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Gene expression of heat shock protein (hsp90) in *Plodia interpunctella* and *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae parasitized by *Bracon hebetor* wasp (Hymenoptera: Braconidae)

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ABSTRACT

Venoms of parasitoid wasps injected into the host may play vital roles in successful parasitism. It could manipulate the host physiology and suppress its immune response. Heat shock proteins (hsps) could be induced by a variety of physiological stresses and might play roles in modulating the host-parasitoid relationship. Quantitative real-time transcription PCR was used to determine the changes of heat shock protein90 gene in *Polidia interpunctella* and *Ephestia kuehniella* larvae post parasitization by *Bracon hebtor* wasp. The results indicated that hsp90 gene expression level showed different behavior in both hosts, at all-time intervals post parasitism. Expression of the gene in *P. interpunctella* larvae was significantly down regulated at 12h. A high significant down regulated was observed at 24 and 72h after parasitization. Meanwhile, a high significant up-regulation was recorded after 48 h compared with control. On the other hand, the levels of hsp90 in *E. kuehniella* were high significantly down regulated in all treated larvae compared with control. We can conclude that the suppression hsp90 gene could be a component of parasitized hosts' manipulation strategy that regulate the host physiology and suppress the immune response. Hsp90 might play an important role in host paralysis and inhibit its development.

Key words: Hsp90, *Polidia interpunctella*, *Ephestia kuehniella*, *Bracon hebtor*,venom, wasp,parasitism.

INTRODUCTION

Heat shock proteins (hsps) are ubiquitous molecular chaperones found in all eukaryotic cells.Chaperones (stress proteins) are essential proteins to help the formation and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses. Therefore, normal chaperone function is a key factor for endogenous stress (Soti *et al.*, 2005) and it has an important role in protein folding, unfolding, aggregation, degradation adaptation of several tissues.In addition, hsp often employ protective functions in response to a number of stressful environmental conditions including abiotic stresses such as heat shock, ultraviolet radiation, chemical pesticides, as well as biotic stresses such as viruses, bacteria, fungi and parasitoid insects (Zhao and Jones, 2012). Hsps are organized into several families based on their molecular mass (kDa). They are highly conserved families that protect the cell protein against stress (Nguyen *et al*., 2009). They play an important function in correctly folding newly synthesized proteins, stabilizing and refolding denatured proteins after stress, preventing misfolding and aggregation of unfolded or partially folded proteins, and assisting in protein transport across the endoplasmic reticulum and organelle membranes (Young *et al*., 2001).

Hsp90 members have key roles in the maturation of signal transduction proteins, like hormone receptors, various kinases and nitric oxide syntheses (Wegele *et al*., 2004). Hsp90s are usually represented with four major types, two cytosolic forms, (alpha and -beta forms),

endoplasmic reticulum and mitochondrial homologues(Felts *et al*., 2000). The "hsp90 client proteins" are directly associated with hsp90 and they include a wide variety of signal-transducing molecules that regulate cell growth and differentiation, protein kinases and transcription factors are more essential(Krishna and Gloor, 2001).

The Indian meal moth, *Polidia interpunctella*(Hübner) and the Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) were insect pests of stored-products and processed food commodities throughout the world. These pests induce considerable losses to cereal, legumes grains and other high value crops such as cocoa beans and dried fruits (Mohandass *et al*., 2007). Parasitoids play an important role in integrated pest management programs due to their capability to keep pest populations under economic thresholds (Belda and Riudavets, 2013). In addition, *Habrobracon hebetor*wasp is a gregarious ectoparasitoid of Pyralid moth larvae. Its Female parasitises the larvae of several species of stored-product moths, including Indian meal moth *P. interpunctella*(Hübner), Mediterranean flour moth *E. kuehniella*, warehouse moth *Ephestia elutella*(Hübner), and the tropical warehouse moth *Cadra cautella*Walker(Schölle,2010).

The ability of a parasitoid to grow within the host is influenced directly by its ability to overcome the host immune system with different strategies. Hymenopteran parasitoids have developed host-specific strategies to overthrow the host immune system, such as the use of venom, endosymbiont virus, or mimicking the host tissue (Caron *et al*., 2008). *Bracon hebetor* Say (Hymenoptera: Braconidae) is ectoparasitoid that is an important active parasitoid against several stored product moths like the Mediterranean flour moth *E. kuehniella*, warehouse moth *Ephestia clutella*, Indian meal moth *P. interpunctella* and rice moth *Corcyra cephalonica* (Darwish *et al*., 2003; Maafi and Chi, 2006; Chen *et al*., 2011). It parasitizes the last instar larvae and host-larvae become paralyzed by venom a few minutes after intoxication (Sláma and Lukáš, 2011).

Several studies have demonstrated that hsps are involved in host-parasitoid interactions and immune response by the host. For example, the expression analysis of hsp20, hsp75 and hsp90 from *Pieris rapae* responsiveness to parasitization by the endoparasitic wasp *Pteromalus puparum* indicated that these three genes were influenced (Zhu *et al*., 2013). With respect to *B. hebetor*, Shim *et al*. (2008) found that the level of hsp90 mRNA in *P. interpunctella* was not influenced by the parasitoid envenomation. Shafeeq *et al*., (2017) showed that hsp90 gene in *P. interpunctella* had different levels of expression in response to *B. hebetor* envenomation under different stress conditions.

The current study aimed to compare the transcription-level of hsp90 gene by quantitative real time PCR in *P. interpunctella* and *E. kuehniella* in response to parasitization by the ectoparasitoid *B. hebtor* wasp.

MATERIALS AND METHODS

1.Experimental insects:

1.a.*Plodiainterpunctella* and *Ephestia kuehniella* rearing:

Adult insects used in this study were obtained from infested stored products. They were reared under laboratory conditions at temperature of 28 ± 2° C with a relative humidity of 70 ± 5 % and a photoperiod of 16:8 L: D (Rharrabe *et al.*, 2010). Adults were kept in plastic jars (17×11×7 cm). They were reared on a diet consisting of cracked wheat (54%), wheat bran (18%), Brewer's yeast (6%), honey (7%), glycerin (12%), powdered milk (2%) and powdered sugar (1%).

1.b.Parasitoid*Braconhebetor* rearing:

Strain of *Bracon hebetor*was collected from wheat infested with *P. interpunctella*. The parasitoid hosts were maintained on larvae of *E.kuehniella* or *P. interpunctella*at 27°C and 50–55% humidity (Buyukguzel*et al.,* 2011).They were also fed on a 50% honey solution (wt:v) soaked in cotton pad. After 24 h, parasitized host larvae were transferred to another clean plastic cup and kept under controlled conditions (28±2°C, 70%RH, and 16 h photophase) until adult emergence. Newly emerged wasps were used for the life history experiments.

1.c.Parasitism:

Adult parasitoids were released into plastic jars containing Five-instar larvae of *E.kuehniella* or *P. interpunctella* for 3 h. After parasitism these larvae were quickly removed from the jarswiped with 70% ethanol and kept on thePetri dishes with paper discs until testing.All measurements were carried out using the *E.kuehniella* or *P. interpunctella* larvae.All experimental and control insects were maintained under rearing conditions according to Kryukova*et al.*(2011).

2.Molecular analysis:

2.a.RNA extraction:

The extraction was performed using RNA Mini Kit (Ambion, 850 Lincoln Centre Drive, Foster City, United States, CA 94404, cat. no.12183018A). After isolating the RNA, the quality of RNA for each individual sample was visualized on a denaturing agarose gel. RNA concentrations were determined using spectrostar Nanodrop (BMG LAB Tech, serial number 601-04550).

Total RNA was extracted as performed in the cloning of *hsp* 90 genes, and 2 µg of RNA per 20 µl. Reaction was used to synthesize first-strand cDNA. The reaction was performed in 20 µl and prepared by adding 10 µl of 2x Rt master mix into each well. Reaction mixtures were incubated for 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 s at60 °C, melting curve from65.0 °C to 95.0 °C, read every 5 °C, held for 10 s.

2.b. cDNA synthesis:

Reverse transcription was done first-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA, Part Number 4368814). The kit contains reagents that when combined, form a 2x reverse transcription (RT) master mix. An equal volume of RNA sample should be added to avoid Rnase contamination.

2.C. Quantitative real time PCR (qRT-PCR):

Real-time PCR was run by using 2xg PCR/RTD- PCR Master Mix E3 (SABiosciencesTM, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA) (2X) Kit (Thermo Scientific #K0223). Real-time PCR is widely used for quantification of mRNA levels and is a fundamental tool for basic research, biotechnology. References genes are expressed in a wide variety of tissues and cells with minimal variations in their expression levels, and thus are used to normalize data of mRNA quantification which help control for internal differences and reduce error between samples, (De Lima Rebouças *et al*., 2013 ;Sun *etal.*,2015). β-actin primers were designed using the online internet based interface (http://frodo.wi.mit.edu/primer3).
Forwardwas 5’ TAACGAGAGGTTCCGTTGCC ’3and reverse was5’ CGGT GGT GGTGAACGAGTAA’3.

Expression profile of hsp90 (GenBank Accession No. DQ988682) mRNAs of *P. interpunctella* and *E. Kuhenilla*,one primer matching the genome of the two insects (because they are belonging to one family pyralidae)

was designed to amplify the complete coding sequence (CDS) of hsp90 gene from total genomic DNA by using the online internet based interface (http://frodo.wi.mit.edu/primer3).
Sequence of hsp90 forward Primer was: 5'ATGATTGGGCAGTTCGGTGT'3 and reverse primer was: 5'CGGACGCACAGTGAATGAAC'3.
Triplicate samples were used in all measurements of qRT-PCR.

2.d. Quantitative real time PCR (RT-qPCR) analysis:

Data analysis from real time PCR was achieved using relative quantification (Livak and Schmittgen, 2001). Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments.

Delta Ct = $Ct_{\text{gene test}} - Ct_{\text{housekeeping gene}}$

Delta Delta Ct = $\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$

RQ = Relative quantification = $2^{-\Delta\Delta Ct}$

Where Ct means cycle threshold and defined as the number of cycles required for the fluorescent signal to cross the threshold.

3. Statistical analysis:

Data of the experiments were made using SPSS program, Version 20.0, expressed as mean ± standard error (SE). Levels of significance for differences of means were determined using Student's t-testfor independent. The level of significance for each experiment was set at $P \leq 0.05$and $P \leq 0.01$.

RESULTS AND DISCUSSION

Heat shock proteins (hsps) have been widely studied in many fields of biology and a large number of publications describe their molecular and physiological functions. These were highly conserved and ubiquitous proteins that are best known for the responsiveness to different stresses (Yi *et al*., 2018). Success in analysis of gene expression by qRT-PCR depends on the appropriateness of the designed primer and probes to be used. The specificity technique is internally linked to annealing of the primer to their complementary targets. For this selection of nucleic acid sequence is an essential step in designing primers for qRT-PCR (Sobhy and Closon, 2012). In the current study, the expression changes of hsp90 gene in 5th larval instars of *P. interpunctella* and *E. kuehniella* parasitized by *B. hebetor* were followed up to 72h after the parasitism. Significant downregulated of hsp90 gene was observed at all interval periods in response to the parasitism. With exception, a high significant up regulation of the gene was recorded after 48h of parasitism in *P. interpunctella* host larvae. The level of hsp90 gene in parasitized *P. interpunctella* larvae (Fig. 1) was significantly down regulated at 12h,it recorded (0.469 ± 0.10) and high significantly upregulated at 48 h, it recorded (1.824 ± 0.128) post parasitization compared with control. While a high significant down regulation of the gene was found at 24 and 72 h post parasitization and recorded (0.327±0.07) and (0.112 ± 0.04),respectively compared with control.

There was obvious positive relationship between hsp90 level and the time of post parasitism in case of parasitized *E. kuehnilla* larvae (Fig. 2).The level of hsp90 gene was highly significant downregulated in all treated larvae at 12, 24, 48 and 72h after parasitization compared with control. They recorded(0.08 ± 0.04, 0.131 ± 0.05, 0.213 ± 0.07, 0.395 ± 0.05), respectively.However, the rate of gene expression was significantly decreased in all time intervals of post parasitism compared with the control.

In the present result the down-regulation of hsp90 was unexpected because parasitization induced host

cellular stress, disrupted physiology and induced immune defense reaction, in haemolymph. This agrees with the finding of Caron *et al*.(2008) who reported that parasitization induces imune reaction by production of reactive oxygen species (ROS) which lead to protein denaturation or proteotoxicity in the host cell itself, and is toxic to the parasitoid offspring of the host larvae. Meanwhile, Wang *et al*. (2016) ; Shafeeq *et al*.(2017) concluded that hsp90 suppresses the immune development and protect the damage of cellular structure and function. But despite the upregulation of hsp90, the opposite was observed in the current study. The down-regulation of hsp90 of all treatment of the two host larvae except at 48 in case *P.interpunctella* might suggest that, subsequent parasitization by *B.hebetor* wasp, on host larvae causes a decrease in hemolymph immunity and down regulate the hsp90 gene.

The upregulation of hsp90 gene in *P.interpunctellae* host larvae at 48h after envenomation due to stress of parasitism, might increase immune response of host hemolymph to protect the host larvae from the venom of this wasp. Subsequent parasitization by *H. hebetor*, on host larvae causes a decrease in hemolymph immunity and down regulate the hsp90 gene in other treatments in*P. Interpunctella* and *E.Kuhenilla.* Many researchers reported that there wasa significant down regulation of the level of hsp90 host-parasite interaction in different insects. In addition,Rinehart *et al*. (2002) found that the level of hsp90 was slightly down regulated in *Sarcophaga crassipalpis* injected by *Nasonia vitripennis* venom,when compared to unenvenomated controls. Zhu *et al*. (2013) reported similar interactions between *Pieris rapae* (Lepidoptera: Pieridae) and its parasitoid *Pteromalus puparum* (Hymenoptera:Pteromalidae) which lead to down-regulation of hsp90 in parasitized pupae. The level of hsp70 in *P. interpunctella* larvae was gradually increased with a high level until 4 days after envenomation by *B. hebetor* (Shim *et al*., 2008).

Gene expression of the hsps in *Bombyx mori* eggs parasitized by *Telenomus theophilae*was differentially influenced by parasitization. Upregulation of hsp genes may play essential roles in silkworm eggs against parasitoids (Wang *et al*., 2016).In addition,Shafeeq *et al*. (2017) reported that the genes associated with the host larvae of *P. interpunctella* were differentially influenced by *B. hebetor* envenomation, and stress and immune responses were upregulated,after envenomation. While those associated with metabolism and development were downregulated. Meanwhile, Fang *et al*.(2016) cleared that only venom components from endoparasitoid *Pteromalus puparum* produced antimicrobial peptides (AMPs). Moreover, Calreticulin venom component, suppress cellular immune responses in its host *Pieris rapae.*Other venom components from this parasitoid wasp suppress the host genes cecropin and lysozyme involved in humoral immune responses. In addition, Rivers *et al*. (2009) stated that the venoms of several ectoparasitoids contain paralytic factors and developmental suppression that interact with hormones and prevent molting of the host so that the parasitoids are not removed from the host's external surface. Moreover, Beckage and Gelman (2004) concluded that the venoms of several ectoparasitoids contain endocrine disruptors that reprogram the development. In early study, Lezzi (1996) suggested that there is a functional connection between hormones and heat shock regulatory systems. Therefore hsps play an essential role in the biological process of development. Moreover, some authors studied the gene expression under different biotic stress. They noticed that the envenomation of the wandering fifth instar

larvae remarkably increased hsp90 levels by 2.8-folds. Its level was highly elevated from the first day after envenomation then slightly downregulated after 4 days. However, the levels of hsp90 did not change in the feeding or starved envenomated larvae compared to the control over the experimental period. Nonetheless, hsp90 gene up regulation was observed in several insects subjected to biotic or abiotic stresses. The expression of hsp90 in *Sitophilus Zea maize* exposed to different stress factors of heat shock and cold shock (Tungjitwitayakul *et al*., 2015) or ultraviolet-C and microwave irradiation(Tungjitwitayakul *et al*., 2016) was significantly increased in responses to the stress. This increase in the gene expression indicates that the chaperone actions of hsp90s are involved in tolerance in stressed cells (Morano *et al*., 2012). Hsp90s appears to participate in the maintenance of muscle structures and down regulation of the hsp90 system causes defects in muscle cells and lead to paralysis (Gaiser *et al*., 2011). Zhu *et al*. (2013) concluded that the transcription of hsp could be a component of the syndrome of parasitized hosts. The regulation of the hsps expression in an insect after parasitization could protect the host's tissues and the developing parasitoid from noxious agents produced by the host's immune response.

In conclusion, the present data indicated that parasitism had a significant impact on suppression of hsp90 genes. As the gene down regulation might be play an important role in host paralysis and inhibit its development to create the appropriate conditions for parasitoid offspring growth.

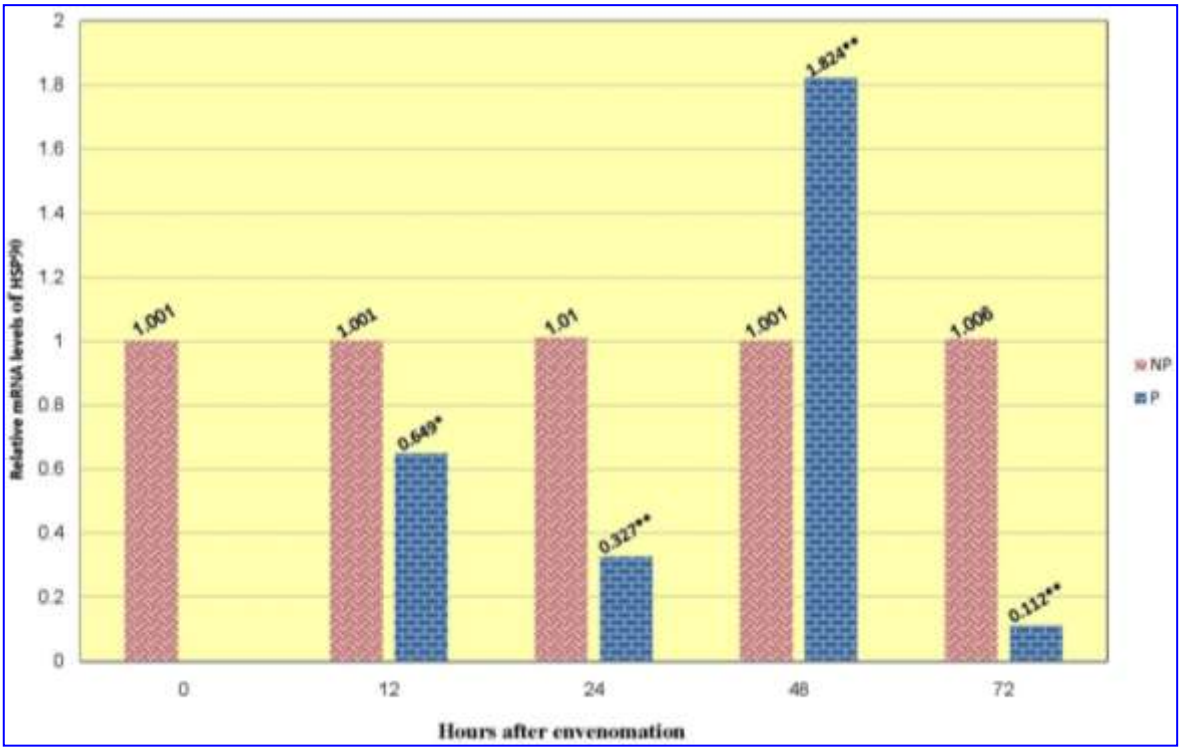

Fig. 1.The expression levels of hsp90 gene in parasitized *P. interpunctella* 5th larval instar by *B. hebtor* at different time intervals post parasitization. The mRNA expression levels for all samples were assessed by qRT-PCR.

* Significance at $P \leq 0.05$.** High Significance at $P \leq 0.01$.

NP: non parasitized larvae, P: parasitized larvae

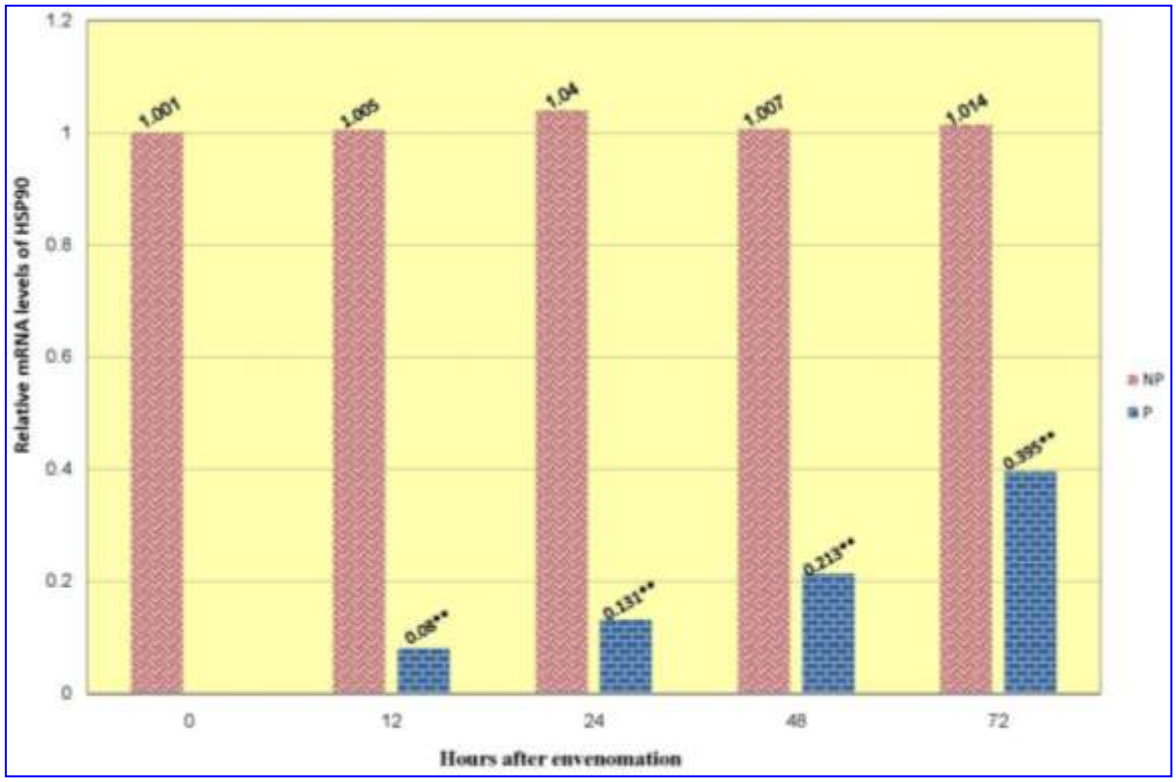

Fig. 2.The expression levels of hsp90 gene in parasitized *E. kuehniella* 5th larval instarby *B. hebtor* at different time intervals post parasitization. The mRNA expression levels for all samples were assessed by qRT-PCR".
* Significance at P ≤ 0.05.** High Significance at P ≤ 0.01.
NP: non parasitized larvae, P: parasitized larvae

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parasitization by *Pteromalus puparum*. Ins. Sci. 20: 183-193.

التعبير الجيني لبروتين الصدمة الحرارية 90 في يرقات *البوليديا إنتربنكتيلا* و*الإيفستيا كيونيلا* (حرشفية الأجنحة - بيريليدي) المتطفلة بدبور *البراكون هيبيتور*(غشائية الأجنحة - براكونيدي)

عايدة سعيد كامل ممتاز- روحية حسن رمضان – فاتن فريد أبو الدهب – مني فوزي عبد العزيز – هبة عبد الخالق
قسم علم الحشرات-كلية العلوم-جامعة بنها

المستخلص

تلعب سموم الدبابير المتطفلة التي تحقن في العائل دورا حيوي ا في نجاح التطفل ويمكن أن تؤثر علي العائل فسيولوجيا فتسبط الإستجابة المناعية لة .كما تحث مجموعة بروتينات الصدمة الحرارية ضغوطا فسيولوجية للعائل وتلعب دورا هاما في تنظيم العلاقة بين العائل والطفيل . وبإستخدام تفاعل البلمرة المتسلسل الكمي الحقيقي لتعيين التغيرات في بروتين الصدمة الحراريه 90 في يرقات *البوليديا إنتربنكتيلا* و *الإيفستيا كيونيلا* بعد التطفل بدبور *البراكون هيبيتور* وأشارت النتائج إلي أن مستوي التعبير الجيني لبروتين الصدمة الحرارية 90إختلف في كل من العائلين علي مدار كل الأوقات (12 , 24 , 48 و 72 ساعة). بينما الإرتفاع المعنوي لمستوي الجين سجل عند 48 ساعة مقارنة باليرقات الغير معاملة (كنترول)ولوحظ أن التعبير الجيني لمستويات بروتين الصدمة الحرارية 90 ليرقات *الإيفستيا كيونيلا* كانوا مرتفعين معنويا في كل المعاملات مقارنة بالحشرات غير المعاملة (كنترول) ونستنتج مما سبق أن قمع جين الصدمة الحرارية 90 من إستراتيجيات العائل, القادرة علي تنظيم فسيولوجيا العائل وإحباط الإستجابة المناعية لة. وأن بروتينات الصدمة الحرارية 90 يمكن أن تلعب دورا مهما في شلل العائل وتثبيط نموة لخلق ظروف مناسبة لنمو نسل الطفيل *براكون هيبتور* في مناخ جيد .