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Identification and coordinated expression of perilipin genes in the biological cycle of sunn pest, *Eurygaster maura* (Hemiptera: Scutelleridae): Implications for lipolysis and lipogenesis

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ABSTRACT

The sunn pest, *Eurygaster* spp., is one of the most destructive pests of grains in Asia, Europe and Africa. The nymphs and adults feed voraciously in the field by late-spring, followed by migration of adults into mountains for diapause, which includes estivation by late summer and hibernation during winter. Adults migrate back to the field by the end of diapause in mid-spring, where they mate and lay eggs. To understand how sunn pest survives and maintains basic metabolic functions without feeding for 7 months during diapause, this study focused on lipid metabolism as the major source of energy production, and the primary organ of lipid metabolism, the fat body. Studies on lipid metabolism revealed two major factors referred to perilipin protein family, Lipid Storage Droplet Protein 1 (LSD1) and Lipid Storage Droplet Protein 2 (LSD2), which are involved in hydrolysis and accumulation of lipids, respectively. In this study, two LSD (EmLSD1-2) orthologues in the hemimetabolous *Eurygaster maura* were identified. *EmLSD1* and *EmLSD2* genes were expressed in multiple tissues, but primarily in fat body. Both genes were continuously expressed throughout the insect's life cycle but peaked in the 4th nymphal stage. Their expression patterns were in accordance with the biological roles of LSDs. *EmLSD1* expression peaked in non-feeding stages supporting its lipolytic role, while the highest level of *EmLSD2* expression was in feeding stages supporting its lipogenetic role. Expression patterns of both genes differed in females and males. Overall, expression patterns of *EmLSDs* provide clues to understanding the interesting life cycle of sunn pest.

1. Introduction

Eurygaster spp. (Hemiptera: Scutelleridae), commonly known as the sunn pest, are one of the most destructive pests of wheat and barley in West and Central Asia, Northern Africa, and Southeast Europe (Critchley, 1998). The insect feeds on both the vegetative and generative stages of the plant. During feeding, they also release digestive enzymes that greatly reduce the baking quality of the flour. Sunn pest infestations can lead to 100% crop loss in the absence of control measures.

Sunn pest is a hemimetabolous insect, which completes one generation per year with two biological forms denoted as active (feeding) and inactive (non-feeding) (Critchley, 1998). Active forms represent the sunn pest nymphs and adults feeding on plants in the field in late spring and early summer; a period which lasts around 3–4 months. Following harvest (mid-July in central Anatolia in Turkey), adults migrate to the higher altitudes of the mountains for estivation to escape from hot temperatures. Estivation is followed by a pre-hibernation period characterized by migration to the lower mountain altitudes in preparation for hibernation. These individuals then hibernate beneath the snow blanket or soil which offers protection from the severe cold during the core winter. Both estivation and hibernation are essential parts of the obligatory diapause. Inactive forms represent the non-feeding estivating adults present in late summer and early fall, as well as the pre-hibernating and hibernating adults around mid-fall. The inactive period lasts 7–8 months at which time the adults emerge from diapause in mid-spring and migrate back to the field. The adults feed for a brief period of time on grains, and then mate and lay eggs by early summer. It has been a mystery how the inactive forms survive and maintain basic biological functions without feeding for 7–8 months during diapause. To address this question, it is essential to understand how nutrient storage and energy metabolism occur in sunn pest.

Lipids and carbohydrates are the two primary energy reserves for animals. In insects, most of the lipid and carbohydrate metabolism occurs in the fat body, a situation analogous to the vertebrate adipose

Abbreviations: LDs, lipid droplets; LSD, lipid storage droplet.

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tissue and liver (Arrese and Soulages, 2010). The fat body serves as a storehouse of energy reserves, but also synthesizes various proteins, such as storage proteins that serve as an amino acid reservoir for morphogenesis, lipophorins responsible for the lipid transport, vitellogenins required for egg maturation, and several antimicrobial peptides (Arrese and Soulages, 2010). Lipids are the major component of the fat body and are stored exclusively in the form of triglycerides, which serve as the major reserve to meet the energy demand for reproduction, diapause, starvation, and prolonged periods of flight (Arrese and Soulages, 2010). It is noteworthy that sunn pest adults migrating to the mountains for diapause are rich in lipids (Amiri and Bandani, 2013). This stored fat reserve is critical for sunn pest survival during the non-feeding overwintering period since the adults will exhibit lower survival rates if fat reserves are inadequate (Critchley, 1998). Therefore, it is essential to understand how lipids are synthesized and converted into energy in the sunn pest.

Lipid research in insects has been inspired from research in mammalians as both share common pathways for lipid metabolism. These studies have focused on an intracellular storehouse for triglycerides, the lipid droplets (LDs), which are common to mammalian and insect adipocytes (fat cells), and in particular the proteins associated with the LDs (Arrese and Soulages, 2010). The first mammalian LD protein described was "perilipin" (PLIN1) (Greenberg et al., 1991), followed by the discovery of four other LD proteins; i) Adipose differentiation-related protein-ADRP (PLIN2), ii) Tail-interacting 47-kDa protein-TIP47 (PLIN3), iii) S3-12 (PLIN4) and iv) OXPAT/MLDP (PLIN5) (Bickel et al., 2009). These proteins are commonly referred to as the perilipin protein family and play a central role in lipid and energy metabolism by regulating the balance between triglyceride synthesis and hydrolysis (Bickel et al., 2009). For example, PLIN1 inhibits triglyceride hydrolysis in its non-phosphorylated (basal) state and stimulates lipolysis upon phosphorylation by protein kinase A (PKA) during times of energy deficit (Bickel et al., 2009). In contrast, PLIN2 promotes fat storage (Bickel et al., 2009). Studies on insect LD proteins, in particular in *Drosophila melanogaster* (Diptera: Drosophilidae) and *Manduca sexta* (Lepidoptera: Sphingidae), identified two perilipin orthologues, the Lipid Storage Droplet Protein 1 (LSD1), an ortholog of PLIN1, and the Lipid Storage Droplet Protein 2 (LSD2), an ortholog of PLIN2 (Grönke et al., 2003; Teixeira et al., 2003; Patel et al., 2005; Arrese et al., 2008b). In accordance with the role of mammalian PLINs, LSD1 is involved in the activation of lipolysis, whereas LSD2 inhibits lipase activity to favor lipid accumulation (Grönke et al., 2003; Teixeira et al., 2003; Patel et al., 2005; Arrese et al., 2008a,b).

Studies on insect LSDs have provided important clues as to their roles; however, these studies are mainly laboratory-based and may not reflect lipid metabolism in the insects in their natural environment. Also, it is unknown how lipid metabolism is maintained during physiological events such as diapause, and biological events such as migration or reproduction. Additionally, the current data were derived from holometabolous systems which have clearly distinct developmental stages exhibiting entire physiological restructuring. However, this may not be indicative of the situation for hemimetabolous insects like sunn pest which has physiologically similar nymphal and adult stages. In the current study, two LSD orthologues from the hemimetabolous sunn pest, *Eurygaster maura*, were characterized. The site-specific and developmental expression patterns of these LSDs were examined. Finally, expression analyses of each gene from six key time points (estivation, pre-hibernation, hibernation, pre-migration at the overwintering areas, migrated and active-feeding stages in the field) in the sunn pest biological cycle were conducted. These analyses revealed a coordinated pattern of LSD gene expression related to biological and physiological processes, such as migration, diapause and reproduction in the sunn pest life cycle. This information was used to generate a model for the putative roles of EmLSD proteins in sunn pest biology, which provided clues about lipid-based energy metabolism in insects and how sunn pest survives without feeding for extended periods.

2. Material and methods

2.1. Insects

E. maura eggs were collected from wheat fields (Haymana Fields). Nymphal stages (1st to 5th stage) and one-week old adults were obtained from the hatched eggs for developmental expression analyses. These insects were reared on wheat pots in a rearing room set at 20 °C and 16:8 h light–dark photoperiod.

Individuals from 6 key points of the sunn pest life cycle were also collected from wheat fields (Haymana Fields) or overwintering areas (Beynam Forests) in Ankara Province, Turkey for time-course studies. These time points included 1) Estivation — estivating individuals in the higher mountain altitudes in September, 2) Pre-hibernation — individuals preparing for hibernation in the lower mountain altitudes in October, 3) Hibernation — hibernating individuals under the soil/snow blanket in the mountains in December, 4) Pre-migration — individuals preparing for migration from the mountain in April, 5) Migrated — newly migrated individuals into the field in May and 6) Feeding — new generation, actively feeding individuals in the field in July. Time-course studies were conducted with adult females and males separately due to the physiological differences between them (e.g. maturation processes of ovaries in premating females). Females and males were separated according to the genital segment on the abdomen (Critchley, 1998).

2.2. Construction of the E. maura fat body cDNA library, sequencing and identification of cDNAs encoding proteins with perilipin domains

A fat body cDNA library was constructed from actively-feeding, new generation *E. maura* adults and sequenced by using a 454 GS FLX (Roche). The fat body was dissected from approximately 50 adults and collected in a tube containing 1.0 mL of TRIzol reagent (Invitrogen). Tissue was homogenized and total RNA was extracted according to the manufacturer's protocol (Invitrogen). Total RNA was re-suspended in 100 μL of sterile DEPC-treated H_2O and quantified by measuring the absorbance at 260 nm and 280 nm using a Nanodrop spectrophotometer (Fisher-Thermo). The RNA was then enriched by using the "RiboMinus Eukaryotic kit" (Invitrogen) following the manufacturer's instructions. The mRNA-enriched fraction was precipitated with ethanol and re-suspended in 12 μL of sterile DEPC-treated H_2O and quantified by using a Nanodrop spectrophotometer. The sample was then quantified and checked for mRNA quality by using a "RNA 6000 Nano Kit" and a Bioanalyzer 2100 (Agilent). The cDNA library construction involved the fragmentation of mRNA by using the high temperature Zn^{++} method and fragments were purified with selection for 300–1000 NT size fragments by using a "cDNA Rapid Library Prep kit" (Roche). The RNA fragments were used to prepare libraries for 454 pyrosequencing according to the manufacturer's instructions (Roche). The output from the GS FLX titanium sequencing runs was assembled into contigs by using Newbler assembler (version 2.3) and annotated via a BLAST algorithm. The cDNA library was scanned for proteins with perilipin domains by using NCBI Conserved Domain Search (Marchler-Bauer et al., 2011).

2.3. Dissection of insect tissues and total RNA extraction

Fat body, midgut, ovary, Malpighian tubules, trachea, head, muscles and nervous system (brain in the head and ganglia in each body segment) were dissected from 25 actively-feeding, new generation adult females (time point 6) using sterilized tweezers under a dissecting microscope in ice-cold phosphate buffered saline (pH 7.4). The tissues were collected in TRIzol reagent (Invitrogen) in a 1.5 mL centrifuge tube on ice and total RNA was isolated according to the manufacturer's instructions.