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Effects of Tetracycline and Temperature on *Drosophila melanogaster* Infected with *Wolbachia* Inducing the Popcorn-Effect

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The expression of cytoplasmic incompatibility, parthenogenesis, and feminization in many hosts of *Wolbachia* is influenced by environmental factors such as temperature and antibiotics. Therefore, it might also affect *Wolbachia* inducing popcorn-effect. To examine the effects of temperature and antibiotic treatment on the life span of *Drosophila melanogaster* harbouring the popcorn-effect inducing strain of *Wolbachia*, flies were reared in different temperature such as at 20 and 29 °C, and cultured (from egg to adult stage) on a medium containing tetracycline. The tetracycline-treated *Wolbachia* was established by placing 0.25 mg mL^{-1} of tetracycline in the media. The result showed that there was no difference in the life span of *D. melanogaster* infected with *Wolbachia* popcorn-effect under untreated and treated condition with tetracycline at 20 °C. Therefore, there is no popcorn-effect in the *D. melanogaster* at low temperature (20 °C). While the life span of *D. melanogaster* at 29 °C, where infected flies have a shorter life span than treated flies. Popcorn-effect (shorter life span) was found at 29 °C.

Key words: cytoplasmic incompatibility, feminisation, parthenogenesis, popcorn-effect, tetracycline

Ekspresi ketidakserasian sitoplasma, partenogenesis, dan feminisasi pada beberapa inang yang terinfeksi *Wolbachia* dipengaruhi oleh faktor lingkungan seperti suhu dan antibiotik. Oleh karena itu, faktor-faktor tersebut kemungkinan juga berpengaruh pada *Wolbachia* penyebab efek-popcorn. Untuk menguji pengaruh suhu dan antibiotik terhadap lama hidup *Drosophila melanogaster* yang mengalami efek-popcorn yang disebabkan oleh infeksi *Wolbachia*, lalat-lalat tersebut dipelihara pada suhu 20 °C dan 29 °C, kemudian dikultur dari stadium telur sampai menjadi dewasa pada media baik yang mengandung tetrasiklin (0,25 mg mL^{-1}) maupun yang tidak mengandung tetrasiklin. Hasil menunjukkan bahwa antara lalat terinfeksi *Wolbachia* efek-popcorn yang diberi pengobatan maupun yang tidak diobati pada suhu 20 °C, tidak ada perbedaan dalam lama hidupnya. Oleh karena itu, tidak ada pengaruh efek-popcorn terhadap *D. melanogaster* pada suhu 20 °C. Lama hidup *D. melanogaster* pada suhu 29 °C yang terinfeksi *Wolbachia* lebih pendek dibandingkan dengan yang diberikan tetrasiklin. Dengan kata lain terjadi efek-popcorn pada suhu 29 °C.

Kata kunci: efek-popcorn, feminisasi, ketidakserasian sitoplasma, partenogenesis, tetrasiklin

Wolbachia pipientis is common bacterial endosymbiont of arthropods and filarial nematodes; they are maternally inherited, obligate, and intracellular bacteria (McGraw *et al*. 2002). Interest in *Wolbachia* stems not only from its widespread distribution in arthropods, but also from the alterations of the mode of reproduction of its hosts caused by the bacteria uch as cytoplasmic incompatibility (CI), feminization (F), parthenogenesis (P), male killing and the popcorn-effect (Yu Peng *et al*. 2008; Carrington *et al*. 2010). The expression of cytoplasmic incompatibility, feminization, and parthenogenesis also density in many hosts of *Wolbachia* is influenced by environmental factors such as antibiotic and temperature (Mouton *et al*. 2007; Zhukova *et al*. 2008; Jia *et al*. 2009; Suh *et al*. 2009).

Breeuwer and Werren (1993) reported that tetracycline treatment (1 mg mL^{-1} in 10% sucrose) on female *Nasonia vitripennis* infected with *Wolbachia* induced cytoplasmic incompatibility which had a dramatic effect on bacterial densities in their eggs. On the first day after treatment, tetracycline had effectively eliminated cytoplasmic bacteria and caused reduction or absent of the microorganism in the eggs. Werren and Jaenike (1995) reported that in *Drosophila recens*, *Wolbachia* was lost with 0.2 and/or 0.25 mg tetracycline per ml within two to three generations. However, 0.1 mg tetracycline per ml apparently was not effective in curing the flies of *Wolbachia*. Kambhampati *et al*. (1993) showed that tetracycline treatment of *Aedes albopictus* results a partial, but significant restoration of compatibility.

Stouthamer *et al*. (1990) investigated *Trichogramma* harbouring parthenogenesis-inducing *Wolbachia*. When it was cured by antibiotics tetracycline hydrochloride, sulfamethoxazole or rifampicin (100mg mL^{-1} honey) the result was the appearance of male offspring because the microorganisms responsible for

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the lytoky were killed. However, treated with gentamycin, penicillin G and erythromycin, *Trichogramma* male offspring did not occur. When *Trichogramma* reared at an elevated temperature >30 °C, the females produced male offspring as well. Stouthamer *et al*. (1993) reported that antibiotic treatment of *Trichogramma deion*, *T. pretiosum*, *T. cordubensis,* and *Mucidifurax uniraptor* resulted in reversion to sexual reproduction and elimination of the microorganisms. Stevens (1989) reported that when larvae of *Tribolium confusum* were reared at 37 °C, the compatibility was partly restored because some larvae become cured of the infection with *Wolbachia*. Subsequently Stevens and Wicklow (1992) reported that tetracycline-producing *Streptomyces* and *Penicillium* can cure *Tribolium flour* beetles of infection with *Wolbachia*. Fujii *et al*. (2001) reported feminisation-inducing *Wolbachia* in *Ephestia kuehniella*. Those cured by tetracycline produced male offspring. In original work on the *w*MelPop strain in *Drosophila melanogaster* by Min and Benzer (1997), all infected flies held at 29 °C died within 14 d. However, when longevity was assessed at 25 °C, infected flies lived twice as long, suggesting that the virulence of *w*MelPop depended on temperature (Carrington *et al*. 2009)

Trpis *et al*. (1981) reported evidence for suppression of CI with high temperature in several species. For example, in the mosquito *Aedes scutellaris* crosses between strains that are normally incompatible became compatible when larvae were raised at a temperature of 32.5 °C. Hoffmann *et al*. (1986) reported that rearing larvae at 28 °C the incompatibility of *Drosophila simulans* R (riverside) males and W (watsonville) females could be suppressed. Stevens (1989) reported that when larvae of *T. confusum* were reared at 37 °C, the compatibility was partly restored because some larvae become cured of the infection with *Wolbachia*.

Previous experiments showed that reproduction abnormalities such as cytoplasmic incompatibility, feminization, and parthenogenesis in many hosts of *Wolbachia* are influenced by environmental factors such as temperature and antibiotics. Therefore, it might also affect *Wolbachia* inducing popcorn-effect. To determine the possible role of the environment on the popcorn-effect inducing *Wolbachia*, *D. melanogaster* infected with *Wolbachia* popcorn-effect will be cured with 0.25 mg mL^{-1} tetracycline (as in the previous experiment by Min and Benzer, 1997) and reared at 20 and 29 °C. The temperature of 20 °C is a close approximation of the optimal physiological temperature of *D. melanogaster* and the temperature at which the originally popcorn-effect was found is 29 °C.

MATERIALS AND METHODS

Analyses of the Effect of Antibiotic Treatment. *D. melanogaster* infected with *Wolbachia* inducing popcorn-effect obtained from the lab of Henk R Braig, School of Biological Sciences, University Of Wales, Bangor, United Kingdom. To examine the effect of antibiotic treatment, flies were cultured (from egg to adult stage) on a medium containing tetracycline. The tetracycline-treated strain was established by placing 0.25 mg mL^{-1} of tetracycline in the media. The strain was treated for three generation and tested for infection status with PCR, utilizing 81F and 691R primers, specific for the *wsp* gene of *Wolbachia* (Braig *et al*. 1998). After adults emerged the male and female flies were reared separately at a density of 15 flies per vial (2.3 cm diameter and 9.5 cm in length) with 10 replicates. To examine the effect of temperature, flies were reared in different temperature such as at 20 and 29 °C.

Statistical Analysis. An effect of tetracycline and temperature in *D. melanogaster* infected with *Wolbachia* popcorn-effect would be manifest by its life-span. Univariate analysis of variance (SPSS 10.1) was used to determine the influence of temperature and tetracycline treatment. In order to apply ANOVA, the experimental data need to show a normal distribution and an equal variability. This is not the case in these experiments. The data therefore had to be transformed before a valid statistical analysis was possible. Several transformation methods were tested including using the square root of the raw data. Only the transformation of the raw data to their decimal logarithm was able to satisfy the requirements of ANOVA.

RESULTS

The effect of tetracycline on the *Wolbachia* inducing popcorn-effect was analysis by PCR and the results showed the untreated sample has band that positive for *Wolbachia* (lane 1 and 2 of Fig 1, Fig 2). However, treated treated samples has no visible band (lane 3, 4, 5, and 6, Fig 1), which indicates that *Wolbachia* was not exist any longer .

When *D. melanogaster* larvae untreated and treated with tetracycline were reared at different temperature, the life-span varied. Flies reared at 29 °C exhibited a shorter life-span when they were infected with *Wolbachia* than

flies treated with tetracycline (Fig 3). The difference in life-span was highly significant. However, there was no significant difference in sex (male and female).

Fig 4 shows that there is no difference in the life span of *D. melanogaster* between untreated and treated. Unlike the life span of *D. melanogaster* at 29 °C where infected flies have a shorter life span than treated flies. Therefore, there is no popcorn-effect in the *D. melanogaster* at low temperature (20 °C).

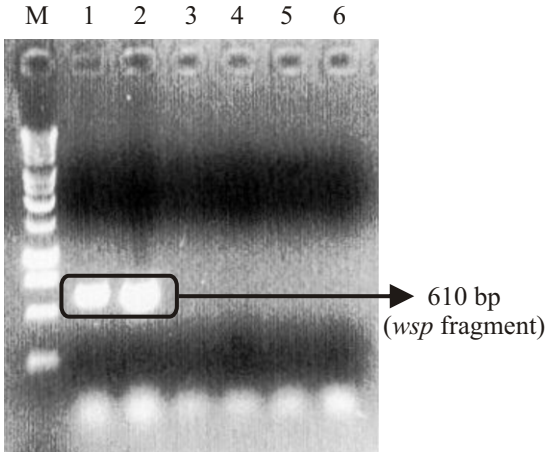

Fig 1 Detection of infection of *Drosophila melanogaster* (W1118) by *Wolbachia* untreated and treated with tetracycline. M: one kilobase ladder; 1 and 2: *D. melanogaster* (W1118) untreated; 3, 4, 5, 6: *D. melanogaster* (W1118) treated with tetracycline.

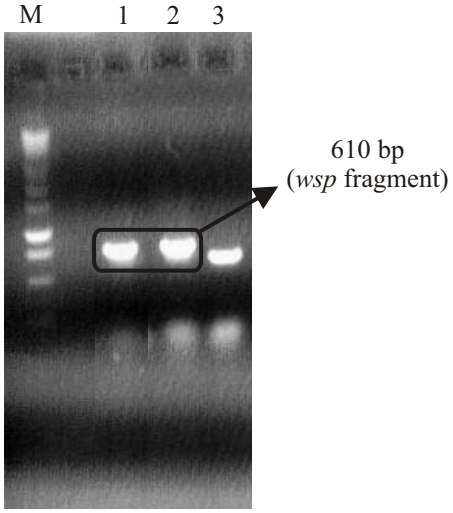

Fig 2 Detection of infection of *Drosophila melanogaster* (W1118) by *Wolbachia* untreated with tetracycline at 29 and 20 °C. M: one kilobase ladder; 1: *D. melanogaster* infected with popcorn-effect maintained at 29 °C; 2: *D. melanogaster* infected with popcorn-effect maintained at 20 °C; Line 3: positive control (amplification of ribosomal of nucleus of *D. melanogaster* infected with popcorn-effect at 20 °C).

The analysis shows highly significant difference (< 0.001) between treatment (treated and untreated) at temperature (29 and 20 °C). However, there is no significant difference between treatment and sex (male and female). At 20 °C untreated and treated are not significant different. However, at 29 °C untreated and treated are significant different.

DISCUSSION

When *D. melanogaster* larvae untreated and treated with tetracycline were reared at different temperature, the life-span varied. Flies reared at 29 °C exhibited a shorter life-span when they were infected with *Wolbachia* than flies treated with tetracycline (Fig 3). The difference in life-span was highly significant. However, there was no significant difference in sex (male and female).

Tetracycline eliminated *Wolbachia* and restored the life-span of male and female flies at 29 °C. This was the same temperature for which the popcorn-effect of *Wolbachia* was originally described (Min and Benzer, 1997). Whereas when reared at 20 °C (Fig 4) the life span of the flies was no longer significantly different between untreated and treated, although *Wolbachia* could still be detected when the flies were reared at 20 °C (Fig 2). Therefore, apparently there was no popcorn-effect at 20 °C. It was probably that at 20 °C both host and bacteria function optimally, therefore no changes in life history parameters of fitness. In other words, the host perfectly controlled the bacteria under optimal physiological condition and *Wolbachia* did not disrupt the symbiotic relationship. Werren (1997) and McGraw *et al*. (2001, 2002) tried to explain the virulence of the popcorn-effect as an 'over-replication' of these bacteria. McGraw *et al*. (2002) investigated the question whether the popcorn-effect might reflect the initial increased virulence commonly observed in new host-parasite associations. They transferred the popcorn-effect *Wolbachia* into a new, naive host, *D. simulans*, where indeed early on reproductive fitness costs were noted caused by initial high densities of *Wolbachia* in the ovaries that were in excess of what was required for perfect maternal transmission. These fitness costs rapidly declined in the generations after transinfection. However, the popcorn-effect was practically independent of the observed attenuation of virulence. The authors reared the transinfected *D. simulans* at 26 °C in order to observe the popcorn-effect. In our hands, a temperature of 29 °C have been deadly already for uninfected *D. simulans* flies. The authors failed to make any observations at physiological temperatures.

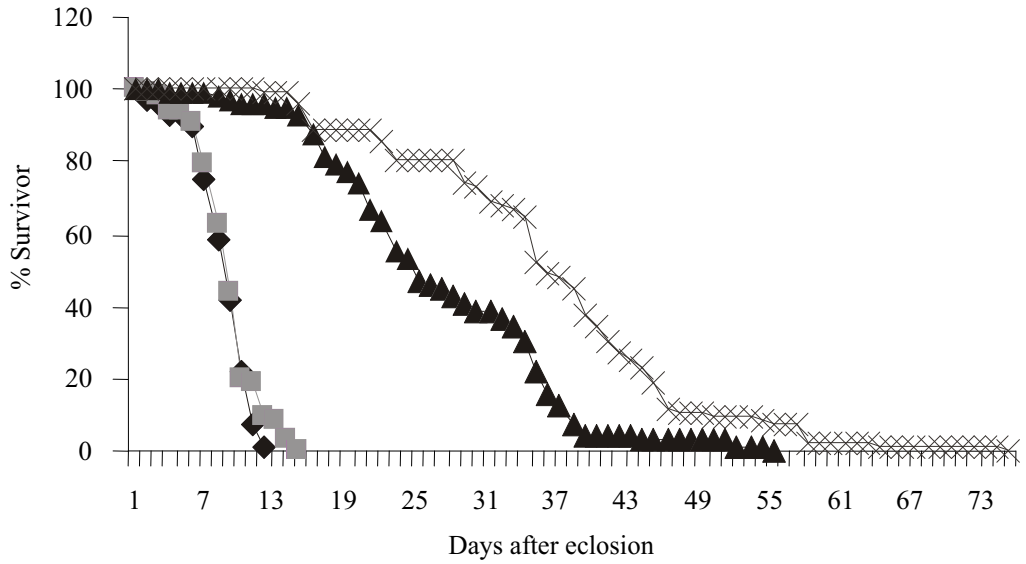

Fig 3 Percentage of adult survivors of *Drosophila melanogaster* (w1118) infected with the popcorn-effect inducing *Wolbachia* when untreated and treated with tetracycline and maintained at 29 °C. It's shows the life span of treated *D. melanogaster* is longer than that of untreated. : % Survivor of untreated adult male w1118; % Survivor of untreated adult female w1118; % Survivor of adult male w1118 treated with tetracycline; % Survivor of adult female w1118 treated with tetracycline.

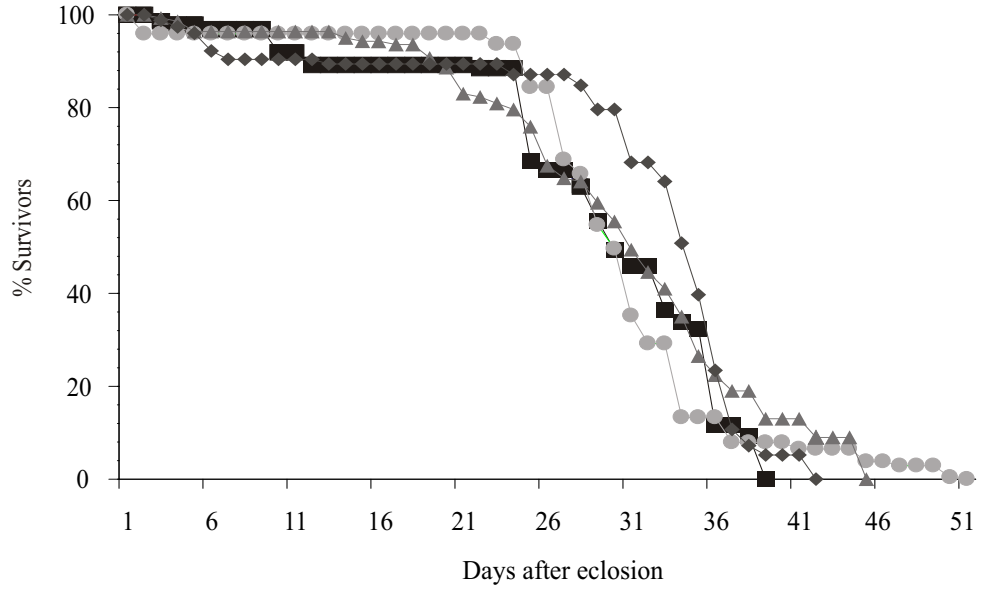

Fig 4 Percentage of adult survivors of *Drosophila melanogaster* (w1118) infected with the popcorn-effect inducing *Wolbachia* when untreated and treated with tetracycline and maintained at 20 °C. : % Survivor of adult untreated male W1118; : % Survivor of adult untreated male W1118; : % Survivor of adult male W1118 treated with tetracycline; : % Survivor of adult female W1118 treated with tetracycline.

The potential of the popcorn-effect for the manipulation of vector insects have been repeatedly stressed, e.g., Bourtzis and Braig (1999). The ideal would be to express the early deaths associated with the popcorn-effect, for example, in *Anopheles mosquitoes* to prevent the transmission of malaria parasites. The finding reporter here that the popcorn-effect was in effect a laboratory artefact and was not expressed under physiological conditions put an early end to these hopes.

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