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# Population Density of *Wolbachia* Bacteria and the Induction of the Popcorn-Effect in *Drosophila melanogaster*

ENDANG SRIMURNI KUSMINTARSIH

*Faculty of Biology, Universitas Jenderal Soedirman, Jalan Dr. Soeparno No 63 Grendeng, Purwokerto 53122, Indonesia*
*Phone: +62-281-638794, Fax: 62-281-631700, E-mail: endang_sk@lycos.com*

*Drosophila melanogaster* is known to harbour *Wolbachia* bacteria that cause the early death of its adult host. The death occurs early as a result of cell rupture caused by the proliferation of *Wolbachia* within it. However, the number of *Wolbachia* causing the early death in *D. melanogaster* is unknown. In order to determine whether the number of *Wolbachia* is related to the early death of its host, the quantity of *Wolbachia* and nuclei were examined using the real-time PCR procedure. The results showed that an egg cell contains at least 2-3 bacterial cells. A 4 day old larva contains at least 45 cells. The number of *Wolbachia* in adult flies of 1, 5, 8, and 12 days old were 422, 535, 964, and 610 per host cell, respectively.

Key words: *Wolbachia pipientis*, real-time PCR, *Drosophila melanogaster*

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*Wolbachia* are maternally inherited, intracellular, and obligate bacteria known to infect a wide range of arthropods. These bacteria were discovered by Hertig and Wolbach (1924) in the ovary of the mosquito *Culex pipiens.* Hertig (1936) formally named them as *Wolbachia pipientis* in honour of his collaborator. Recent surveys indicate that around 16-20% of all insect species may be infected with *Wolbachia* (Werren and Jaenike 1995). *Wolbachia* are also widespread in filarial nematodes. Infections with *Wolbachia* have been associated with host reproductive abnormalities such as cytoplasmic incompatibility, feminization, parthenogenesis, male killing (dying males caused by bacteria infectious in the ovaries of females) and the *Popcorn-effect*. It is named *Popcorn-effect* because when the bacteria multiply inside the fly's cells they cause a sudden massive degeneration of host cells, which resemble the baking process of "Popcorn" in a microwave (Min and Benzer 1997). The *Popcorn-effect* of *Wolbachia* has so far only been detected in one laboratory strain of *Drosophila melanogaster.* It is characterised by a sudden onset of widespread degeneration in somatic tissue such as the brain, retina, and thoracic muscle of *D. melanogaster.*

Min and Benzer (1997) and Werren (1997) explain that death occurs early as a result of cell rupture caused by the proliferation of *Wolbachia* within it. They note that *Wolbachia* are present at a small population level during development through the embryonic, larval, and pupal stages. As soon as the adults emerge, the bacteria start to multiply rapidly and this leads to an abrupt death during the adult's halfway lifespan. It might be possible to make use of the *Popcorn-effect* induced by *Wolbachia* to control insect vectors of important human diseases in which the pathogen or parasite is transmitted at the end of the insect's lifespan after it has taken three to four blood meals. In the future, this may play a vital role in the prevention of arthropod-borne diseases. Disease transmission depends on the older insect and *Wolbachia* infection has been found to shorten the lifespan of insects.

However, the cell population size of *Wolbachia* to cause the *Popcorn-effect* in *D. melanogaster* is unknown. Up to recent days, there has been no *in vitro* system for culturing *Wolbachia* within insect cells. The fastidious nature of *Wolbachia,* together with the difficulty in counting bacterial numbers inside tissues, has made it difficult to assess *Wolbachia* density within its host. This has severely impeded progress in the study of *Wolbachia*. In recent years, more sophisticated methods have been developed in which amplified DNA is quantified during the exponential phase of the PCR. These methods, some of which have been automated in real-time PCR, have eliminated much of the variation associated with end-point measurements. Based on the above reasoning, real-time PCR was used to study *Wolbachia* in order to determine whether the population density of *Wolbachia* is related to the early death of its host.

There are several steps required to work with real-time PCR. Firstly, primers need to be designed to provide a product of less than 200 bp. The reason for using the short product is that a straight-line relationship of the starting amount versus threshold cycle needs to be obtained. A short sequence is needed to design these primers. Therefore, we cloned and sequenced fragments of the *wsp* gene of *Wolbachia* and the $Na^+$ pump á subunit of nuclei. These genes were used because they are present as single copies in the genome (Lebovitz *et al*. 1989; Bensaadi-Merchermek *et al*. 1995; Braig *et al.* 1998). In the present study, the cell population density of *Wolbachia* which could induce the *Popcorn-effect* in *D. melanogaster* was determined from eggs, larvae, and adult flies of 1, 5, 8, and 12 days old. This is based on previous observations that the survival of adult of *D. melanogaster* ($w^{1118}$) infected with popcorn-effect inducing *Wolbachia* was to a maximum of 13 days, while adult survivors of *D. melanogaster* ($w^{1118}$) uninfected with Popcorn-effect inducing *Wolbachia* could survive up to 45 days at 29°C.

McGraw *et al.* (2002) reported in their research that after transinfection of the over-replicating *Wolbachia popcorn* strain from *D. melanogaster* to *D. simulans* they showed that initial high densities in the ovaries were in excess of what was required for perfect maternal transmission, and were likely causing reductions in reproductive fitness. Both

densities and fitness costs associated with ovary infection rapidly decline in the generations after transinfection. The early death effect in *D. simulans* attenuated only slightly and was comparable to that induced in *D. melanogaster.* Kondo *et al.* (2005) reported that *Wolbachia* density was determined through a complex interaction between host genotype, symbiont genotype, and other factors. Dutton and Sinkins (2004) studied the strain-specific quantification of *Wolbachia* density in *Aedes albopictus* and effects of larval rearing conditions and showed that larval crowding always reduced adult size, but reduced the density of *Wolbachia* strains relative to uncrowded conditions only if crowding was accompanied by restricted nutrient availability. Crowded rearing conditions never resulted in strain segregation or in a reduction in the penetrance of cytoplasmic incompatibility (CI), however. The rate of maternal transmission and the penetrance of CI are the two most important variables that determine relative *Wolbachia* population invasion dynamics, and both are considerably higher here than have been reported in the *D. simulans* model system. Mouton *et al.* (2007) studied the interaction between three strains of *Wolbachia* in two divergent homozygous lines of the wasp *Leptopilina heterotoma* at two different temperatures and showed that *Wolbachia* density varied between the two host genotypes at only one temperature, and the host genotype plays an important role in *Wolbachia* density. They also highlighted its interaction with environmental conditions, making the evolution of local adaptations for the regulation of *Wolbachia* density possible.

## MATERIALS AND METHODS

**DNA Extraction from *D. melanogaster.*** Total genomic DNA was extracted from 25 whole flies, 50 whole larvae, and 100 eggs.

**Amplification of *wsp* and $Na^+$ Pump á Subunit Genes, Cloning, Purification, and Restriction of Plasmids.** Primers used in the PCR were as follows: 610 bp for *Wolbachia* primer, *wsp* 81F (5' TGGTCCAATAAGTGATGAAGAAAC 3') and *wsp* 691R (5' AAAAAT TAAACGCTACTCCA 3'); and 545 bp for nuclei primer, $Na^+$ pump á subunit S 1124 (5' AGCGTATGGC(C/A)TC(A/G)AAGAACTG 3'), and A 1669 (5' AG(C/T)TCC ATGTAGGCATTGTTGA 3'). The conditions for the PCR were as follows: denaturation for 5 min at 95°C, followed by 35 cycle of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, and finally 4°C for an infinite amount of time. PCR products from *D. melanogaster* with bright bands were directly cloned into a TA cloning vector Kit (Invitrogen, USA) according to the manufacturer's directions (TOPO TA Cloning Version L 01261 25-0184). Plasmids were extracted from bacteria using the miniprep DNA purification Kit (QIAGEN, USA). In order to verify the presence of an insert, the plasmids were digested using *Eco*R1 (Promega, USA), and the products were run on 0.7% agarose gel.

**Sequencing.** DNA of plasmids with an insert was sequenced using Dye Terminator Cycle Sequencing on CEQ 2000 with Quick Start Kit (QIAGEN, USA).

**Primer Design.** Primer Select software provided by the DNA STAR program was used for designing the primers. The value in determining a primer, set as the primary selection criterion of the software, was the length of amplicon (< 200 bp). The use of this software resulted in a series of best-fit suggestions for the primer set. The program checks for primer location, primer dimer, melting temperature ($T_m$), annealing temperature, and GC percentage value within primer sets.

**Measurement of Standard DNA, of *Wolbachia* Bacteria and Nuclei Copy Number using Real-time PCR.** The standards used were the *wsp* plasmid for *Wolbachia* numbers and the sodium pump á subunit plasmid for nuclei numbers. These plasmids in real time PCR was calculated using Pico Green dsDNA quantification reagent (Molecular Probe, Netherlands). Pico Green is an ultrasensitive fluorescent nucleic acid stain for quantifying double-stranded DNA (dsDNA) in solution. Pico Green dsDNA quantification reagent enables quantification of as little as 25 $pgml^{-1}$ of dsDNA (50 pg dsDNA in a 2 ml assay volume) with a standard spectrofluorometer (Vector).

**Quantification of Standard DNA Copy Number.** The standard DNA was calculated based on the equation:

$$\text{Copy number} = \frac{\text{standard DNA sample}}{\text{MW}} \times \text{Avogadro Number}$$

**Quantification of *Wolbachia* and Nucleus Copy Number.** *Wolbachia* and nucleus amount (the number of *Wolbachia* and nucleus) in unknown samples was calculated based on a single egg, larva, or adult fly.

**Real-Time PCR Amplification Efficiencies.** Real-time PCR efficiencies were calculated from the given slopes in the *i*Cycler (BIO-RAD, USA). The corresponding real-time PCR efficiencies (E) of one cycle in th□ □xponential phase was calculated according to the equation :

$$E = 10^{(-1/\text{slope})} - 1.$$

The new *Wolbachia* and nucleus primers were used to amplify the genes. For *i*Cycler reaction, a master mix of reaction components was prepared as follows: 12.5 µl PCR Master Mix, 2X (Promega), 0.5 µl forward primer (20 µM), 0.5 µl reverse primer (20 µM), 0.5 µl SYBR Green 1/dye (1:10 000 dilution), 5 µl of template was added to the standard or unknown (for negative control no template was added), 6 µl nuclease free water. Finally, 25 µl aliquots of each mix were pipetted into rows of a 96-well thin-well PCR plate. The plate was covered with a piece of optically clear sealing tape and placed in the *i*Cycler IQ detection system. PCR was carried out at 95°C for 2 min 18 sec; followed by 40 cycles of 15 sec at 95°C, 20 sec at 55°C, and 30 sec at 72°C. Fluorescent data were collected during the 72°C step. Three and four standards were set up (2 replicates of each) of *wsp* and $Na^+$ pump á subunit and unknowns were investigated [eggs, larvae, adult flies (day 1), adult flies (day 5), adult flies (day 8), and adult flies (day 12)].

**Statistical Analysis.** The density of *Wolbachia* and nuclei in D. melanogaster infected with *Wolbachia* popcorn-effect in eggs, larvae, and adult flies was analysed by using one-way ANOVA. All of the data were transformed to logarithmic data before performing one-way ANOVA.

## RESULTS

**Restriction of Plasmids using *EcoR*1.** Plasmids from two different amplifications were found to contain the expected inserts of approximately 610 bp for *wsp* and 545 bp for the Na+ pump á subunit gene fragments. The gel electrophoresis of the restriction product is shown in Fig 1.

**Sequence from *Wolbachia* Surface Protein (*wsp*) Gene and Sodium (Na+) Pump á Subunit Gene.** The sequences reported here (Table 1 and 2) showed similarity to GenBank database (http://www.ncbi.nlm.nih.gov) accession no. 13173392 and 18487812 for *wsp* and Na+ pump á subunit sequences. The sequences had identities of 98% and 97%, respectively.

A standard curve was created and used to calibrate the PCR assay. The standard curve gives a correlation coefficient of 0.980 and slope of – 3.176 (Fig 2). The efficiency was calculated using the equation:

$$E = 10^{(-1/slope)} - 1 = E = 10^{(-1/-3.176)} - 1$$

and resulted in a high real-time PCR efficiency rate (100%). The correlation coefficient indicated how well the standards fit on the curve. Given known starting amounts of the target nucleic acid, a standard curve can be constructed by plotting the log of the starting amount versus the threshold cycle. The efficiency was good when the value ranged 90% to 110%

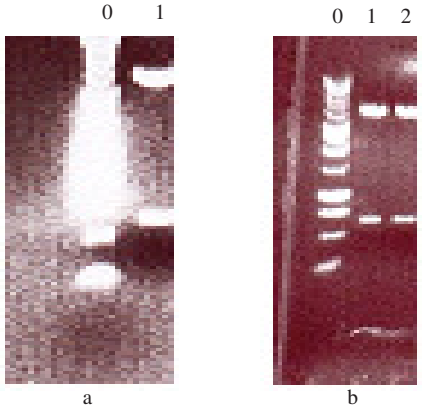


Fig 1 Restriction digest of plasmids containing *wsp* and Na+ pump á subunit fragments. o: one kilobase ladder (Promega, USA); a, lane 1: restriction enzyme treatment of *Wolbachia* surface protein fragment; b, lane 1 and 2: Restriction enzyme treatment of Na+pump á subunit fragment.

Table 1 Sequence of *wsp* fragment

| | |
|---|---|
| 1 | GAATTTTTAC CTCTTTTCAC AAAAGTTGAT |
| 31 | GGTATTACCT ATAAGAAAGA CAAGAGTGAT |
| 61 | TACAGTCCAT TAAAACCATC TTTTATAGCT |
| 91 | GGTGGTGGTG CATTTGGTTA CAAAATGGAC |
| 121 | GACATCAGGG TTGATGTTGA AGGAGTTTAT |
| 151 | TCATACCTAA ACAAAAATGA TGTTAAAGAT |
| 181 | GTAACATTTG ACCCAGCAAA TACTATTGCA |
| 211 | GACAGTGTAA CAGCAATTTC AGGATTAGTG |
| 241 | AACGTGTATT ACGATATAGC AATTGAAGAT |
| 271 | ATGCCTATCA CTCCATACAT TGGTGTTGGT |
| 301 | GTTGGTGCAG CGTATATTAG CACTCCTTTG |
| 331 | GAACCCGCTG TGAATGATCA AAAAAGTAAA |
| 361 | TTTGGTTTTG CTGGTCAAGT AAAAGCTGGT |
| 391 | GTTAGTTATG ATGTAACTCC AGAAGTCAAA |
| 421 | CTTTATGCTG GAGCTCGTTA TTTCGGTTCT |
| 451 | TATGGTGCTA ATTTTGATGG AAAAAAAACA |
| 481 | GATCCTAAAA ATTCAACCGG ACAGGCTGCT |
| 511 | GATGCAGGCG CATAC 526 |

Table 2 Sequence of Na+ pump α subunit fragment

| | |
|---|---|
| 1 | TTTGTTTGGT GGAGTTTGAA GGGGCACCTC |
| 31 | GGGCAATCTT CTTTATTACG GCTTGGCGAA |
| 61 | TGGTTCATCA CATCGCCCAG AGCCAGGTTC |
| 91 | CATGCACTTG AGCAGAGCAG CCTCGGAGGC |
| 121 | ATCTCCACTG ACTTCTTTCT TGAGGATTGG |
| 151 | GACGCCATCT TGGCCTCCCT TGAACTCGGC |
| 181 | ACGGTTACAG AGAGTGGCAA TGCGAGAGAG |
| 211 | CGCCTTGAAT CCAGGGCTGG TTCTATCGTA |
| 291 | TTGAACACCC GACTGATCCT CAGTTGTGTC |
| 241 | GGCCTCGATG ATCTGATTAT CGAACCACAT |
| 271 | GTGGGCGACC GTCATTCGGT TCTGGGTGAG |
| 301 | GGTGCCGGTC TTATCGGAGC AGATGGTCGA |
| 331 | TGTGGAGCCA AGGGTCTCCA CGGCCTCCAG |
| 361 | ATTCTTCACC AGACAGTTCT TCGATACCAT |
| 391 | ACGCTAAGGG CGAATTCTGC AGATATCCAT |
| 421 | CACACTGGCG GCCGCTCGAG CATGCATCTA |
| 451 | GAGGGCCCAA TTCGCCCTAT AGTGAGTCGT |
| 481 | ATTAC |

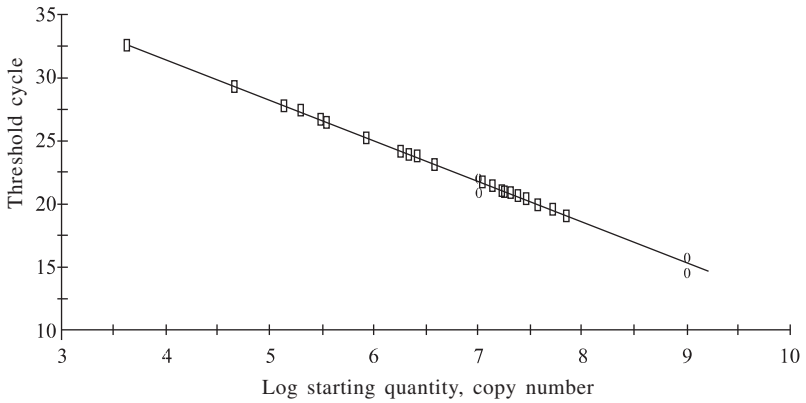


Fig 2 The standard curve of *Wolbachia* surface protein: correlation coefficient of 0.980 and slope of -3.176.

Fig 3 showed that the melting temperature resulted in one peak of the product at 79°C. This indicated that the melting temperature of all DNA templates, both standard and unknown, were the same. It also indicated that the amplified products were homogeneous and reassured that the correct product had been specifically amplified. The colours indicate different templates.

**Copy Number of *Wolbachia* in *Drosophila melanogaster* Infected with Popcorn-Effect Inducing *Wolbachia*.** Fig 4 shows an increase in the number of *Wolbachia* from eggs, larvae to adults at day 1, 5, 8, and 12. The analysis of bacterial cells (copy number) of *Wolbachia* in eggs was significantly different with that of larvae day 4. The copy number of *Wolbachia* in larvae at day 4 was also significantly different

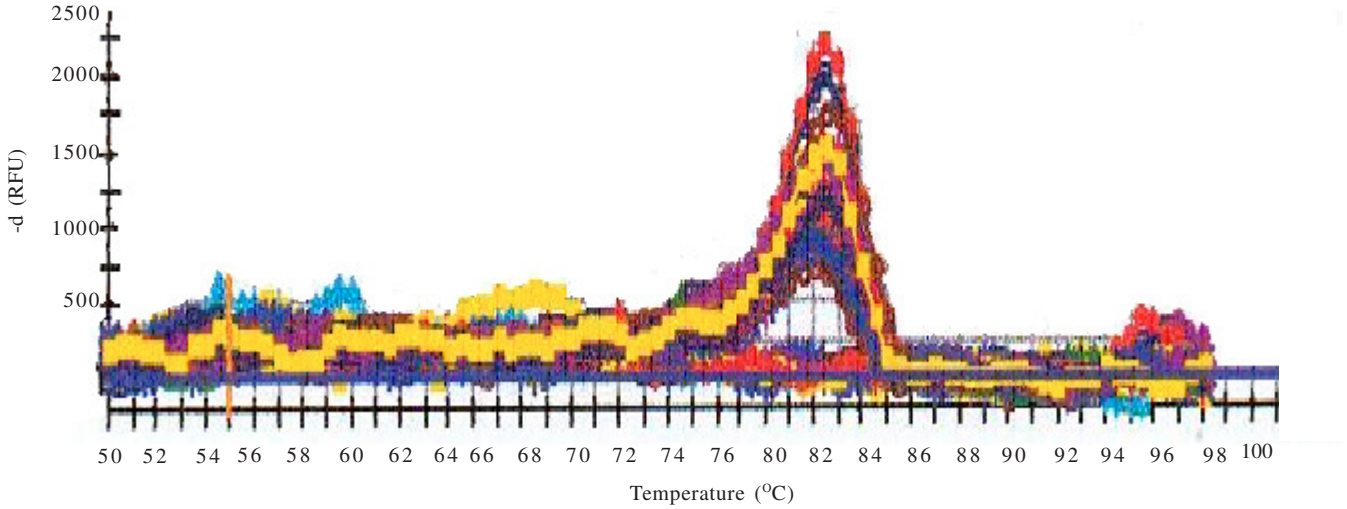


Fig 3 Melting curve of *Wolbachia* surface protein product in real-time PCR.

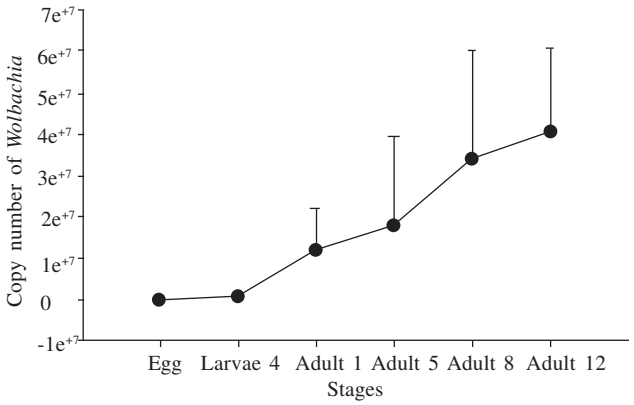


Fig 4 Copy number of *Wolbachia* in *Drosophila melanogaster* infected with *popcorn-effect* inducing *Wolbachia*.

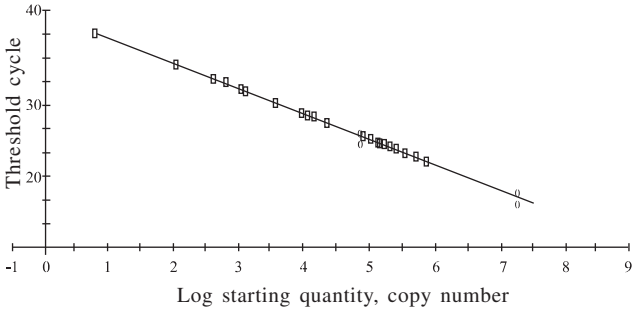


Fig 5 Standard curve of the *Ne* primers in real-time PCR. Correlation coefficient of 0.952 and slope of -2.685.

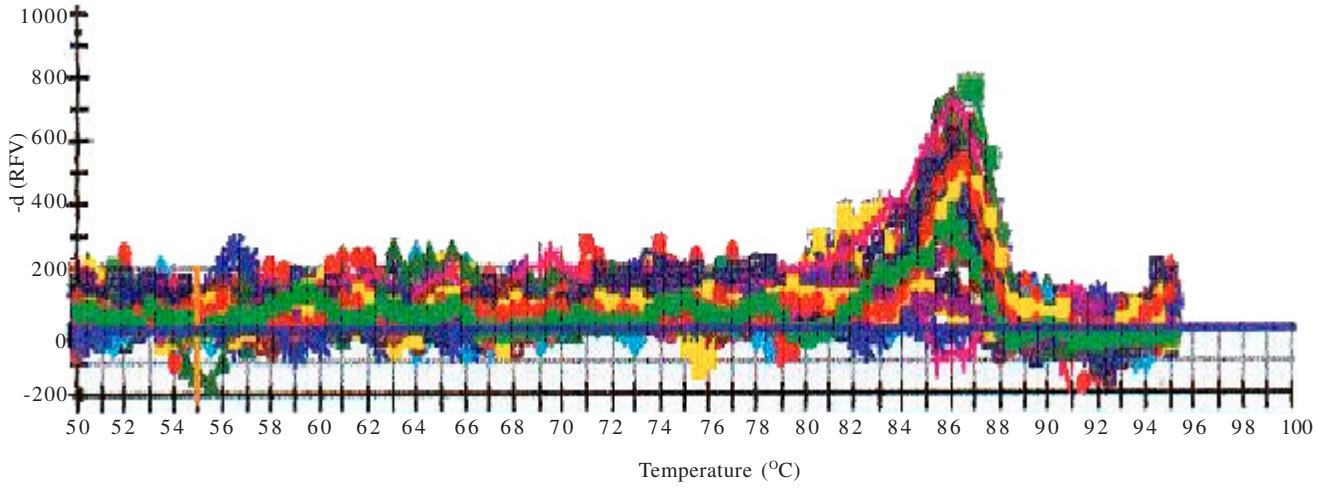


Fig 6 Melting Curve of *Ne* product in real-time PCR.

(P< 0.001) with that of adults at day 1, 5, 8, and 12. How ever, there was no significant difference in the copy number of *Wolbachia* found in adults (at day 1, 5, 8, and 12).

The standard curve gave a correlation coefficient of 0.952 and slope of – 2.685 (Fig 5). The efficiency was calculated using the equation mentioned above and resulted in a real-time PCR efficiency rate (135%).

Fig 6 showed that the melting temperature was obtained in one peak of the product at 86°C. This indicated that the melting temperature of all DNA templates, both standard and unknown, were the same. It suggested that the amplified products might be homogeneous and the $T_m$ provided reassurance that the correct product had been specifically amplified.

**Copy Number of Nuclei in *Drosophila melanogaster.*** Fig 7 shows an increase in the number of nuclei from eggs, larvae to adults day 1, 5, 8, and 12. The copy number of nuclei in eggs was significantly different with the copy number of nuclei in adults day 8 and 12 and the number of nuclei in the larvae day 4 was significantly different with the number of nuclei in adults day 12.

## DISCUSSION

The mean copy number of *Wolbachia* present in egg and 4-day-old larva were 20 x $10^3$ and 524 x $10^3$, respectively. After the adults emerged, the bacteria reached 12 x $10^6$, 18 x $10^6$, 34 x $10^6$, and 41 x $10^6$ in adults aged 1, 5, 8, and 12 days, respectively. The copy numbers of *Wolbachia* in the egg determined by confocal microscopy have been reported by Boyle *et al*. (1993). They showed that there were 20 x $10^3$ *Wolbachia* in eggs, which is similar to the present findings. The number of *Wolbachia* increase significantly from eggs, larvae, and adults. This was similar to the finding of Min and Benzer (1997), who reported that as soon as the adult flies emerge the bacteria started to multiply rapidly, causing the sudden death of their host. However, statistical analysis in our research showed that the copy number of *Wolbachia* found in adults was not significantly at each day.

The copy number of nuclei was 8 x $10^3$, 18 x $10^3$, 28 x $10^3$, 33 x $10^3$, 35 x $10^3$, and 67 x $10^3$ in eggs, larvae (day 4), adults (day 1), adults (day 5), adults (day 8), and adults (day 12), respectively. Statistical analysis showed that there was a significant difference between eggs and the adults at day 8 or 12 and between larvae day 4 to adults day 12. It is assumed that some of cells in the adult fly at day 12 are apoptotic and therefore, slightly higher. Cell division mainly occurred between immature stages and practically will not occur in adults other than in the reproductive tissue.

The number of nuclei in eggs was 8 x $10^3$ whilst the number of *Wolbachia* in the egg was 20 x $10^3$ and therefore, it is assumed that a cell contains at least 2-3 *Wolbachia* bacteria cells. In larvae on day 4, the number of nuclei was 12 x $10^3$, whilst the number of *Wolbachia* was 52 x $10^3$ and therefore, it can be said that a cell contains at least 45 *Wolbachia* bacteria. Furthermore, the number of *Wolbachia* in a cell of adults at day 1, 5, 8, and 12 were 422, 535, 964, and 610, respectively. The differences observed in adult flies were statistically insignificant. The significant increase in the density of *Wolbachia* from eggs to larvae and adult flies may be attributed to the increase in their total volume per cell and consequently in the population of the *Wolbachia* population.

The quantity/density of *Wolbachia* causing cytoplasmic incompatibility (CI) in *D. simulans* was detected using competitive/quantitative PCR by Sinkins *et al.* (1995). Comparisons were initially made between three strains of *D. simulans*: DSR (*D. simulans* Riverside), DSCH (*D. simulans* Coffs harbour), DSH (*D. simulans* Hawaii), and between three strain of *Aedes albopictus* (Houston, Koh Samui, and Mauritius). The result showed that *Wolbachia* density in DSR was the highest: this strain shows the strongest expression of CI. *Wolbachia* density in DSCH was an intermediate between that of DSR and DSH (the relative densities being 100, 60, and 20, respectively). In this method, the number of *Wolbachia* bacteria could not be measured absolutely, and therefore the exact number of *Wolbachia* was unknown.

Recently, McGraw *et al*. (2002) reported that the density of *Wolbachia* in ovaries of *D. simulans* with Riverside *Wolbachia* and *D. melanogaster popcorn-effect Wolbachia* was low and increased very little during the lifespan of the flies (7.37 and 2.7 copies per cell). In contrast, *Wolbachia* density in ovaries of *D. simulans* with *popcorn-effect Wolbachia* (resulted from artificial transfer of *D. melanogaster popcorn-effect* to *D. simulans*) rose rapidly (24.2 copies per cell). As a result, the number of *Wolbachia* in *D. melanogaster* causing the *popcorn-effect* was higher than the number of *Wolbachia* causing CI in *L. triatellus* or in *S. furcifera* (Noda *et al.* 2001). This is reasonable since *Wolbachia* causing CI did not destroy the cells. However, *Wolbachia* in *D. melanogaster* cause the *popcorn-effect* in which cells rupture as a result of massive multiplication of bacteria within them. Mouton *et al.* (2007) studied the interaction between three strains of *Wolbachia* in two divergent homozygous lines of the wasp *Leptopilina heterotoma* at two different temperatures, and showed that *Wolbachia* density varied between the two host genotypes at only one temperature, and the host genotype played an important role in *Wolbachia* density. They also highlighted its interaction with environmental conditions, making the evolution of local adaptations for the regulation of *Wolbachia* density possible.

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