al., 1990,1988,1993). They provide energy in the form of amino acids essential for insect metamorphosis, diapause, egg development (Han et al., 2017), etc. Hexamerins may transport the hormones (Braun and Wyatte, 1996) and also possess certain immune activities, such as the inhibition of apoptosis (Lee et al., 2015; Rhee et al., 2007), antioxidant activity (Lee et al., 2017) and antifungal activity (Li et al., 2019; Ujita et al., 2005). The SP-2 is also characterized by the high content of aromatic amino acids as arylphorins storage proteins (Chen et al., 2015; Fujii et al., 1989; Mine et al., 1983). In

Lepidopteran species, both vitellogenin and production of eggs happen entirely within the adult stage. Hence, these processes are dependent on stored amino acid reserves, while others enhance their fecundity through amino acids and other nutrients obtained from their adult diet or males during mating (Boggs, 1997). For example, *Heliconius* butterflies obtain a large proportion of amino acids required for oogenesis from adult feeding as well as male donations (Boggs, 1987).

Rafaeli et al., (2013), using a proteomic based method, identified the BJHSP-2 and SP-2 proteins in the MAG of *H. armigera* and reported its function in metabolism. Recently Wang et al. (2019) reported the presence of BJHSP-2 and SP-2 proteins in hemolymph and the fat body of *Bombyx mori*, suggesting that these proteins were absorbed by the fat body from hemolymph, which might prevent excessive oxidative conditions and maintain the fitness of fat body.

The SP-2 found in the virgin MAG of *L. orbonalis* may be transferred to the females during copulation involved in egg development-related functions. However, it is challenging to say the same for BJHSP-2.

Conclusion

Some of the identified MAG proteins in *Leucinodes orbonalis* revealed the possibility of their transfer to females and inturn altering female physiology and behavior. As this is the first report on proteomics of *L. orbonalis* MAG, the work is of high significance, especially with the emerging trend of considering MAG proteins as future biopesticides that could be safe, specific, and eco-friendly.

Declarations

Compliance with Ethical Statements : This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest: The authors declare that they have no conflict of interest.

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Ethical approval

This article does not contain any studies with animals performed by any of the authors.

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Figures

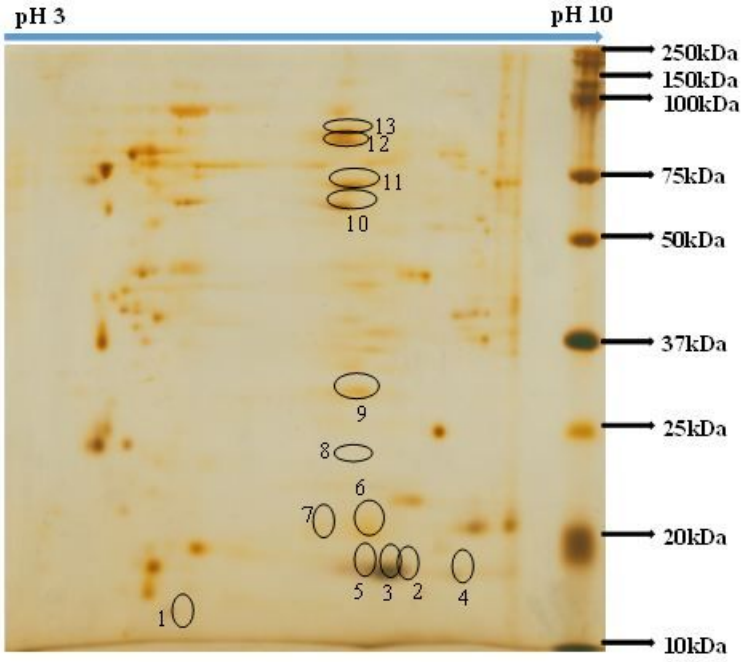

Figure 1

Silver stained 10% 2-D gel Electrophoresis of male accessory gland proteins of L. orbonalis with the pH 3-10 containing virgin male sample. Block colour circles indicate protein spots that yielded protein identification by MALDI-MS analysis.

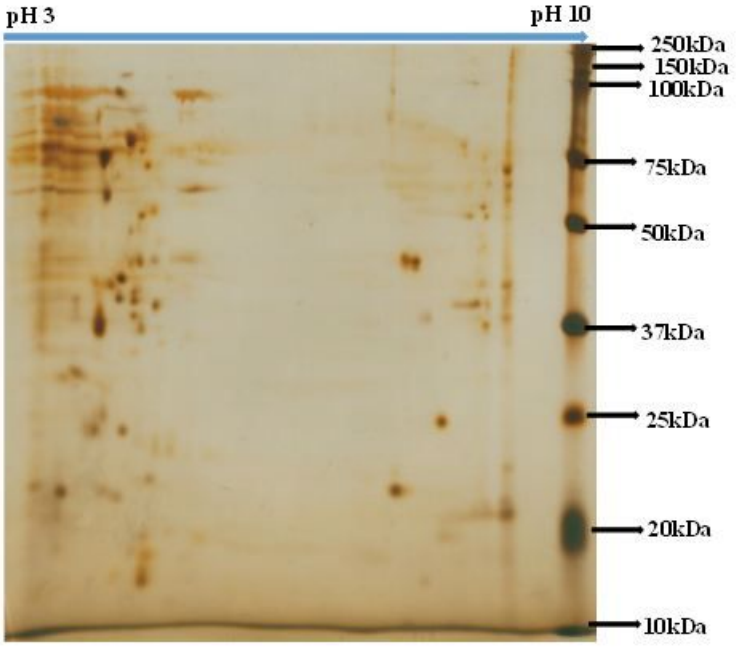

Figure 2

Silver stained 10 % 2-D gel Electrophoresis of male accessory gland proteins of L. orbonalis with the pH 3-10 containing mated male sample.

Supplementary Files

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- Supplementarytable.1.xlsx