REAL-TIME PCR REVEALS ENDOSYMBIONT TITER FLUCTUATIONS IN *METASEIULUS OCCIDENTALIS* (ACARI: PHYTOSEIIDAE) COLONIES HELD AT DIFFERENT TEMPERATURES

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Real-time PCR (Polymerase Chain Reaction) can estimate *Wolbachia* endosymbiont population density in mosquitoes (Wiwatanaratanabutr & Kittayapong 2009). The COS (Carbaryl-OP-Sulfur resistant) colony of the phytoseiid *Metaseiulus* (=*Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) harbors several endosymbionts, including *Cardinium, Wolbachia, Enterobacter,* and *Bacteroidetes* in their gut and reproductive tissues (Johanowicz & Hoy 1996; Hoy & Jeyaprakash 2005). Johanowicz & Hoy (1998) observed mating incompatibility when females from a heat-treated (33°C) COS colony (a treatment presumed to eliminate *Wolbachia*) was crossed with males from a room-temperature (23°C) COS colony containing *Wolbachia,* resulting in the production of shriveled eggs that did not hatch. It was not clear whether the *Wolbachia* population was reduced or completely eliminated from the heat-treated mites because a standard PCR protocol was used, which is relatively insensitive compared to high-fidelity PCR (Jeyaprakash & Hoy 2000). In this paper, we estimate the endosymbiont population density in COS colonies of *M. occidentalis* held under different temperatures in the laboratory using real-time PCR.

One colony of *M. occidentalis* was maintained at room-temperature (23-25°C) and 2 colonies initiated from this line were maintained at 32 and 34°C for More than 1 year in growth chambers prior to experiments. Female mites (100) were isolated from each colony and their genomic DNA extracted by Puregene and QIAGEN methods (Jeyaprakash & Hoy 2007), and resuspended in 50 µL of sterile water. Four different plasmids carrying endosymbiont *16S rRNA* sequences; pAJ233 (*Cardinium*), pAJ234 (*Bacteroidetes*), pAJ235 (*Wolbachia*) and pAJ239 (*Enterobacter*), were extracted and their yield estimated with a BioPhotometer Plus (Eppendorf AG, Hamburg, Germany). Species-specific primers were designed with Primer 3 software (http://frodo.wi.mit.edu/primer3/) and a probe was designed with Primer X software (Applied Biosystems, Foster City, CA) from the variable regions for each species (Table 1). Real-time PCR was performed in a 20-µL reaction volume containing genomic DNA from 2 females (1 µL) or serially-diluted plasmid DNA (100 µg to 1 fg) or a no DNA control (1 µL), forward and reverse primers (400 pM), probe (100 pM) and TaqMan master mix (10 µL). Two linked profiles: (1) one cycle of 50°C for 2 min and 95°C for 10 min, and (2) 60 cycles consisting of denaturation at 95°C for 15 s, annealing at 59°C (*Cardinium*) or 57°C (*Wolbachia* or *Enterobacter* or *Bacteroidetes*) for 30 s and extension at 72°C for 30 s, were used. Each treatment was replicated 3 times. The starting copy number of all plasmid DNA dilutions used in the real-time PCR was obtained with an open-source software (http:/

TABLE 1. LIST OF SPECIES-SPECIFIC PRIMERS AND PROBES DESIGNED FOR REAL-TIME PCR.

Species	Primer and probe sequence	Product size
Cardinium	MoCardRT-F2 5'-GCCGGCGACCGGCGAATG-3' MoCardRT-R1 5'-CGGAGGCTATTCCCCAGTGT-3' MoCardProbe1 6FAM-TGCGTAATGCACATGC-MGBNFQ	66 bp
Wolbachia	MoWolRT-F1 5'-GCAACGCGAAAAACCTTACCAC-3' MoWolRT-R1 5'-CCGACCCTATCCCTTCGAAT-3' MoWolProbe 1 6FAM-TCTTGACATGAAAATC-MGBNFQ	65 bp
Bacteroidetes	MoBactRT-F1 5'-AGCGGCAGGCCTAATACATG-3' MoBactRT-R1 5'-CTCACCATCTTCGAGCAAGCT-3' MoBactProbe 1 6FAM-AAGTCGGACGGGATC-MGBNFQ	65 bp
Enterobacter	MoEnterRT-F1 5'-TGCCAGCAGCCGCGGTAAT-3' MoEnterRT-R1 5'-TTTACGCCCAGTAATTCCGATT-3' MoEnterProbe 1 6FAM-CGGAGGGTGCAAGC-MGBNFQ	59 bp

/molbiol.edu.ru/eng/scripts/h01_07.html). A standard regression curve was generated with the Ct-value (Number of PCR cycles required for sample fluorescence to reach the threshold level) obtained from all 9 serial plasmid DNA dilutions. The Ct-value obtained from the 2 female mites was then used in the regression analysis to estimate the copy number of bacterial cells present, which was divided by 2 to obtain the copy numbers in a single female.

The titer of *Cardinium* and *Wolbachia* was much higher than that of the gut endosymbionts *Enterobacter* and *Bacteroidetes* in the room-temperature *M. occidentalis* COS colony (Table 2). The *Cardinium, Wolbachia* and *Enterobacter* titer was reduced, but not eliminated, in both heat-treated colonies. By contrast, *Bacteroidetes* had a higher titer in the heat-treated colonies, but their density was below detectable levels (1 or 2) in the room-temperature colony, and could not be reliably amplified by real-time PCR (Table 2).

We think that the titer of *Wolbachia* or *Cardinium* could play a role in mating compatibility when making crosses between heat-treated (uninfected) females and room-temperature (infected) males. However, it is not clear which endosymbiont actually causes mating incompatibility in the COS colony because this requires generating colonies infected with only *Cardinium* or *Wolbachia*. Weeks & Stouthamer (2004) reported that *Wolbachia* infection in another colony of *M. occidentalis* is lost when fed a *Wolbachia*-free two-spotted spider mite diet. During the summer of 2009 we determined, by high-fidelity PCR, that the two-spotted spider mite culture in our greenhouse had inadvertently lost *Wolbachia* after being heated to 40-42°C for several months (3-6) due to a maintenance problem and, subsequently, the 23-25, 32 and 34°C COS colonies that were fed this *Wolbachia*-free spider mite diet were determined to have lost *Wolbachia* (data not shown), suggesting that the *Wolbachia* in these colonies of *M. occidentalis* was obtained from their spider mite prey.

To determine whether the *Wolbachia*-free COS colonies reared at 23-25 and 34°C exhibited incompatibility several inbred lines were generated. They displayed no fluctuation in *Cardinium* and *Enterobacter* titers (Table 2). A total of 7 or 8 reciprocal single-pair and control crosses were performed on 3 separate dates. Equal numbers of reciprocal and control crosses were successful and produced unshriveled eggs that subsequently hatched, suggesting that *Cardinium* is not involved in causing cytoplasmic incompatibility in the COS colony at these titers (Table 2) and that *Wolbachia* might be involved. It appears that this COS colony is obtaining *Wolbachia* by feeding on its *Wolbachia*-infected prey. These results are consistent with earlier data indicating that there are no differences in *16S, ftz* and *wsp* sequences from *Wolbachia* in the COS and *T. urticae* colonies (Hoy & Jeyaprakash 2005).

The standard PCR protocol previously used by Johanowicz & Hoy (1996) we now know is not sufficiently sensitive to be certain that endosymbionts truly are lacking (Jeyaprakash & Hoy 2000) and should not be used when comparing heat-treated and room-temperature colonies. Real-time PCR is an efficient tool to quantify endosymbiont titer based on this study and that by Wiwatanaratanabutr & Kittayapong (2009). This research was supported in part by the Davies, Fischer and Eckes Endowment in Biological Control to M. A. Hoy.

Summary

Real-time PCR amplification of *16S rRNA* sequences from 4 *M. occidentalis* endosymbionts; *Cardinium, Wolbachia, Enterobacter,* and

Table 2. Mean number (S.D.) of endosymbionts estimated per adult female *Metaseiulus occidentalis* maintained at 3 different temperatures[1].

Date	Colony	Rearing temperature	*Cardinium*	*Wolbachia*	*Enterobacter*	*Bacteroidetes*
8/10/09	COS	23-25°C	26,666 ± 23	13,585 ± 22	379 ± 12	BDL
	COS	32°C	127 ± 16	68 ± 15	10 ± 2	543 ± 12
	COS	34°C	138 ± 16	74 ± 15	15 ± 2	499 ± 12
9/25/09 COS inbred lines	C10-BaBA	23-25°C	18,516 ± 5	ND	264 ± 5	BDL
	C10-BA	23-25°C	19,199 ± 5	ND	274 ± 5	BDL
	C10-CB	23-25°C	20,892 ± 5	ND	295 ± 5	BDL
	F10-FAB	23-25°C	16,711 ± 5	ND	278 ± 5	BDL
	F10-BAA	23-25°C	19,907 ± 5	ND	322 ± 5	BDL
	F10-IOO	23-25°C	19,315 ± 5	ND	329 ± 5	BDL

[1]Means were obtained based on 3 reactions per condition.

BDL = Below detectable level (1 or 2); COS = Carbaryl-OP-Sulfur resistant; ND = Not detected (0) and had been fed two-spotted spider mite prey lacking *Wolbachia*.

Bacteroidetes, provided an estimate of their titer in colonies reared under 3 temperatures. This work also revealed, for the first time, that the *Wolbachia* and *Cardinium* titer were reduced in heat-treated COS colonies, but not completely eliminated. However, feeding *M. occidentalis* colonies the *Wolbachia*-free two-spotted spider mite diet did eliminate *Wolbachia* from this predator. No cytoplasmic incompatibility was detected when crosses were made between males reared at room-temperature (23-25°C) containing *Cardinium* (and lacking *Wolbachia*) and females reared at 34°C containing a much lower titer of *Cardinium* (and lacking *Wolbachia*), indicating that *Wolbachia* may be involved in causing previously observed incompatibilities rather than *Cardinium*.

References Cited

HOY, M. A., AND JEYAPRAKASH, A. 2005. Microbial diversity in the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae) and its prey, *Tetranychus urticae* (Acari: Tetranychidae). Biol. Control 32: 427-441.

JEYAPRAKASH, A., AND HOY, M. A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9(4): 393-405.

JEYAPRAKASH, A., AND HOY, M. A. 2007. The mitochondrial genome of the predatory mite *Metaseiulus occidentalis* (Arthropoda: Chelicerata: Acari: Phytoseiidae) is unexpectedly large and contains several novel features. Gene 391: 264-274.

JOHANOWICZ, D. L., AND HOY, M. A. 1996. *Wolbachia* in a predator-prey system: 16S ribosomal analysis of two phytoseiids (Acari: Phytoseiidae) and their prey (Acari: Tetranychidae). Ann. Entomol. Soc. America 89: 435-441.

JOHANOWICZ, D. L., AND HOY, M. A. 1998. Experimental induction and termination of non-reciprocal reproductive incompatibilities in a parahaploid mite. Entomol. Expt. Appl. 87: 51-58

WEEKS, A. R., AND STOUTHAMER, R. 2004. Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. Proc. R. Soc. London B (Suppl.) 271: S193-S195.

WIWATANARATANABUTR, I., AND KITTAYAPONG, P. 2009. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. J. Invert. Path. 102: 220-224.