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INFLUENCES OF *WOLBACHIA* (RICKETTSIALES RICKETTSIACEAE) ON THE CELLULAR RESPONSE TO COLD STRESS IN *DROSOPHILA MELANOGASTER* (DIPTERA DROSOPHILIDAE)

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Camerota M., Simoni S., Di Giaimo R., Bouneb M., Roversi P.F. – Influences of *Wolbachia* (Rickettsiales Rickettsiaceae) on the cellular response to cold stress in *Drosophila melanogaster* (Diptera Drosophilidae)

Wolbachia pipiensis (Hertig et Wolbach, 1924) is known to manipulate the expression of genes implicated in the metabolism, immunity and reproduction in *Drosophila melanogaster* (Meigen, 1830). Under stress, cells activate the cellular stress response (CSR). The CSR is a conserved network of pathways regulating identification, check and response to stress, preserving the cellular homeostasis. The CSR involves the unfolded protein response, autophagy, the heat shock response and other subcellular pathways. How *Wolbachia* affects the CSR has not yet been investigated. Here, we report the influence of *Wolbachia* infection and cold stress on the expression of the *Heat-shock-protein-70Aa* (*Hsp70Aa*), *Autophagy-related gene-1* (*Atg1*) and *X box binding protein-1* (*Xbp1*) genes and the influence of cold stress on the *Wolbachia surface protein* gene (*wsp*). The *Hsp70Aa*, *Atg1*, and *Xbp1* genes were affected by *Wolbachia* infection since they were found to be up-regulated in the *Wolbachia*-free flies. After cold stress, the *Wolbachia*-infected flies showed high expression of the *Atg1* and *Hsp70Aa* genes in comparison to the *Wolbachia*-free flies. Moreover, cold stress negatively influenced the expression of the *wsp* gene.

KEY WORDS: *Wolbachia* infection, cold stress, *Heat-shock-protein-70Aa*, *Autophagy-related gene-1*, *X box binding protein-1*.

INTRODUCTION

Insects activate the cellular stress response (CSR) when exposed to environmental stress such as high and low temperatures (KÜLTZ, 2003). The CSR, which plays an important role in preserving cellular homeostasis, is a highly conserved network of pathways involving the unfolded protein response (STEVENS and ARGON, 1999), autophagy (CUERVO, 2004), the heat shock response (MORIMOTO, 2008) and other subcellular stress response pathways (KOURTIS and TAVERNARAKIS, 2011). Under stress conditions, the endoplasmic reticulum (ER) functions of protein synthesis, folding and post-translational modifications are highly stimulated, generating unfolded or misfolded proteins that remain in the ER and compromise its homeostasis (LAI *et al.*, 2007). The ER then activates the unfolded protein response (UPR^{ER}) pathway in which *X box binding protein-1* (*Xbp1*) is the transcriptional factor regulating the transcription of genes involved in ER homeostasis and promoting the breakdown of misfolded proteins (KOURTIS and TAVERNARAKIS, 2011).

The autophagy pathway is involved in the normal turnover of long-lived cellular components and damaged proteins, in cellular development and differentiation (KOURTIS and TAVERNARAKIS, 2011). *Autophagy-related gene-1* (*Atg1*), the most conserved gene in all organisms including *Drosophila melanogaster* (Meigen, 1830) (MEIGEN *et al.*, 1869), plays a central role in the autophagy response (SCOTT *et al.*, 2007).

A key aspect of the CSR is the heat shock response, i.e. the production of heat shock proteins (Hsps) involved in protein transport and folding and the demolition of protein aggregates (SØRENSEN *et al.*, 2003). In arthropods, many Hsps are up-regulated in response to heat and cold stress. The *Hsp70* family includes the most widely studied Hsps under cold stress conditions; in particular, the isoform *Hsp70Aa* has been shown to play an important role in the response to thermal stress in *D. melanogaster* (COLINET *et al.*, 2009).

Wolbachia pipiensis (Hertig et Wolbach, 1924) (HERTIG and WOLBACH, 1924) is an endosymbiotic maternally transmitted α-proteobacterium found in arthropods (WERREN *et al.*, 2008) and influences the expression of genes involved in metabolism, immunity and reproduction in *D. melanogaster* (ZHENG *et al.*, 2011). *Wolbachia* is also found in nematodes, many of which are pathogenic for humans (RAO *et al.*, 2002). These nematodes show an obligate relationship with *Wolbachia* and its removal causes the inhibition of nematodes development and their death (RAO *et al.*, 2002). Despite the great importance of the interaction between *Wolbachia* and cell symbiosis, the influence of *Wolbachia* on CSR pathways has not yet been investigated.

The aim of the present study was to evaluate the influence of *Wolbachia* infection on cellular responses to cold stress in *D. melanogaster* by transcriptional analyses of the *Hsp70Aa*, *Xbp1* and *Atg1* genes, as well as the influence of cold stress on the expression of *wsp* gene, encoding for a surface protein of *Wolbachia*.

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MATERIAL AND METHODS

Fly culture

Drosophila melanogaster Oregon R-C, a *Wolbachia* infected wild-type line, was obtained from the Bloomington Drosophila Stock Center, Indiana University. Flies were reared in 42 ml bottles on standard medium (COLINET *et al.*, 2009) at 25 °C under a 12/12 h light-dark cycle in a climatic chamber (Binder KBWF 720, Tuttlingen, Germany).

Antibiotic treatment

To obtain a genetically identical *Wolbachia*-free *D. melanogaster* line from the *Wolbachia D. melanogaster*-infected stock, we isolated a pool of males and females flies on standard medium to which was added 0.25 mg/ml tetracycline antibiotic (FRY *et al.*, 2004). The treatment was carried out for two generations. To confirm that the treated *D. melanogaster* were free of *Wolbachia,* we extracted DNA (BOUNEB *et al.*, 2014) and performed PCR using wsp primers (BRAIG *et al.*, 1998) that amplify a *wsp* gene region of 590-632 bp coding for a surface protein of *Wolbachia*. The *Wolbachia*-free *D. melanogaster* line was then maintained on standard medium to ensure full recovery of the flies after the antibiotic treatment (FRY *et al.*, 2004).

Cold stress and recovery treatment

Four-day-old virgin *D. melanogaster* females, both *Wolbachia*-infected (W^+) and *Wolbachia*-free (W^-), were placed in 0.5 ml tubes, immersed in 10% of glycol solution precooled, and cold stressed at 0 °C for 9 h (COLINET *et al.*, 2009). The cold stressed flies (W^+ S and W^- S) were then allowed to recover at 25 °C for 2 h in 42 ml tubes containing the standard food (COLINET *et al.*, 2009).

In addition, the effect of cold stress on *Wolbachia* was evaluated by monitoring the *wsp* gene expression at 30 min, 2 h, 4 h, 6 h and 8 h of recovery at 25 °C.

Transcriptional analyses

For each treatment, a single *D. melanogaster* female was used for RNA extraction and purification. Four biological replicates were considered in the analysis. RNA extraction was carried out using the PureLink® RNA Mini Kit (Life Technologies) following the manufacturer's protocol. The purified RNA was treated with DNase (TURBO DNA-free™ Kit, Life Technologies). Three hundred nanograms of total RNA were considered for cDNA synthesis with the SuperScript® VILO cDNA Synthesis Kit (Life Technologies).

The *Xbp1* and *Atg1* coding sequences reported in Flybase (http://flybase.org/) were used to design primers. *Xbp1*: Xbp1_F: GGATGACGATAACATGGCTG, Xbp1_R: TCTCGTAGTCCATCTCCTCCAT; *Atg1*: Atg1_F: GGGTTTGCGCGATTCCTG, Atg1_R: GCCAGGTTAGCATTCTGC. The primers for the *Ribosomal protein S20* (*Rps20*) housekeeping gene, used as reference gene, and the *Hsp70Aa* were the same used by COLINET *et al.* (2009).

Real time-PCRs were carried out in a LightCycler® 480 system (Roche Diagnostic) in 96-well plates (Applied Biosystems®, Life Technologies) in reaction volumes of 20 µl containing: 10 µl of Power SYBR Master Mix (Applied Biosystems, Life Technologies), 0.3 µM of each primer and 1 µl of cDNA. The qRT-PCR conditions were 10 min at 95 °C followed by 39 cycles of 95 °C for 15 s, 56 °C for 30 s, 60 °C for 30 s. A melting curve analysis was performed to confirm the specificity of each primer pair. The relative quantification of gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ calculation method (LIVAK and SCHMITTGEN, 2001) than normalized using the *Rps20* reference gene.

Cold stress influence on the *Wolbachia* wsp gene

RT-PCR was performed to investigate on the expression of the *Wolbachia* wsp gene in infected flies after cold stress. RNA extraction and cDNA synthesis were carried out as described above. For PCR, 1µl of cDNA was used in 25 µl total volume containing: 0.2 µM of each dNTP, 0.3 µM of wsp primers (BRAIG *et al.*, 1998), 1X reaction buffer, 1.5 mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Invitrogen). Cycling conditions were: 5 min of denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 54.1 °C for 50 s, 72 °C for 50 s followed by post-amplification extension at 72 °C for 5 min. PCR products were loaded on 2% agarose gel and visualized under UV light.

Statistical analyses

One-way ANOVA was applied to the fold change in gene expression in order to evaluate the effect of *Wolbachia* infection on the CSR. Post-hoc comparison (Tukey test, $P<0.05$) was performed to analyse the difference in gene expression between treatments. All statistical analyses were carried out with SPSS v.13.0 (SPSS, 2004).

RESULTS

The effect of the cold stress and recovery treatment was significantly changed for all three genes (*Atg1*: $F_{1,3}= 27.37$, $P< 0.05$; *Xbp1*: $F_{1,3}= 322.79$, $P< 0.01$; *Hsp70Aa*: $F_{1,3}= 277.80$, $P< 0.01$). Prior to cold stress, all the genes were found to be up-regulated in the *Wolbachia*-free *D. melanogaster* line (W^-) in comparison to the *Wolbachia*-infected line (W^+) (Fig. I).The difference between the W^+ and W^- lines could be explained only by the effect of *Wolbachia* and not by the antibiotic treatment (FRY *et al.* 2004).

The response to cold stress in the *Wolbachia*-infected line was up-regulation of *Atg1* (1.90 fold) and *Hsp70Aa* (4.1 fold) and down-regulation of *Xbp1* (0.03 fold). The *Xbp1* gene expression was similar in the W^+ and W^- stressed lines, probably it was less responsive to cold stress than *Hsp70Aa* and *Atg1* genes. In the *Wolbachia*-free line, we observed a lesser extent of up-regulation of *Hsp70Aa* (2.5 fold) and down-regulation of *Atg1* (0.03 fold) after cold stress.

We also evaluated the transcriptional changes of the *Wolbachia wsp* gene by means of RT-PCR after 30 min, 2, 4, 6 and 8 h of recovery at 25 °C following a cold stress treatment of 9 h at 0 °C. This treatment negatively influenced the expression of the *wsp* gene throughout the time course. The *wsp* gene mRNA could not be detected for up to 8 hours of recovery after cold stress (fig. II), indicating that the *wsp* gene expression is very sensitive to cold stress and that its activity is hampered even after recovery of the host.

DISCUSSION AND CONCLUSION

The cellular stress response (CSR) is a universal network of pathways regulating identification, check and response to stress preserving the cellular homeostasis. The CSR includes the unfolded protein response (STEVENS and ARGON, 1999), the autophagy (CUERVO, 2004), and the heat

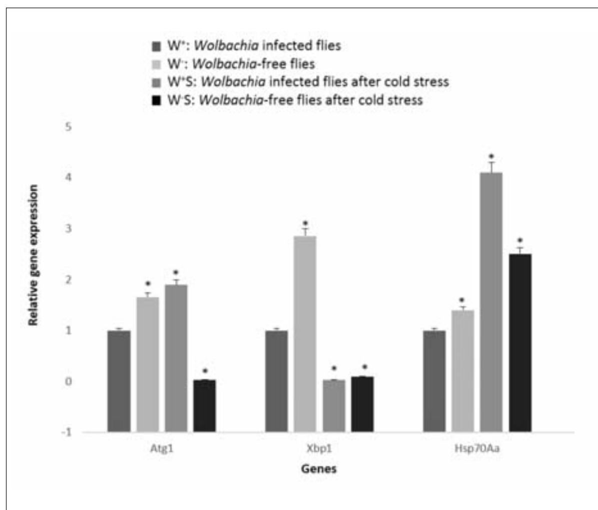

Fig. I – Relative *Atg1*, *Xbp1* and *Hsp70Aa* gene expression after antibiotic and cold stress treatments. Four biological replicates were considered in the analysis. W^+: *Wolbachia* infected flies; W^-: *Wolbachia*-free flies; W^+S: *Wolbachia* infected flies after cold stress; W^-S: *Wolbachia*-free flies after cold stress. An aterisk (*) indicates when the level is significantly different from the control (ANOVA post-hoc test, Tukey; P< 0.05).

shock response (MORIMOTO, 2008). Key proteins involved in these pathways are conserved in all organisms through the evolution (JOLLY and MORIMOTO, 2000; KÜLTZ, 2005). It has been also reported that *Wolbachia* infection influences the expression of genes involved in metabolism, immunity and reproduction in *D. melanogaster* (ZHENG *et al.*, 2011). In this study, aimed to evaluate the influence of *Wolbachia* infection on cellular response to cold stress, it was evidenced that *Wolbachia* infection affect the expression of *Xbp1*, *Atg1* and *Hsp70Aa* genes. In fact they are up-regulated in the *Wolbachia*-free flies. After the cold stress, the *Wolbachia*-infected flies exhibit high expression of the *Atg1* and *Hsp70Aa* genes compared to the *Wolbachia*-free flies. However the reason of this molecular response is still not clear, and more studies are needed to deepen the molecular mechanism of the interesting *Wolbachia* influence on the genes studied. Furthermore, it was assessed that the cold stress treatment affects negatively the expression of *Wolbachia wsp* gene even where the host was recovered, indicating that *Wolbachia* is more susceptible to cold stress than its host. These results can be interesting in view to test: a) the effects of cold stress on nematodes infected by *Wolbachia*, b) if other bacterial infections influence the response to various stresses. Further studies are also necessary to study: the possible involvement of *Wolbachia* in the manipulation of other cellular response pathways and the mechanism underlying the regulation of *Wolbachia wsp* gene expression after cold stress.

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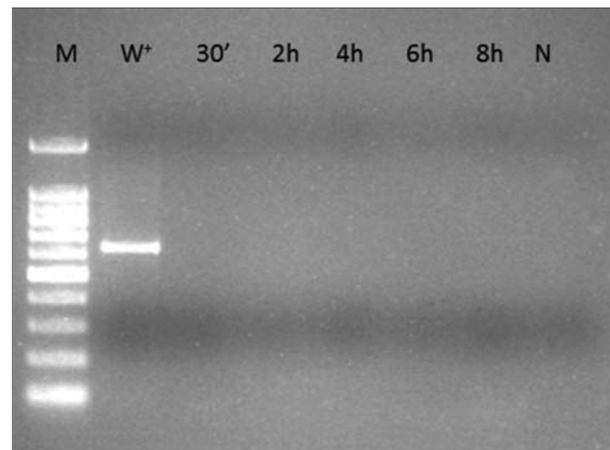

Fig. II – Gel electrophoresis showing the PCR product of the *Wolbachia* wsp gene. Lane 1 (M): 5 µl of marker (100-1500 bp, 5 PRIME); lane 2 (W^+): 10 µl of wsp PCR product (620 bp) of a W^+ fly; lane 3 (30'): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 30 min at 25 °C; lane 4 (2h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 2 h at 25 °C; lane 5 (4h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 4 h at 25 °C; lane 6 (6h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 6 h at 25 °C; lane 7 (8h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 8 h at 25 °C; lane 8 (N): 10 µl of negative control.

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