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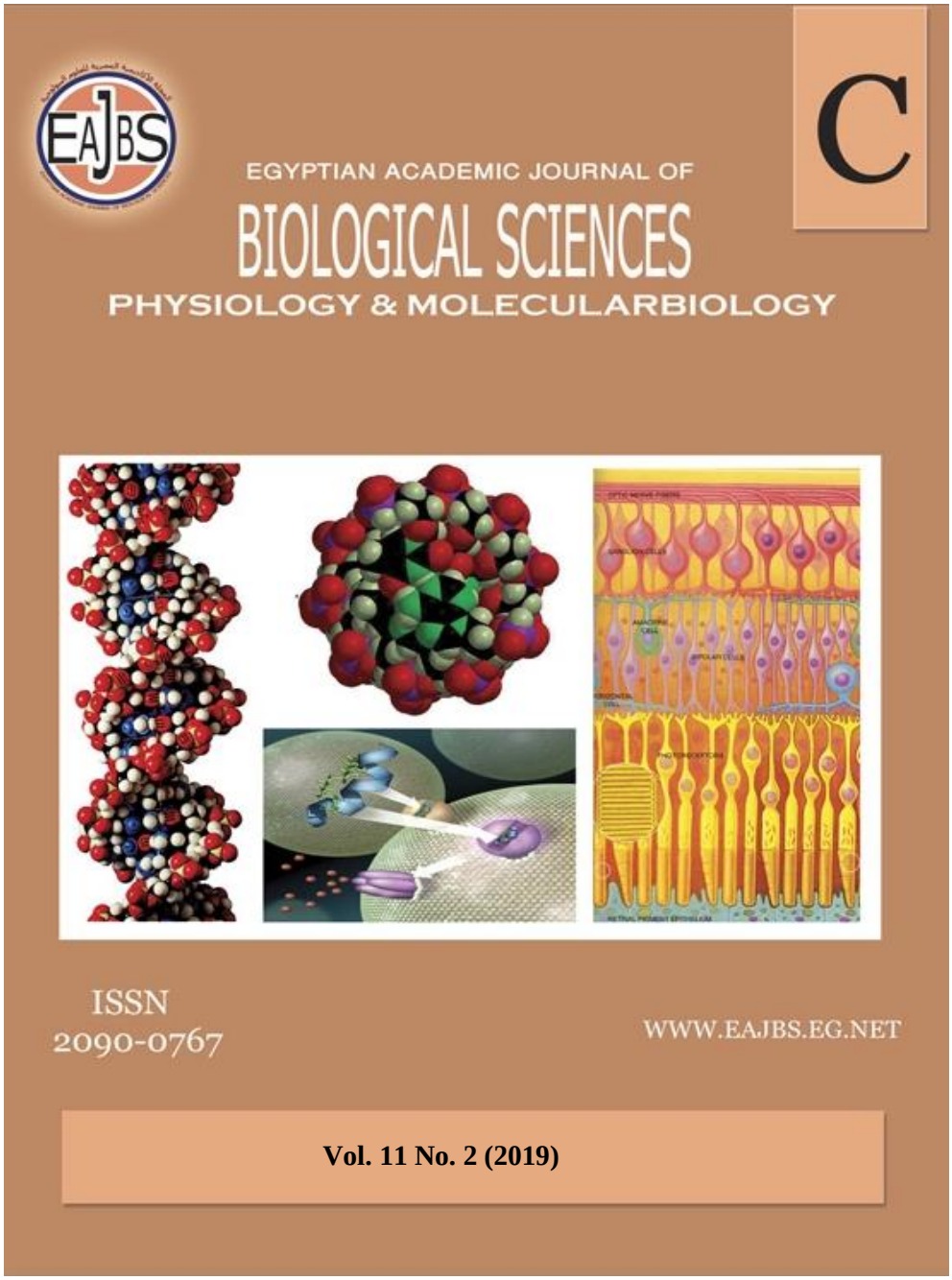

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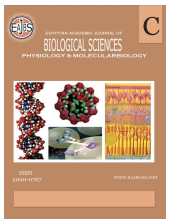

Molecular Changes in Salivary Glands of *Culex pipiens pipiens* (Diptera: Culicidae) at Different Feeding Stages

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ABSTRACT

This investigation was conducted to detect genetic variations between male and female *Cx. pipiens pipiens* during different feeding stages using DD-PCR technique. Salivary glands were collected at 3, 12, 24, 48, 72 h post-sugar administration for both males and females, at 3, 12, 24, 48, 72 h post-blood meal, and post-oviposition for females. Unfed, starved and sugar-fed individuals were used as controls. DD-PCR technique was employed using 4 arbitrary primers separately. Several common bands were detected in all samples. Other hands were stage-specific. Many bands were up-regulated or down-regulated at different feeding stages. Our results showed the powerfulness of DD-PCR to discover changes of gene expression in salivary glands of male and female *Cx. pipiens pipiens* at different feeding stages. Sequencing of the differentially displayed bands would be very useful in the identification of the genes involved in the feeding process of mosquitoes.

INTRODUCTION

Mosquitoes are considered the most dangerous vectors of human and animal diseases worldwide (Brown, 1993). Many harmful infections such as malaria, yellow fever, Chikungunya, West Nile Virus, dengue fever, filariasis, Zika virus, Rift valley fever and other arboviruses are transmitted by subtropical mosquito species, especially, *Culex pipiens* (Taylor *et al*., 1953; Hurlbut *et al.*, 1956; Southgate, 1979; Curtis & Feachem, 1981; Meegan *et al*., 1980; Tawfick, 1990; Morsy *et al*., 2003; Luisa *et al*., 2015).

Salivary glands are paired organs located on either side of thorax flanking the esophagus. The structure of mosquito's salivary glands is sexually different (Jariyapan & Harnnoi, 2002; Dhar & Kumar, 2003; Prasad *et al.*, 2016). An additional difference in feeding habit is female mosquito has to uptake blood as an extra protein source for egg development (James, 1994; Stark & James, 1996a; Rawal *et al*., 2016). Pathogens (viruses, bacteria, protozoa or filaria) are transmitted horizontally when female injects saliva via biting or taking a blood meal from a host (Rawal *et al*., 2016).

Studying mosquito salivary gland clarified that it contains many bioactive molecules such as; digestive enzymes, anticoagulant peptides, vasodilators, inhibitors of platelet aggregation and others (Ribeiro *et al*., 1984a; Grossman & James, 1993;

Champagne *et al.*, 1995; Ribeiro, 1995; Smartt *et al*., 1995; Ribeiro & Francischetti, 2003). Discovery of candidate peptides became a focus of research for the development of epidemiological techniques (Remoue *et al.*, 2006; Drame *et al.*, 2010), transmission-blocking vaccines (Titus *et al.*, 2006), or novel therapeutic agents (Remoue *et al.*, 2006; Titus *et al.*, 2006; Drame *et al.*, 2010; Mans & Francischetti, 2011; Seufi *et al.*, 2016; Arcá *et al*., 2017; Seufi *et al.*, 2017).

Differential display polymerase chain reaction (DD-PCR) is considered one of the most efficient techniques for the detection of differentially expressed genes (Liang & Pardee, 1992; Liang, 2002). DD-PCR is sensitive, highly specific, and fast, it does not require any prior knowledge on sequences and allows direct information on changes in mRNA. A big shortage in studies focusing on molecular changes of salivary glands associated with the feeding of mosquitoes has been observed. Thus, the present work describes the screening of genetic differences between male and female *Cx. pipiens pipiens* during different feeding stages using DD-PCR technique.

MATERIALS AND METHODS

Insect Colonization:

A laboratory colony of the mosquito, *Culex pipiens pipiens* was originally obtained from the Research Institute of Medical Entomology, Dokki, Giza, Egypt. This colony was maintained in the insectary of the Department of Entomology, Faculty of Science, Cairo University under controlled conditions; 27 ± 2 °C and 70 ± 5% RH and 14L: 10D photocycle (Adham *et al.*, 2003).

Dissection of Salivary Glands:

Mosquitoes were anaesthetized on ice for dissection of salivary glands. Mosquitoes were dissected in phosphate-buffered saline (PBS); 10 mM Na_2PO_4, 145 mM NaCl (pH7.2) under a stereoscopic microscope (Leica, Germany). Samples were collected at the following times: (i) sugar-fed stage at 3, 12, 24, 48 and 72 h after feeding, (ii) starved stage (iii) newly emerged un-fed stage (males and females), (iv) blood-fed stage at skin exploring time, at 3, 6, 12, 24, 48 and 72 h after blood meal and (v) after oviposition (approximately after 10 days from adult emergence). At each dissection time, 30 salivary glands were transferred to a microcentrifuge tube containing a suitable volume of PBS and stored at -80 °C until processed.

Differential Display Technique (DD-PCR):

Total RNA was extracted from salivary glands using Biozol reagent (Bioflux, Germany) according to the manufacturer's instructions. RNA was dissolved in DEPC-treated water, quantified using a BioPhotometer 6131 (Eppendorf, USA) and visualized on 2 % denatured agarose gel to ensure its integrity. The 260/280 and 260/230 ratios were examined for protein and solvent contamination. Two µg of DNA-free total RNA were converted into cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo-Fisher, USA) according to the manufacturer's instructions. Synthesis of the first cDNA strand was carried out using thermal cycler (PeQlab, USA) programmed at 42 °C for 1 h, 72 °C for 10 min and a soak at 4 °C. The cDNA was aliquoted and stored at -80 °C until used (within a weak).

Total PCR volume was 25 µl containing 12.5 µl PCR master mix (Promega, USA), 7 µl of 10 pmol primer (Table 1), 1 µl cDNA, 4.5 µl H_2O. To assess DNA contamination, a no–reverse transcription control was carried out. Thermal cycler was programmed for one cycle at 95 °C for 5 min followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C, and 1 min at72 °C. A single step of the final extension was done at 72 °C for 10 min. PCR product was visualized on 2 % agarose gel and photographed using a gel documentation system.

Table (1): List of names and nucleotide sequences of the arbitrary primers used in this study

No	Primer name	Primer sequence
1	R1	5`CCCGACTCGTGGATCATGTA3`
2	R2	5`CCATCTTTTCCATCGGCCAG3`
3	R3	5`TCCCAAGAGAGCAATGACCA3`
4	R4	5` TGGTGACATTATCGAGGCCG3`

Data Scoring and Analyses:

Reproducible and clear bands produced by the four arbitrary primers were scored as present (1) or absent (0). Binary matrices of the profiles were assembled. Percentage polymorphism (PP%) was calculated.

RESULTS

The main objective of the present work was to investigate molecular changes in salivary glands of male and female *Cx. pipiens pipiens* during different stages of the feeding process. To achieve this objective DD-PCR technique was used. Figures (1- 4) show the results of differentially displayed cDNAs using 4 arbitrary primers (R1, R2, R3 and R4). All used primers matched with cDNA of both male and female *Cx. pipiens pipiens*, detecting the decreased and increased gene expression. A total of 331 DNA fragments were amplified and resolved on 2% agarose gel. The molecular size of the amplified bands ranged from 80 to 1550 bp. Several common bands were observed. Meanwhile, some stage-specific bands have been recorded. Many bands exhibited irregular patterns of expression without obvious relation to the stage. Generally, DD-PCR technique revealed that many down-regulated (turned off) and up-regulated genes (turned on) were observed in the present work (Figs. 1- 4).

Using R1 primer, 155 bands (100% polymorphic bands) have resolved on agarose gel for all samples (250 – 700 bp). Using R2 primer, 54 bands (50% polymorphic bands) have resolved on agarose gel for all samples (200 – 750 bp). Using R3 primer, 49 bands (100% polymorphic bands) have resolved on agarose gel for all samples (80 – 1550 bp). Using R4 primer, 73 bands (86% polymorphic bands) have resolved on agarose gel for all samples (200 – 950 bp). The average number of bands detected in each time interval ranged from 1 to 5 bands with different molecular sizes. About 90% of total polymorphism has been detected (Figs. 1- 4).

At the unfed stage (just after emergence), dimorphic RNA patterns have been displayed for salivary glands of males and females using the four primers (Figs. 1- 4; Lanes: 13, 14).

At the sugar feeding stage, polymorphic RNA patterns have been displayed for males and females salivary glands at 3, 12, 24, 48 and 72 h after administration of sugar to mosquitoes, respectively (Figs. 1- 4; Lanes: 1-10).

In addition, starving males and females showed dimorphic RNA patterns using the four primers (Figs. 1- 4; Lanes: 11, 12).

Using sugar-fed, unfed and starved females as controls for blood-feeding stage, the skin exploring females showed similar RNA pattern to starved female in the case of both R1 and R3 primers (Figs. 1 & 3; Lanes: 15- 18), similar to both unfed and starved females in the case of R2 primer (Fig. 2; Lanes: 15- 18) and similar to unfed female in the case of R4 primer (Fig. 4; Lanes: 15- 18). Excitingly at 3, 12, 24, 48 and 72 h post-blood-feeding, the females showed polymorphic RNA patterns which were definitely different from controls and previous blood-feeding stage in the case of R2, R3 and R4 primers (Figs. 2, 3 & 4; Lanes: 19- 23). R1 primer showed additional up-

regulated and down-regulated bands for the above-mentioned post-blood-feeding intervals (Fig. 1; Lanes: 19-23).

After oviposition, female salivary glands have exhibited RNA patterns very similar to sugar-fed females in the case of R1 and R2 primers (Figs. 1 & 2; Lane: 24), similar to starved females in the case of R3 primer (Fig. 3; Lane: 24) and definitely different pattern in the case of R4 primer (Fig. 4; Lane: 24).

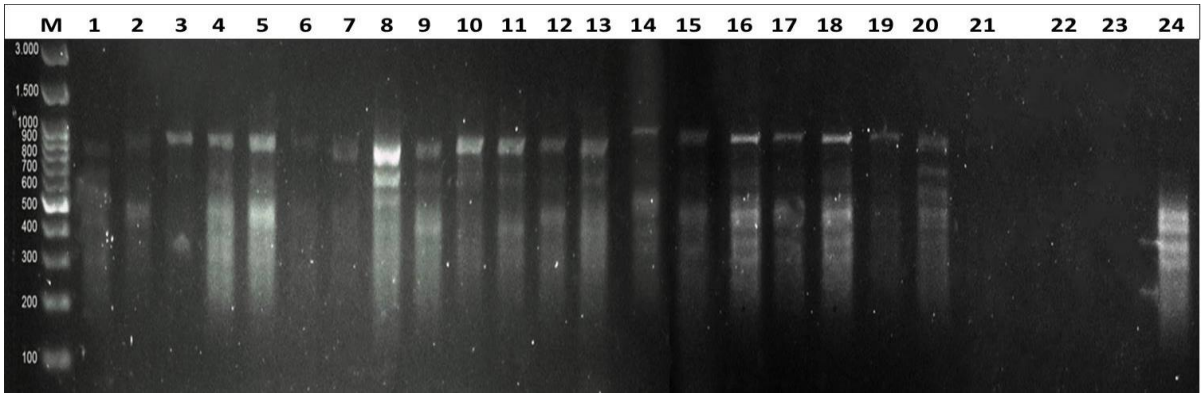

Fig. (1): 2% agarose gel of DD-PCR patterns generated by primer R1 stained with ethidium bromide. Lane M: DNA Ladder, Lanes 1-14: Sugar feeding stages: 3 h male, 3 h female, 12 h male, 12 h female, 24 h male, 24 h female, 48 h male, 48 h female, 72 h male, 72 h female, starved male, starved female, unfed male, unfed female. Lanes 15-17: Control females: sugar-fed, unfed, starved. Lanes 18-24: Blood feeding stages: 3, 12, 24, 48 and 72 h post-blood-meal, post-oviposition female.

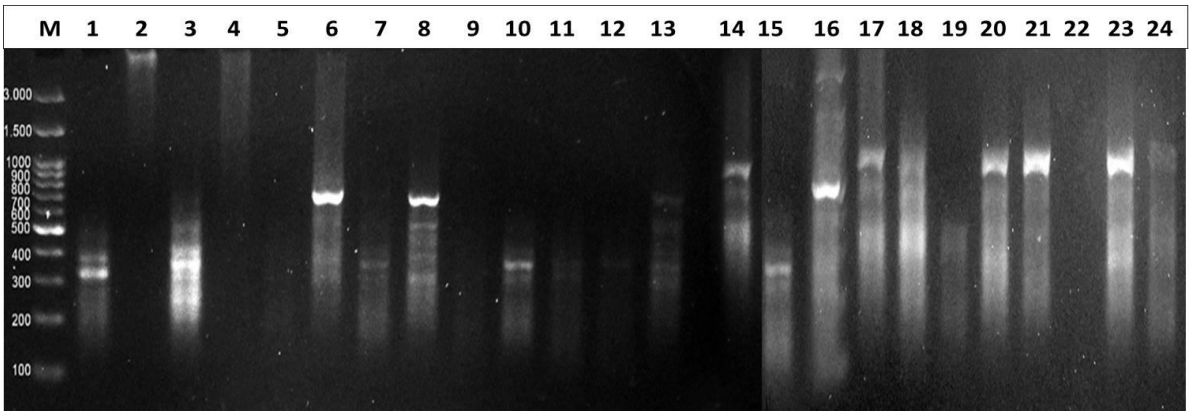

Fig. (2): 2% agarose gel of DD-PCR patterns generated by primer R2 stained with ethidium bromide. Lane M: DNA Ladder, Lanes 1-14: Sugar feeding stages: 3 h male, 3 h female, 12 h male, 12 h female, 24 h male, 24 h female, 48 h male, 48 h female, 72 h male, 72 h female, starved male, starved female, unfed male, unfed female. Lanes 15-17: Control females: sugar-fed, unfed, starved. Lanes 18-24: Blood feeding stages: 3, 12, 24, 48 and 72 h post-blood-meal, post-oviposition female.

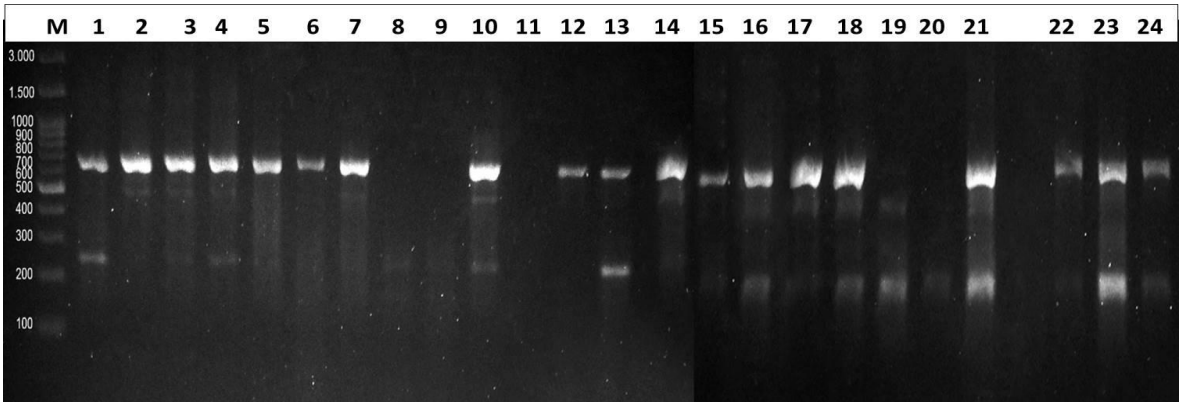

Fig. (3): 2% agarose gel of DD-PCR patterns generated by primer R3 stained with ethidium bromide. Lane M: DNA Ladder, Lanes 1-14: Sugar feeding stages: 3 h male, 3 h female, 12 h male, 12 h female, 24 h male, 24 h female, 48 h male, 48 h female, 72 h male, 72 h female, starved male, starved female, unfed male, unfed female. Lanes 15-17: Control females: sugar-fed, unfed, starved. Lanes 18-24: Blood feeding stages: 3, 12, 24, 48 and 72 h post-blood-meal, post-oviposition female.

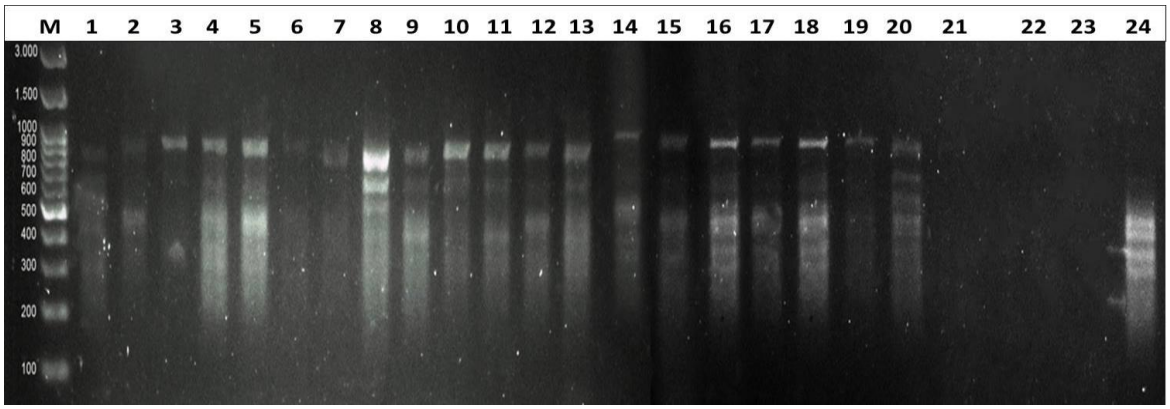

Fig. (4): 2% agarose gel of DD-PCR patterns generated by primer R4 stained with ethidium bromide. Lane M: DNA Ladder, Lanes 1-14: Sugar feeding stages: 3 h male, 3 h female, 12 h male, 12 h female, 24 h male, 24 h female, 48 h male, 48 h female, 72 h male, 72 h female, starved male, starved female, unfed male, unfed female. Lanes 15-17: Control females: sugar-fed, unfed, starved. Lanes 18-24: Blood feeding stages: 3, 12, 24, 48 and 72 h post-blood-meal, post-oviposition female.

DISCUSSION

The present investigation assured the powerfulness of DD-PCR technique in discovering up and down-regulated genes in male and female mosquitoes during different feeding stages. Many have been published on using RNA fingerprinting to identify transcripts which are regulated in human tumors (Liang & Pardee, 1992), differentially expressed during mouse brain development (McClelland *et al.*, 1993), or differentially expressed during peroxide stress in *Salmonella* (Wong & McClelland, 1994). Meanwhile, others reported that DD-PCR is a powerful tool for the characterization of altered gene expression in eukaryotic cells (Mong *et al.*, 2002). DD-PCR has been recommended for the isolation of mRNAs of oncogenes (Gonsky *et al.*, 1997), novel genes expressed in human lung and wound healing (Lurton *et al.*, 1999; Soo *et al.*, 2002) and key genes involved in development of *Melipona scutellaris* (Santana *et al.*, 2006). Sturtevant (2000) reviewed many applications of DD-PCR in medical mycology.

Our results present the amplification of 331 DNA fragments (80 to 1550 bp) with 90% polymorphism. Several common

bands, some stage-specific bands and bands exhibiting irregular patterns of expression have been isolated. The common bands observed in all feeding stages are possibly general enhancement of the mosquito's immune system or genes involved in regular cellular processes. However, the stage-specific and up-regulated bands may be a result of specific induced genes. These genes could be attributed to the change in salivary gland function with the feeding stage. Down-regulated and disappeared bands could be interpreted as function-related inhibition of some genes. Agreeable results have been previously presented; Dimopoulos *et al.* (1996) have successfully used DD-PCR technique to screen for mRNAs specifically expressed in adult males, females mosquito. In addition, they studied the midgut tissues of blood-fed and unfed females. Furthermore, Ramalho-Ortigão *et al.* (2001) applied DD-PCR and RAPD-PCR to characterize 32 differentially expressed mRNAs from sugar, blood-fed and *Leishmania*-infected sand fly.

Conclusively, our results have confirmed the powerfulness of DD-PCR for screening induced and inhibited genes in different feeding stages of *Cx. pipiens* mosquitoes. Additional sequencing of the differentially expressed bands would be informative at the level of structure and function of up and down-regulated genes during different feeding stages.

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