Transcriptomic and proteomic analysis of the most important olive pest, the insect *Bactrocera oleae*, with emphasis on sex differentiation and insecticide resistance systems

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The olive fruit fly, *Bactrocera oleae* (Tephritidae), is the most important enemy of cultivated olives, causing around 30% of damages per annum. Control of the fly is mainly based on chemical insecticides. In our country, organophosphate and pyrethroid insecticides are primarily used, however, the naturalyte spinosad has been introduced in recent years. Intensive as well as improper use of insecticides has hazardous effects, such as ecological disturbances, establishment and spread of resistance, dangerous toxicological effects and consequences on human health. More environmentally friendly approaches have also been proposed, such as the sterile insect technique (Sterile Insect Technique, SIT), which involves the mass rearing and releasing of sterilized insects in nature. This method has been successfully used to control other insects (eg. Mediterranean fly) in many regions worldwide, however the SIT has not been successfully applied in the olive fly yet. As has been shown in many cases, the successful application of SIT presupposes good knowledge of the insect's biology and ecology, as well as the availability of effective molecular and genetic tools. Given the minimal information about olive fly's genome, this research aimed at the development of proteomic and transcriptomic tools in order to identify and isolate genes that are involved in pathways of sex differentiation or and spinosad resistance.

Transcriptomic analysis: In order to identify genetic loci that are involved either in spinosad resistance or that are implicated in sex differentiation, RNA was isolated either from heads of sensitive and resistant to spinosad insects, or from the reproductive systems of male and female flies. RNA was converted into cDNA and subsequently massively sequenced in the SOLiD platform of the Fleming Institute in Athens (Table I). The bioinformatic analysis resulted in a number of genes with differential expression in spinosad sensitive and resistant flies, as well as in male and female reproductive systems of *B. oleae*. Processing and comparison of more than 13,000 genes identified the statistically significant upregulation of nine genes and statistically significant downregulation of ~40 genes between sensitive and resistant to spinosad insects. Similarly, 330 genes were upregulated in the female reproduction system while 1238 were downregulated in male testes flies (Fig.1). Several genes were further selected for validation through qRT-PCR (Figures 2 & 3).Twelve reproductive loci were considered: *kl2 (male fertility factor, kl2), kl3 (male fertility factor, kl3), kl5 (male fertility factor, kl5), ory (occludin-related Y protein), fem-1 (sex-determining protein fem-1), gas8 (growth arrest specific protein 8), lobo (lost boys), ix (intersex), pbl (pebble)* and *hcf (host cell factor C1)* that were overexpressed in males and the two genes *sox* and *pcp (pupal cuticle protein 78E)* that were overexpressed in female flies. The results confirmed significant overexpression in male testes for genes *kl2, kl3, kl5, ory, fem-1, gas8, lobo, pbl, hcf* and significant overexpression of *pcp* in female tissues while minimal differences were observed in *ix* expression between males and females. qRT-PCR did not validate the expression profile of the *sox* gene obtained from RNA-seq (Figure 4).Furthermore, validation of the genes implicated in spinosad resistance was performed on two resistant and two sensitive populations of *B. oleae*. The resistant populations were: a laboratory strain (SPIN) selected by continually increasing amounts of the insecticide in its diet; and a wild population from California (w-CAL) which has a 13 times greater resistance compared to the laboratory sensitive strain. The sensitive populations were: the laboratory strain (LAB) which has been reared in laboratory conditions over the past ~40 years; and a population derived from olives of the region of Volos-Agria (w-GR) where insecticides are not used. In total, sixteen loci were studied, many of which belong to energy metabolism groups: *Yolk protein 2* (*Yp2*), *ATP synthase FO subunit 6* (*ATP synthase*), *Low affinity cationic amino acid transporter 2* (*CAT-2*), *Serine protease 6* (*SP6*), *4-nitrophenylphophatase* (*pNPPase*), *Salivary Cys-rich secreted peptide-vWF* (*SalCys*), *Cytochrome P450 6a23-like* (*Cyp6α23*) and *Antigen 5 precursor* (*Ant5*) were upregulated in resistant to spinosad insects; and *Heat-shock protein 70* (*Hsp70*), *Heat-shock protein 23* (*Hsp23*), *Larval serum protein 1* (*LSP1*), *HexamerinL1* (*HexL1*), *Chitinase 5* (*Cht5*), *Oxidase/peroxidase* (*oxidase*), *Macrophage mannose receptor 1* (*mmr1*) and *Cell division cycle-associated protein 7* (*Cdc*) were down regulated in resistant flies. The results confirmed a significant overexpression of genes *Yp2, ATP synthase, CAT-2, SP6,*

pNPPase, *SalCys* and *Cyp6α23* in resistant insects and significant overexpression of genes *Hsp70, Hsp23, LSP1, HexL1, Cht5* in sensitive flies. For genes *Ant5, oxidase, mmr1* and *Cdc* the RNA seq result was not confirmed (Figure 5).

Proteomic analysis: For proteomic analysis, a two-dimensional electrophoresis (2D-PAGE) for heads of male and female insects was initially performed, in order to study the differential expression of proteins in the two sexes of the fruit fly. Subsequently, the differentially expressed proteins were identified, isolated, followed by digestion with trypsin and analyzed by mass spectrometry in the Athens Fleming Institute. Finally, the results were analyzed through bioinformatics analysis. In Table II the male and female differentially expressed proteins are shown. These proteins belong to different functional groups such as cytoskeleton, metabolism, synaptosomal, signal transduction and oxidative stress. Moreover, a new peptidomic approach was applied. In order to discover the mechanisms involved in sex differentiation systems, at first tissues were collected from the reproductive systems of male and female insects which were then homogenized in the appropriate lysis buffer and the peptides were isolated. MALDI TOF-TOF analysis was subsequently followed and the outcoming results were analyzed with the MASCOT software. This analysis successfully identified peptides overexpressed in either male or female olive fly tissues.