Effects of LPS and Juvenile Hormone III treatments on cell growth and gene expression in the *Ceratitis capitata* (Diptera Tephritidae) CCE/CC128 cell line.

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Abstract

The Mediterranean fruit fly, *Ceratitis capitata*, is one of the most important insect pest species in the world. It has a high colonization capacity and population variety giving it considerable genetic diversity. Strategies for its control have included the sterile insect technique (SIT) and insect growth regulators (IGRs). Many works have analysed the medfly transcriptome, and along with the medfly genome sequence, the sequences of multiple genes related to sex determination, mating, development, pheromone detection, immunity or stress have been identified. In this work, the medfly CCE/CC128 cell line was used to assess cell growth variation and changes in the expression of genes covering different functions, after lipopolysaccharide (LPS) and Juvenile Hormone III (JHIII) treatments. No significant effects on cell growth and gene expression were observed in the cells treated with LPS. In the cells treated with JHIII, the results showed no significant effects on cell growth, but an overexpression was found of the *Shade* gene, one of the Halloween gene members of the cytochrome p450 family, which is involved in development and the synthesis of 20-hydroxyecdysone (20E). This work shows preliminary results on the insect cell line in combination with whole genome sequencing which can facilitate studies regarding growth, toxicity, immunity and transcriptome regulations as a response to different compounds and environmental alterations.

Keywords: medfly, Mediterranean fruit fly, genome, gene expression, insect cell line, development, cell challenging, transcriptome

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Introduction

Ceratitis capitata (Wiedmann) (Diptera Tephritidae), the Mediterranean fruit fly (medfly), is an insect pest which causes considerable damage to many crops (Liquido et al., 1991). Although its origin is thought to be in sub-Saharan Africa, the medfly has great invasive potential that has led to a wide distribution (Gasperi et al., 1991; Gasperi et al., 2002; Li et al., 2009; Szyniszewska & Tatem, 2014; EPPO, 2018). The invasive capacity of the species has been amplified by human interactions, and multiple introductions have resulted in a high genetic variability among all medfly populations.

Understanding its functional genomics is essential for developing pest control strategies (Malacrida et al., 2007; Scolari et al., 2014). Sterile insect technique (SIT) programs are used in some areas to control the population growth and are based on the use of genetic sexing strains (GSS), i.e. the release of sterile males previously reared in high numbers (Calla et al., 2014).

The genetics of *C. capitata* have clarified the role of many genes involved in various functions. The sex determination transformer-2 gene, responsible for female determination during insect development has been identified (Pane et al., 2002; Pane et al., 2005; Gomulski et al., 2008; Salvemini et al., 2009; Saccone et al., 2011). This knowledge can help in developing strategies to regulate the sex ratio in medfly populations for release, for example, in SIT programs (Liu et al., 2015).

Many other studies have also been performed searching for genes related to key processes in the medfly life cycles in order to understand the mechanisms involved in reproduction and survival. Gomulski et al. (2012) obtained a transcriptome profile of genes related to maturation and mating effect. Genes from early embryos of *C. capitata* have also been identified to increase knowledge of the mechanisms of sexual determination (Salvemini et al., 2014a, 2014b). Other studies have determined chemosensory genes of *C. capitata*,

encoding for various odorant binding proteins (OBPs) which play a key role in the detection of allelochemicals from host plants (Siciliano et al., 2014).

The entire genome sequence of the medfly was reported by Papanicolaou et al. (2016), who provided crucial information for further genomic studies. Genome studies facilitate the identification of multiple genes related to other aspects of medfly biology, such as those involved in detoxification processes or responses to stress or immune-related genes. One of the most interesting gene categories includes genes related to development, which belong to the cytochrome p450 family (Feyereisen, 2006). Some of these genes are related to the synthesis of 20-hydroxyecdysone (20E), the hormone that controls the ecdysis which regulates development and metamorphosis in insects (Petryk et al., 2003). On the other hand, the juvenile hormone (JH) retains immature instars in insects, thus preventing metamorphosis (Goodman & Granger, 2005). A recent study on *Drosophila melanogaster* (Liu et al., 2018) showed the antagonistic actions of JH and 20E within the ring glands together with the existence of cross-talk between these two hormones in the regulation of insect metamorphosis.

The availability of established cell lines for *C. capitata* has led to several studies on aspects related to cell biology. These lines have thus been used for the detection of viruses (Plus et al., 1981), an investigation on the radiosensitivity of cells (Cavalloro et al., 1984), the induction of resistance to malathion (Rossi & Rainaldi, 2000), the evaluation of 20E treatments on cell growth and cell thermotolerance, and the identification of heat shock proteins (Jang, 1988 and 1992). Insect cell cultures are useful for studies in virology, immunity, biochemical mechanisms, toxicity and are an interesting means to boost the development of pest control strategies (Smagghe et al., 2009).

Data on the whole medfly genome and the availability of an established cell line have led to the development of *in vitro* methodologies for testing compounds and stress conditions. Checking the cell response by their growth modifications,

survival and transcriptome alterations via gene expression analyses provides a faster, easier and less harmful way than the one performed *in vivo* assays.

In this work, an established *C. capitata* cell line was exposed to two different treatments, with a lipopolysaccharide from *Escherichia coli* (LPS) and Juvenile Hormone III (JHIII). The aim was to investigate the treatment effects on cell growth and on the regulation of genes related to the immune system, response to stress, sexual determination and development.

The reviewers may comment that your Introduction reads like a history lesson, it doesn't explicitly explain why your approach is better / different than previous approaches. Why does the reader need to read the Introduction if you make no comparisons with your own work?

Materials and Methods

Cell line and identity check

The CCE/CC128 cell line was used for this work. This was established by Cavalloro et al. (1981) from *C. capitata* fertilized eggs. Cells were stored and frozen at the Tissue Culture Service of the University of Murcia (Spain). An aliquot of 3 x 10^6 cells was thawed and cultured with Shields and Sang's culture medium (Sigma-Aldrich®, St Louis, MO, USA) with 10% FBS (PAA) and 100 U/mL penicillin at 25 °C.

DNA from approximately 10^6 cells was isolated using the Invisorb® Spin Tissue Mini Kit (Stratec Molecular GMBH, Berlin, Germany). Cytochrome oxidase subunit I (*cox1*) was amplified by PCR to check the identity of the cell line using the primers from Simon et al. (1994). The PCR protocol was as follows: one cycle at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min; one cycle at 72 °C for 10 min. Amplicons were checked on 1.5% agarose gel and sequenced by Secugen (Madrid, Spain). BLASTn query was used to compare the obtained sequences to those of the sequence database.

Treatments

Two different treatments were conducted on the *C. capitata* cells: a) LPS from *E. coli* treatment at 1 μg/ml and b) JHIII treatment at a concentration of 10 μM. Both treatments were carried out in parallel.

The effect of each treatment was evaluated in relation to: i) the variations in cell growth after the JHIII and LPS treatments; ii) the changes in expression for a selection of genes of interest.

Effects on cell growth

A quantity of approximately 5 x 10^5 cells/well was seeded on 12-well plates with 1ml of fresh medium and incubated overnight. After this, 1 ml of fresh medium containing LPS (2 μg/ml; final concentration of 1 μg/ml) or JHIII (20 μM; final concentration of 10 μM) was added to their respective wells; 1 ml of non-supplemented medium was added to the control wells. Growth differences were assessed at 24h, 48h, 72h and 96h post treatment. Each treatment and time were performed in triplicate and cells were counted with a TC10™ Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA). Two-way ANOVA with Bonferroni post-hoc test analysis was carried out with the software GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA, www.graphpad.com) to assess the statistical significance among samples. The cells were photographed at each experimental time.

Effects on gene expression: RNA extraction, primer design, and RT-qPCR

To assess the expression of the *C. capitata* cell line transcriptome, the same concentrations and experimental procedures already described were used. However, treatment times were different from the cell growth experiment. In this case, the medium was removed after 1h, 3h, 6h, 12h, 24h and 48h and cells were stored in RNAlater (Qiagen, Crawley, UK). Sample homogenization and RNA isolation were performed using TRIzol Reagent (Life Technologies™) following the manufacturer's protocol and stored at -80 °C. The presence of

DNA contamination was removed with the TURBO DNA-free kit (Ambion®, Life Technologies™), RNA was quantified with NanoDrop 1000 from Thermo Fisher Scientific® (Waltham, MA, USA) and first strand cDNA synthesis was performed with the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan) and stored at -20 °C.

Nineteen target genes were checked for their amplification and efficiencies before considering them suitable for qPCR (shown in Table 1). Most were designed with Primer Express 3 software (Applied Biosystems®) directly from mRNA sequences obtained from the exploration of the *C. capitata* genome data viewer from the NCBI database. *FAMet* primers from Vannini et al. (2010) and *CYP6A51* from Arouri et al. (2014) were also used. In addition, five reference genes from Sagri et al. (2017) were selected to test their stability across conditions for use as an internal control in qPCR analyses. All gene efficiencies and specific amplifications were tested with a serial diluted pool of cDNA from *C. capitata* cells using the standard curve method on a StepOnePlus Real-Time PCR system. NZYSpeedy qPCR Green Master Mix (2x), Rox plus (Nzytech, Lisbon, Portugal) was used for the experiment and each dilution was amplified in triplicate. Melting curve analyses were performed to ensure the specificity of the amplification and non-template samples were used as negative controls. The PCR program was as follows: one cycle at 95 °C for 2 min; 40 cycles at 95 °C for 15 s and 60 °C for 30 s.

To assess the most stable and suitable number of reference genes for the qPCR experiment, all four candidate genes were amplified for the 6h samples under all conditions following the procedures described above. Amplification data were obtained with 7500 software v2.0.5 (Applied Biosystems®) and C_T values were transformed to logarithmic scale for stability analyses using two different algorithms: *geNorm* (Vandesompele et al., 2002) and *NormFinder* (Andersen et al., 2004).

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Those genes with a good amplification and appropriate efficiencies were chosen to test their relative expression in all conditions and times using, the previously selected reference genes as the internal control, according to their stability. The experiment was performed as described above, using all three biological replicates and three technical replicates for each condition. All gene efficiencies were considered for relative expression analyses using the $2^{-\Delta\Delta Ct}$ methodology (Livak & Schmittgen, 2001) considering efficiency corrections. Statistical analyses were performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA, www.graphpad.com), running a Student's t-test for the comparison with untreated samples.

Results and discussion

Cell line identity

The CCE/CC128 cell line identity was confirmed by the amplification and sequencing of cytochrome oxidase subunit I (*cox1*) fragment, which resulted in a single fragment which was consequently sequenced and returned a 100% identity after a BLAST search against the NCBI database.

Effects of treatments on cell growth

The number of cells at 24h, 48h, 72h and 96h after the treatment with LPS and JHIII was not significantly different from the control culture, thus the two xenobiotics did not influence the cell growth rate (Figure 1).

Cells treated with LPS grew as the control cells, which differed from the findings of Wittwer et al. (1997) in the moth *Estigmene acraea* cell line, where a concentration of 1mg/mL produced a slowed growth after 48 hours.

A delay in cell growth was observed in cells treated with JHIII, as confirmed by the photographs taken along the treatments (Figure 2).

In insect larvae, the juvenile hormone represses metamorphosis and prevents larval instar from becoming pupae and consequently reaching the adult stage

(Riddiford, 2008; Qu et al., 2018). Thus, an over exposure to this hormone is expected to generate a limitation in cell growth. Results from Soin et al. (2008) reported a cytotoxic effect of juvenile hormone analogs on S2 and Bm5 cell cultures, derived from *Drosophila melanogaster* and *Bombyx mori*, respectively.

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Effects on gene expression

Candidate reference gene expression and primer design

Five reference genes (*α-tub*, *14-3-3zeta*, *RPL19*, *β-tub* and *GAPDH*) were selected to test their stabilities before using them for the quantitative PCR experiment. However, *α-tub* efficiencies were outside the acceptable range and were discarded from the experiment. For the other four genes, *geNorm* and *NormFinder* algorithms were used to assess the stability of these genes after 6h of LPS and JHIII treatment, resulting in the ranking shown in Supplementary Table 1.

Overall, the chosen genes were very stable and were considered acceptable for their use in the RT-qPCR experiment in these conditions. These genes had been previously tested and selected as some of the most stable genes by Sagri et al. (2017) for other conditions such as developmental stages, body tissues, reproductive and olfactory systems, and in the present study they were useful in the condition tested in the *C. capitata* cell line. *geNorm* also provided the average stability values of the remaining genes during stepwise exclusion, thus confirming these results. At the same time it established two genes as the optimal number of reference genes needed for both experiments (Supplementary Figure 1). Thus, *14-3-3zeta* and *RPL19* were chosen as reference genes for further qPCR experiments.

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Nineteen primers for different genes were selected in order to test their expression in RT-qPCR after treatment, involving a broad spectrum of functions related to development, sex determination, immune system or response to

stress. All pairs of primers were designed from the different genes tested except for the juvenile hormone esterase (JHE) gene, for which two different primer pairs were tested. After testing their correct amplification and their efficiencies (Table 1), most of the genes did not produce optimal amplification results, either because they showed poor amplification efficiencies as no or a too late amplification was observed, or because amplification was not specific as shown in the melt curve analyses. Thus, all these primers and their corresponding genes were discarded and only *Hsp83*, *Tra-2*, *Toll7* and *Shade* were selected for the RT-qPCR experiment.

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Effects of treatments on gene expression

Gene expression results are shown in Figure 3. Transformer-2 is a known sex determination gene which triggers female determination when it is active during embryo development (Pane et al., 2005; Gomulski et al., 2008). In previous studies (Pane et al., 2002; Ruiz et al., 2007; Salvemini et al., 2009; Saccone et al., 2010) a putative role has been hypothesized of this gene in controlling medfly by altering its expression in order to produce phenotypic males, however no variations in its expression were found after any of the treatments. JHIII does not seem to regulate the activity of *Tra-2* gene and therefore it would not affect sex determination derived from the regulation of this gene. However, the juvenile hormone can affect the sexual dimorphism of sleep and the down-regulation of the transformer gene in *Drosophila melanogaster* (Wu et al., 2018). At the same time, the relation between JH and sex determination has been observed in female and male mandibles in stag beetles (Gotoh et al., 2014).

Hsp83 expression did not vary either as a response to LPS or JH treatments, although some down-regulation was observed after three hours however these results were not statistically significant. Heat shock proteins are triggered as a response to stress to ensure survival as has previously been reported in a

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different *C. capitata* cell line after a thermal challenge (Jang, 1992). In addition, a small heat shock protein (*Hsp27*) was characterised in this species, and the presence of ecdysone had an effect in its expression in cultured salivary glands (Kokolakis et al., 2008).

Many other heat shock proteins have been identified in the medfly (Thanaphum & Haymer, 1998; Papadimitriou et al., 1998; Theodoraki & Mintzas, 2006; Kokolakis et al., 2009). *Hsp83* has also been observed to be related to the presence of the Juvenile Hormone and the regulation of the JH response region in *Drosophila* (He et al., 2014). A study in *Locusta migratoria* (Luo et al., 2017) showed how two genes from the heat sock protein family *Grp78* were regulated by the Juvenile Hormone and play a role in vitellogenesis and egg production. The Juvenile Hormone does not seem to affect the expression of *Hsp83* in the CCE/CC128 cell line, but it may be involved in other ways in terms of development and protection - as seen in other insect species.

The Toll pathway is one of the signaling pathways present in insect species involved in immune responses and is activated via the Toll-like receptors (Boutros et al., 2002; Valanne et al., 2011). Besides the immune system, Toll-like receptors can play different roles and some have been proven to be related to embryonic development (Halfon et al., 1995; Espín-Palazón & Traver, 2016; Anthoney et al., 2018).

In this work, no signs of alteration in *Toll7* gene expression were found after treatments, suggesting that it is not linked to the response to the presence of LPS from *E.coli* or to an increase in JH. *Toll7* has been found to play an important role in *Drosophila* development as its presence was found at higher levels in embryonic and pupae tissues (Tauszig et al., 2000). *Toll7* might not be affected by LPS and JH in this cell line, however additional experiments comparing its expression with other Toll-like receptors and tissues would help to understand the behaviour and importance of these genes in the immune

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system and development. In addition, a wider study involving the expression analyses of other genes up and downstream of the Toll cascade is necessary to assess the importance of this pathway in the *Ceratitis capitata* embryonic cell line and how it is affected by any external pressure such as an increase in JH in the medium or the presence of pathogens.

Lastly, the only gene that was altered significantly by the Juvenile Hormone treatment was the *Shade* gene, with an increase in its expression after 1h, 6h and 12h. This gene is one of those known as the Halloween genes belonging to the cytochrome P450 family, which play a key role in insect development and are highly related to the synthesis of ecdysteroids and 20-Hydroxyecdysone (20E) (Petryk et al., 2003; Jia et al., 2013; Kong et al., 2014), a hormone involved in insect growth and triggering metamorphosis. This hormone appears to be present in several tissues of many insect species, including *Drosophila* (Gilbert, 2004; Iga and Smagghe, 2010). 20E and the Juvenile Hormone play antagonistic roles in development, as JH inhibits metamorphosis and 20E promotes it.

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Both hormones have been reported to suppress each other's biosynthesis pathways in order to regulate these processes in *Drosophila* mediated via crosstalk (Liu et al., 2018). We found that JHIII seems to cause an up-regulation of the *Shade* gene. This would indicate a positive relationship between the presence of JH and the formation of 20E, in contrast with the antagonistic behaviour that should be taking place. JHIII may possibly also generate a positive response in ecdysone synthesis to ensure normal growth and the survival of the embryonic cell culture. However, the ecdysone biosynthetic pathways are quite complex and not completely understood (Ou et al., 2016; Uryu et al., 2018). They involve many tissues and enzymes, and therefore this information regarding *Shade* expression may contribute to clarifying the role of this gene in the *C. capitata* cell line.

Many other genes and conditions should also be tested in order to have a wider spectrum of how different agents may alter the *C. capitata* transcriptome in this cell line and how this information could be extrapolated to understand the functioning of the whole organism.

This work has provided preliminary results on how having an entire genome sequence and an established cell line of a species helps in assessing the effects of different compounds or treatments on cell growth and the expression of multiple genes. This approach could provide an easy way to test the involvement of numerous genes with a great variety of functions related to the survival and defense of insect species. It could therefore help to generate a selection of genes to be studied on the whole organism in order to develop pest control strategies.

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Acknowledgements

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Table 1. List of primers used for RT-qPCR experiments showing reference work or genome accession number, amplicon length, efficiencies and their final selection for expression analyses based on their correct amplification, efficiencies or stabilities.

Gene	Genome accession/Reference	Primers	Amplicon length	Efficiency (%)	Selected for qPCR
Reference genes					
ribosome protein L19 *(RPL19)*	Sagri et al. (2017)	F=AACAAACGTGTACTGATGG R=CACGTACTTTATGTCGTCTG	103	97.9	Yes
glyceraldehyde 3-phosphate dehydrogenease *(GAPDH)*	Sagri et al. (2017)	F=ATGAAGGTCGTATCTAATGC R=TAGTTGCGTGAACAGTAGTC	115	104.3	
α-tubulin *(α-tub)*	Sagri et al. (2017)	F=GGTGCCCTACCCACGTATTC R=ACCATCTGGTTGGCTGGTTC	132	-	
β-tubulin *(β-tub)*	Sagri et al. (2017)	F=TCTCTACCAGTTGATGCAC R=CCGACAGAATAATGAACAC	105	108.2	Yes
14-3-3zeta	Sagri et al. (2017)	F=GGTCTAGCACTAAACTTTTC R=TGAGTCTTTGTATGAGTCC	138	104.8	
Target genes					
toll-like receptor Tollo	XM_004522201.2	F=GCTCACGCAAATCTCGCATT R=GCCGATGGCAGATTTGCTAT	82	-	
toll receptor 7	XM_012300338.2	F=GCAACTTCCTCTCCGACATCA R=GCCGTGTATGTCCAGCCATT	137	109	Yes
Ceratotoxin A	XM_004523284.2	F=CAATTCACCATGGCCAACCT R=TCGGCTACTACGCATTGCAA	80	-	
Ceratotoxin A (2)	XM_004523285.3	F=TTCCTCATCTGCATTGTTGCA R=AACCGGGAGGGCTTTTTTAA	120	-	
Defensin A	XM_004537571.3	F= TCGGCAAAAACGTTTCACTTG R= TGCAGTAACCGCCACGTATTT	92	-	
cytochrome P450 (*Shadow*)	XM_012307303.2	F=ACCAACTGCGCCTCATAAGC R=ACCACCTTCGACGCGTAGTC	88	-	
cytochrome P450 (*Phantom*)	XM_012305515.3	F=GCTGGGCAATGTGGATGTAGT R=ACCCATTATGCCGTGCGTTA	115	-	
cytochrome P450 (*Disembodied*)	XM_004525241.3	F=CCAGAGGAGCAACTGCCAAA R=CCCTGATCGGTGGGTAGGAT	89	-	
cytochrome P450 (*Shade*)	XM_004518670.2	F=TGGAAGAAAGCAGCCACGTT R=GCTACCAGCGGCTAGCTTTG	75	109	Yes
Juvenile Hormone Esterase	XM_004537487.2	F=AAATCATGATGGGCGTGGAT R=AAGCTCTGGGATTCGCAAAA	102	-	
Juvenile Hormone Esterase	XM_004537487.2	F=GCCGCTGCATCGAAGTTATT R=CTCAGCCGAAGGAGCAAATG	117	-	
Juvenile Hormone Esterase duplication 2	NM_001320386.1	F= GCGAACTTACGTGCGGAATT R=GCGTTCTGTGACTGCAGCAA	108	-	
Transformer-2	NM_001279408.1	F=TGGTGGTGTTCGCTGTTCAG R=CGAGAACGGGAACGTGACAT	90	106.3	Yes
Cell death protein Grim	XM_012300178.2	F=TTGTCATGCGCTACGCTTTT R=CAGCACTTGCCTCACTTTGG	98	-	
Cell death protein Hid	XM_004522085.3	F=ACCGGCGGTAATTTGTTCAA R=CACACGACGATGTCGATGCT	118	-	
Vitellogenin 2	XM_004524927.2	F= GCATACAACGGCCGTGTACA R=TTTGGACGGTTGGAGGATTC	92	-	
Heat shock protein 83	NM_001287826.1	F=TTGGAATTCCGTGCTTTGCT R=CGGCGGACGTACAGTTTGAT	98	102.5	yes
P450 *CYP6A51*	Arouri et al. (2014)	F=CGGAATACTTCCCTGATCCA R=TATGTGAGACCGACCAACGA	95	-	
farnesoic acid O-methyl transferase (*FAMeT*)	Vannini et al. (2010)	F=CAGCGAACTACCACCCTTTG R=TCATGTTTATTCACCTCCTCACC	165	-	

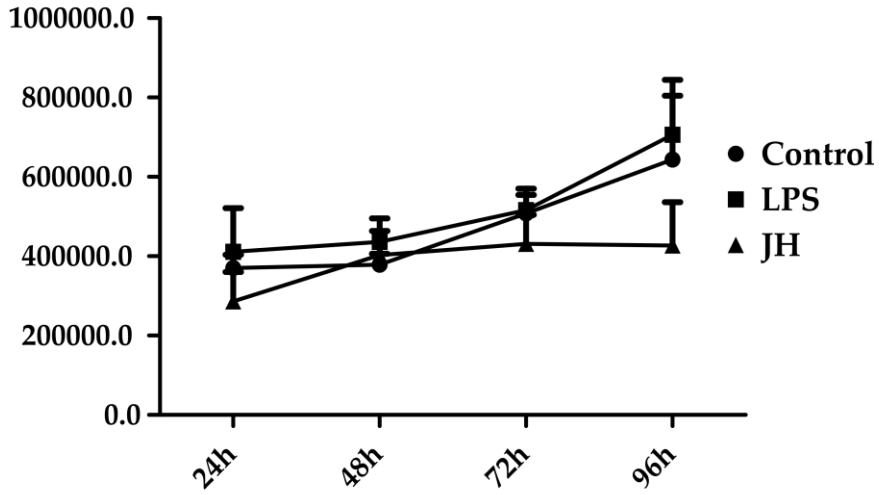


Figure 1. Cell number variation for control, LPS and JHIII cultures over time. Experiments were performed in triplicates; data were plotted as average values with standard deviation bars.

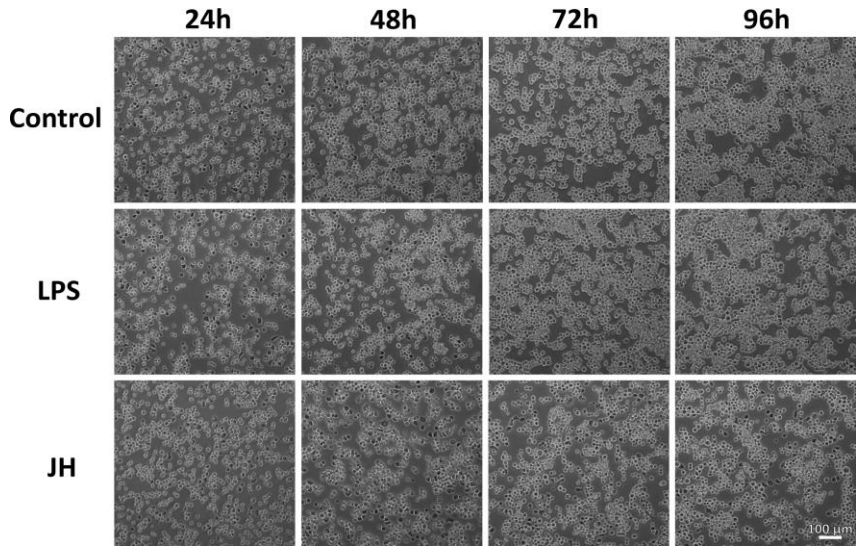


Figure 2. Evolution of the cell culture for control, LPS and JHIII treatments for all times of exposure. X-axis represents treatments, Y-axis represents time from culture initiation.

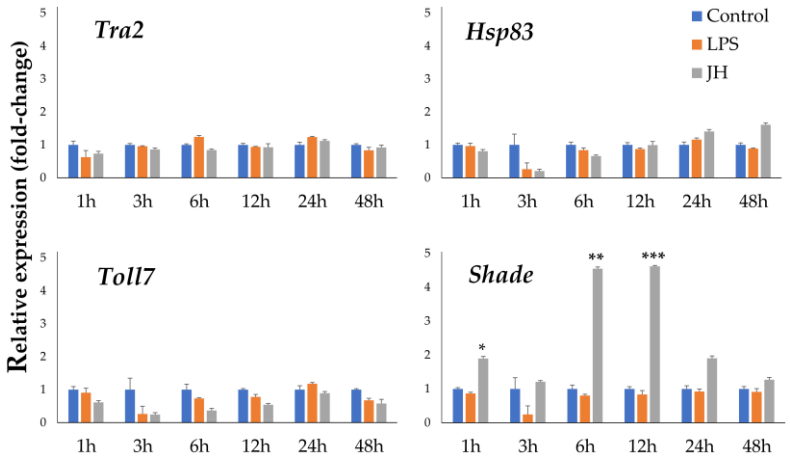
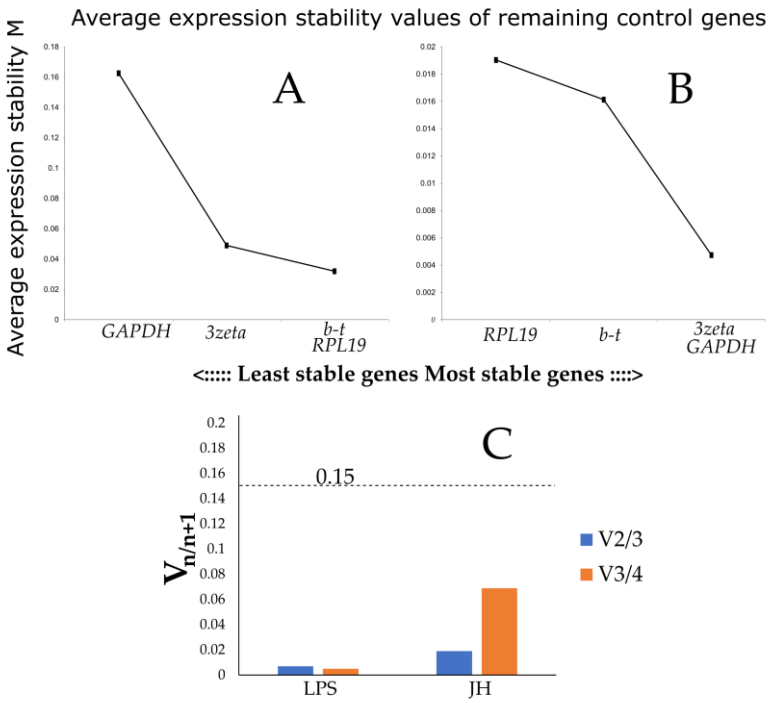


Figure 3: Relative expression of *Tra2, Hsp83, Toll7* and *Shade* genes for the LPS and JHIII treatments. Untreated samples were used as normalizer for each experiment. Error bars represent the Standard Error for the Normalized Relative Quantity. The statistical significance was obtained performing a Student's t-test. Letters indicate difference among tissue samples; * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

Supplementary data

Supplementary table 1. Ranking of candidate reference genes based on stability values obtained with *geNorm* and *NormFinder* for LPS and JHIII treatments in *C. capitata* CCE/CC128 cell line.

Condition		geNorm		NormFinder	
		Stability	**Rank**	**Stability**	**Rank**
LPS	*14-3-3zeta*	0.02	3	0.063	3
	RPL19	0.022	4	0.165	4
	GAPDH	0.017	1	0.039	2
	β-tub	0.017	1	0.017	1
JH	*14-3-3zeta*	0.143	3	0.089	3
	RPL19	0.115	1	0.189	4
	GAPDH	0.276	4	0.015	2
	β-tub	0.115	1	0.013	1



Supplementary Figure 1. Average stability values (M) of remaining reference genes during stepwise exclusion for *C. capitata* CCE/CC128 cell line in: A) LPS treatment; B) JHIII treatment. C) Optimal number of reference genes for normalization in *Ceratitis capitata* cell line. Pairwise variation Vn/n+1 values of candidate reference genes for both treatments tested were calculated with *geNorm* software by analysing the parwise variation (V) between the normalization factors NFN and NFN+1. Values < 0.15 are indicating that additional genes are not necessary for normalization.