

Horizontal Transfer of the ‘Popcorn-Effect’ Strain of *Wolbachia* from *Drosophila melanogaster* to *Stomoxys calcitrans*

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The *Wolbachia* containing haemolymph from *Wolbachia* infected *Drosophila* third/fourth instar larvae was transferred through microinjection into 5-days-old pupae of the blood sucking stable fly, *Stomoxys calcitrans*. There was no previous record of *Wolbachia* being present in *S. calcitrans*. From a total of 682 emerging adults, 236 were females and of these, seven females were tested positive (approximately 3%) for *Wolbachia* infection. Using electron microscopy, it was shown that *Wolbachia* were present in the muscle tissues of *S. calcitrans*.

Key words: *Drosophila melanogaster*, *Stomoxys calcitrans*, ‘popcorn-effect’, haemolymph, microinjection

Wolbachia infection was first discovered in mosquitoes (Hertig 1936). Since then, these intracellular bacteria have been found in a variety of insect species, mites and isopods (Rousset *et al.* 1992; Breeuwer and Jacobs 1996; Werren 1997; Grenier *et al.* 1998). *Wolbachia* can induce different effects in the reproductive organs of its hosts. These include cytoplasmic incompatibility (CI) in isopods, insects and mites (Breeuwer and Jacobs 1996), parthenogenesis/thelytoky (T) in parasitoid insects (Stouthamer *et al.* 1990), feminisation (F) in isopods (Juchault *et al.* 1994), male-linked lethality in *Drosophila bifasciata* and the butterfly *Acraea ecedon* (Hurst *et al.* 1999 and 2000), and the ‘popcorn-effect’ in *D. melanogaster* in which an early death of the insect occurs (Min and Benzer 1997). A sudden massive degeneration of the fly’s cells occurs in response to bacterial multiplication. The bacteria are present in low numbers during development through the embryonic, larval and pupal stages. As soon as the adult flies emerge, the bacteria start to multiply rapidly, causing sudden death of their host. 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 life span 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 life span of insects.

Wolbachia is maternally inherited. However, there is strong evidence for horizontal transfer between species that are distantly related, based on non-congruence of host and bacterial phylogenies (O’Neill *et al.* 1992; Rousset *et al.* 1992; Werren *et al.* 1995). Comparative molecular phylogenies of 20 parthenogenetic *Trichogramma* populations and their symbiotic *Wolbachia* suggest the occurrence of occasional horizontal transmission (Schilthuisen and Stouthamer 1997). This is a good indication that individual *Wolbachia* species or strains are not restricted to a single host. The ‘popcorn-effect’-inducing *Wolbachia* causes a reduction in insect life span, so if this strain could be transferred to insects that are disease vectors, it might be an effective vector control. For example, adult *Anopheles gambiae*, which transmits *Plasmodium* spp. (causing malaria), has a life expectancy of

15 days (Garrett-Jones and Shidrawi 1969) whilst *Plasmodium* has an incubation period of 11 days (Baker 1966). Similarly, adult *Aedes trivittatus* has a life expectancy of about 25 days in the laboratory (Christensen 1978) whilst the parasite it transmits (*Dirofilaria immitis*) has an incubation period of 16 days at 22.5°C (Christensen and Hollander 1978). They will only be infectious and pathogens transmitted after the host has taken at least three to four blood meals. If the insect was infected with the ‘popcorn-effect’-inducing *Wolbachia*, the insect will experience early and sudden death in its adult stage perhaps preventing disease transmission to humans.

Experimental transfers have been performed, in which *Wolbachia* has been transferred by microinjection (within and between species, between families, and between orders (Boyle *et al.* 1993; Braig *et al.* 1994; Chang and Wade 1994; Juchault *et al.* 1994; Karr 1994; Rousset and Stordeur 1994; Rigaud and Juchault 1995; Sinkins *et al.* 1995; Clancy and Hoffmann 1997; Dunn and Rigaud 1998; Grenier *et al.* 1998; Poinsot *et al.* 1998; Van Meer and Stouthamer 1999; Fujii *et al.* 2001). The interfamily transfer of *Wolbachia* was demonstrated by Braig *et al.* (1994). They found that bacteria from the mosquito *Aedes albopictus* could be transferred by microinjection into uninfected embryos of *D. simulans*, conferring complete CI on the adults. Recently, embryonic microinjection protocol for *Wolbachia* transfection have been developed for the mosquitoes *A. albopictus* (Xi *et al.* 2006) and *A. aegypti* (Xi *et al.* 2005), as well as *Wolbachia* transfer protocols based on injection of symbionts directly into adult *Drosophila* and *Aedes* mosquitoes (Frydman *et al.* 2006; Ruang-Areerate and Kittayapong 2006). Grenier *et al.* (1998) successfully transferred the symbiote within the Trichogrammatidae. However, there is no study to date on horizontal transfer of the ‘popcorn-effect’. To investigate whether the ‘popcorn-effect’ phenotype is specific to *Drosophila* or whether it can be transmitted to another species, the popcorn-effect inducing *Wolbachia* was transferred from *D. melanogaster* by microinjection into uninfected *S. calcitrans* pupae. There is no previous record of *Wolbachia* being present in *S. calcitrans*.

To verify the presence of *Wolbachia* in newly infected individuals of *S. calcitrans*, the tissues from *S. calcitrans* which tested positive for *Wolbachia* by PCR were then

examined by electron microscopy. This is to confirm that the positive PCR amplification represented the identification of real *Wolbachia*, and not a fortuitous PCR amplification of genomic DNA.

MATERIALS AND METHODS

Microinjections. *Stomoxys calcitrans* was obtained from Prof Lehane's and *D. melanogaster* larvae harbouring *Wolbachia* 'popcorn-effect' was obtained from Dr. Braig's laboratory School of Biological sciences, University of Wales, Bangor, United Kingdom. Microinjection experiments using cell micro-injector PM 1000, a *D. melanogaster* fourth instar larva, derived from a previously established colony, was placed in front of the microcapillary needle and punctured. Subsequently, the haemolymph was drawn into the needle via capillary action of the filament. A 5-day-old *S. calcitrans* pupa was placed in front of the needle and punctured with the use of the macro- and microcontrols. The *D. melanogaster* haemolymph in the needle flowed into the pupa when the control pedal was pressed. The microcapillary needle was then removed. If the needle broke or if backflow into the needle occurred, then the pupa was discarded. A new needle was used for each transfer. In order to ensure that all pupae used in this experiment were of the same age (5-day-old pupae), all stock used was 17 days old from egg collection. The pupae were kept on moist cotton wool (checked daily, water being added if necessary).

Rearing, Collection, and Killing of Flies. After microinjection the pupae were put in a plastic cup (10.5 cm diameter, 4 cm height) containing moist cotton wool. Humidity was maintained by regular addition of water. The plastic cups were housed in net cages (26 cm length, 26 cm width and 26 cm height) at 25°C. The number of pupae in every plastic cup container depended on the number of pupae that were injected in the same day (187, 69, 173, 48, 30, 184, 199, 119, 87, 169, 109, 109, 109, 116 and 37). The cages were placed on paper in order to facilitate egg collection (next generation). Adults emerged after several days and were allowed to mate. Adult flies were fed daily with pig's blood (by placing cotton wool, which was soaked in the pig's blood, on top of the net cage). Eggs were transferred from the paper into a plastic cup container (10 cm diameter and 4 cm height) containing larval feeding medium after 28 days from emergence, adult flies were killed and the females tested for *Wolbachia* infection. The period of 28 days after emergence, is the longest possible time before risking losing any flies due to natural mortality. The flies were not killed sooner in order to allow the bacteria to grow in the new host. The period of 28 days gave the female flies the possibility to lay eggs so that the line would continue to the next generation. As *Wolbachia* are maternally inherited, the bacteria would be transmitted to the progeny just through the females, not the males.

DNA Extraction, Electrophoresis, and Electron Microscopy. The procedure of DNA extraction was using the DNeasy tissue kit (Qiagen). The extracted DNA from the adult *Stomoxys calcitrans* female was analysed using the polymerase chain reaction (PCR) to determine evidence of *Wolbachia* infection. The procedure was according to the

manufacture instructions (Promega). One master mix was used to detect *Wolbachia* (using *Wolbachia* surface protein *wsp* gene). Forward (*w81F*: 5'-TGG TCC AAT AAG TGA TGA AGAAAC-3') and the reverse primer (*w691R*: 5'-AAAAAT TAA ACG CTA CTC CA-3) were designed to amplify a fragment of 610 bp (Braig *et al.* 1998); as a control to detect mitochondria, forward primer 12SbiF (5'-AAGAGC GAC GGG CGA TGT GT-3') and reverse primer 12SaiR (5'-AAA CTA GGA TTA GAT ACC CTA TTA T-3') were used. The microcentrifuge tubes were then placed in a thermal cycler (Technogene). An initial denaturation at 95°C for 5 minutes was followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes: followed by 72°C for 10 minutes and finally 4°C.

To verify the presence of *Wolbachia* in individuals of *S. calcitrans*, which were infected by microinjection of haemolymph from *D. melanogaster* infected with the popcorn strain of *Wolbachia*, the tissues from *S. calcitrans* tested positive for *Wolbachia* by PCR were further examined by electron microscopy. This is to confirm that the positive PCR amplification represented the identification of real *Wolbachia*, not fortuitous PCR amplification of genomic DNA. The tissues (muscle, testes, ovaries, brain and eyes) were taken from flies dissected in PBS (phosphate buffer saline) and put in Karnofsky's fixative (Glauert and Lewis 1998) consisting of 0.4% paraformaldehyde and 0.16% glutaraldehyde in sodium cacodylate, 10% w/v sucrose, pH 7.2 for 1 h, then washed twice using PBS. The tissues are then put in 1% osmium tetroxide plus cacodylate buffer (1:1) for one hour, washed twice with water and put in 2% uranyl acetate and kept in the fridge (4°C) overnight. After fixation the material was dehydrated using serial ethanol changes, and embedded in the resin. A glass knife was used to shape and cut the embedded sample in the piritome (LKB Leica) and ultratome (LKB Leica). The sections were collected on grids (pioloform coated nickel 200 mesh grids) and then stained with lead citrate for 6 minutes. Sections were observed using a Philips EM 60 kV.

RESULTS

From a total of 682 emerging adults, 236 were females and of these, seven females (approximately 3%) tested positive for *Wolbachia* infection. Fig 1 and 2 show gel electrophoreses of injected female *S. calcitrans* testing positive for *Wolbachia* infection. The first lane was a one kilobase ladder, second lane was *Wolbachia* positive control from *D. melanogaster* infected with 'popcorn-effect'. Therefore, the next lanes, which had the same size of band as the *D. melanogaster* positive control, indicated that *S. calcitrans* were *Wolbachia* positive lane 4, 6, 8, 10, 18 and 20 in Fig 1 and lane 4 in Fig 2. Mitochondrial primers (12SbiF and 12SaiR) were used for checking whether the conditions for the PCR were appropriate in every sample. Using electron microscopy it was shown that *Wolbachia* were present in the muscle tissues of *S. calcitrans* (Fig 3 and 4).

To provide evidence that *S. calcitrans* did not contain bacteria before injection with *Wolbachia*, the muscle tissue was examined by PCR and electron microscope. Fig 5 shows gel electrophoresis of *S. calcitrans* testing negative for

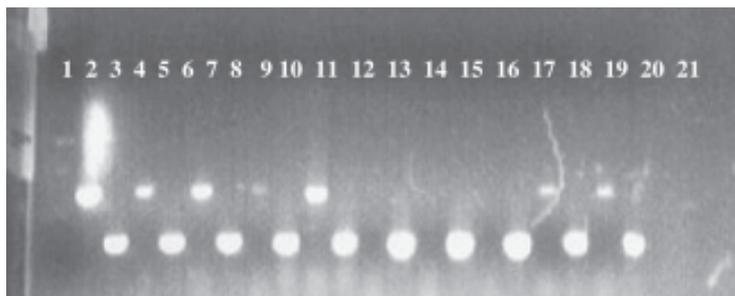


Fig 1 Gel electrophoresis of *Stomoxys calcitrans* testing positive for *Wolbachia* infection. Lane 1, 1 kb ladder; lane 2, positive control for *Wolbachia* from *Drosophila melanogaster*; lane 3, positive control for mitochondria from *D. melanogaster*; lane 4, 6, 8, 10, 18 and 20. *Wolbachia* positive results from *S. calcitrans*; lane 5, 7, 9, 11, 13, 15, 17, 19 and 21, Mitochondria from *S. calcitrans*; lane 12, 14, 16. *Wolbachia* negative results from *S. calcitrans*.

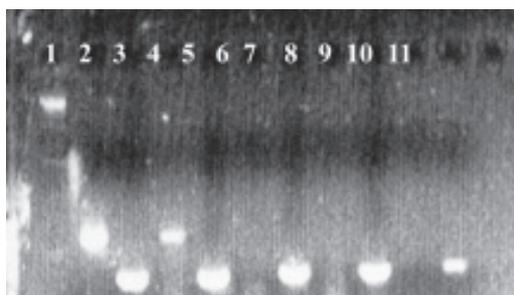


Fig 2 Gel electrophoresis of *Stomoxys calcitrans* testing positive for *Wolbachia* infection. Lane 1, 1 kb ladder; lane 2, positive control for *Wolbachia* from *D. melanogaster*; lane 3, positive control for mitochondria from *D. melanogaster*; lane 4, *Wolbachia* positive result from *S. calcitrans*; lane 5, 7, 9, and 11. Mitochondria from *S. calcitrans*; lane 6, 8, and 10. *Wolbachia* negative from *S. calcitrans*.

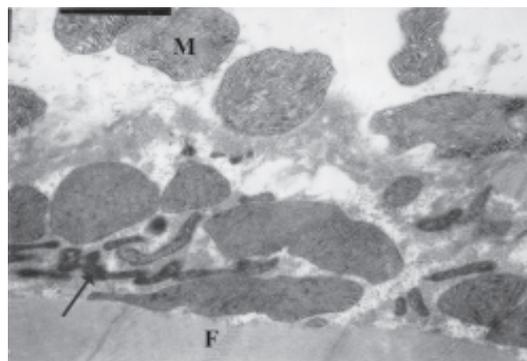


Fig 4 *Stomoxys calcitrans* muscle tissue (19,000 X). M, mitochondria and F, fibril of muscle. Arrow: *Wolbachia*.

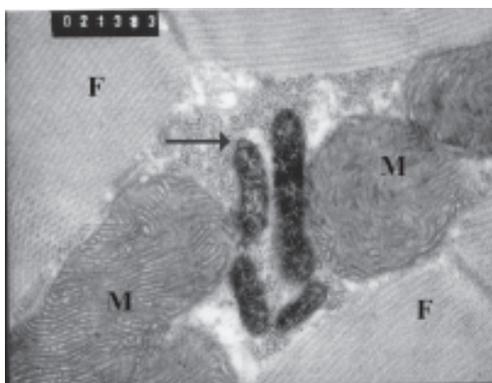


Fig 3 *Stomoxys calcitrans* muscle tissue (51,000 X). Using electron microscopy it was shown that *Wolbachia* were present in the muscle tissues of *S. calcitrans*. M, mitochondria and F, fibril of muscle. Arrow: *Wolbachia*.

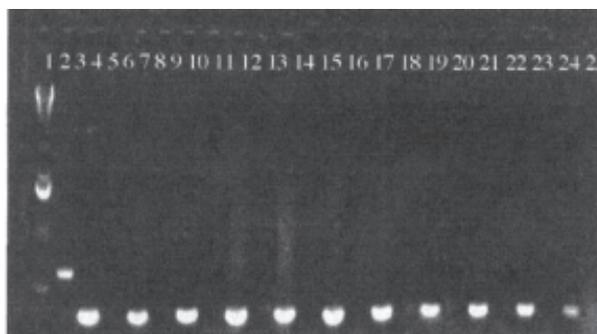
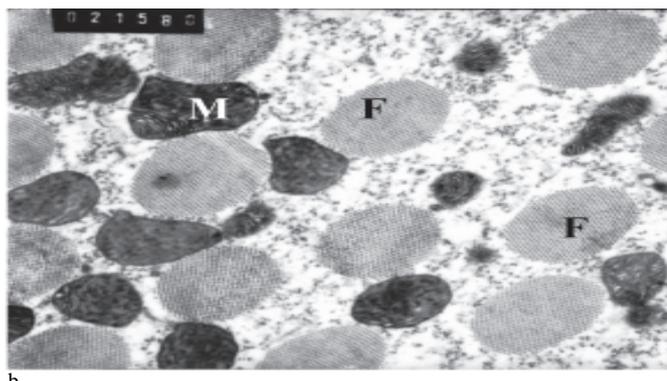
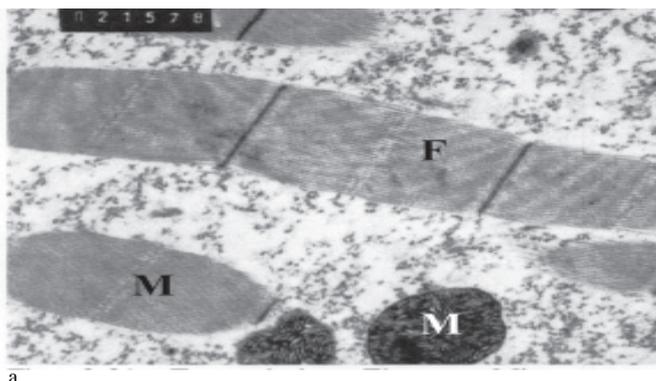


Fig 5 Gel electrophoresis of *Stomoxys calcitrans* testing negative for *Wolbachia* infection before injecting. Lane 1, 1 Kb ladder; lane 2. Positive control for *Wolbachia*; lane 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 mitochondria primer of *S. calcitrans*; lane 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 *Wolbachia* primer of *S. calcitrans* there were no bands indicating negative of *Wolbachia*.



Figs 6 Muscle of uninfected *Stomoxys calcitrans* before injection (20 000 X): a, a horizontal section and b, a transversal section. M, mitochondria and F, fibril of muscle. There is no *Wolbachia* in the tissue.

Wolbachia infection before injecting and Fig 6 show muscle tissues of *S. calcitrans*, where no *Wolbachia* or any other bacterial structures were detected. This indicated that uninjected (normal slide) *S. calcitrans* were negative for *Wolbachia*.

DISCUSSION

From 236 females, only seven females (approximately 3%) tested positive for *Wolbachia* infection. There are several possible reasons for why such a low number of positive results were obtained. Firstly, the *Wolbachia* density might have been too low to produce an infectious dose in the pupae, resulting in elimination of the inoculum. Secondly, that the interaction between *Wolbachia* symbiont from *D. melanogaster* and the new host genome of *S. calcitrans* is unfavourable (Kondo *et al.* 2005). Therefore, the success of transmission for the bacteria are influenced by several factors, including bacterial density and host genotype (Breeuwer and Werren 1993; Bourtzis *et al.* 1996; Grenier *et al.* 1998; Kondo *et al.* 2005; Duron, *et al.* 2006; Mouton *et al.* 2006). Thirdly, the pupae might have been too young. Young pupae have a very up-regulated immune system whereas older pupae (which are protected by an additional exoskeleton) exhibit a highly down-regulated immune system (Braig HR 2009, personal communication). If the pupae are too young at the time of infection, the inoculum might be destroyed by the immune system regardless of its dose. However, if the pupae are much too old, the barrier between haemolymph and germ line tissue might become unpenetrable for *Wolbachia*. Strong somatic infections might result but permanent, inheritable infections might not be established. Even a germ line infection requires a certain threshold density of bacteria before a productive infection in the next generation can be secured. Reaching this threshold for an endosymbiont that has been adapted to a different host for thousands and thousands of generations might constitute an immense barrier to be overcome.

Although PCR is far from quantitative, some of the *Wolbachia* signals from females in lanes 4, 6, 8, 10, 18 and 20 in Fig 1 and lane 4 in Fig 2 were similar the intensity of the *Wolbachia* signal of the infected donor, being the 'popcorn-effect' of *D. melanogaster*. The strong signal of females (aged 28 days) suggests a productive infection. The PCR assay is not sensitive enough to pick up the inoculum. Lane 4 is presumably an example of a weak infection. Unfortunately, even the six strongest infections did not lead to any inheritance. Similar results have been obtained in flour beetles (*Tribolium confusum*) in which the infection has been lost in the second generation as well (Chang and Wade 1994). van Meer and Stouthamer (1999) transferred *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera), which harbours parthenogenesis-inducing *Wolbachia* to *D. simulans* (Diptera) without establishing a stable infection. For *S. calcitrans* that were positive for *Wolbachia* after transferring these bacteria by microinjection, no specific effects on the host were detected and the bacteria were not maintained stably. However, investigation by electron microscopy showed that the bacteria were present in the tissues of infected *S. calcitrans* such as the thoracic muscle.

This indicates that *Wolbachia* develops in the tissues of the new host. It was assumed that the bacteria did not reach the reproductive organs when injected, thereby causing no maternal transmission to the next generation.

Furthermore, host-symbiont interactions are important for success in the establishment of an infection. Another example of a failed transfer of *Wolbachia* has been obtained for the isopods *Chaetophiloscia elongata* and *Armadillidium vulgare*, which are distantly related species. However, *Wolbachia* could be transferred between closely related species of isopods (*A. nasatum* to *A. vulgare*) (Juchault *et al.* 1994). On the other hand, phylogenetic work on *Wolbachia* (Werren *et al.* 1995) provides evidence that, at least in some cases, this bacterium has successfully transferred over large phylogenetic distances in its interspecific movement. It remains unknown what further permits particular inter-family transfers to occur successfully, other than time.

Using electron microscopy it was shown that *Wolbachia* were present in the muscle tissues of *S. calcitrans*. The most common form observed was an elongated cylinder with convex ends typical of bacterial rods; it is the most common shape of bacteria, after all, the word bacterium means rod or staff. The second form of *Wolbachia* that had been encountered was a barrel shape, oval like an egg and showed a maximum diameter along its length. The presence of *Wolbachia* in *S. calcitrans* was reported for the first time in this study. It was confirmed by EM following a positive PCR result. However, although there were positive results in *S. calcitrans*, no permanent establishment of *Wolbachia* from *D. melanogaster* occurred in *S. calcitrans* and the *Wolbachia* could not be detected in the next generation. A reason for the infection not being stable is that *Wolbachia* may not be physiologically adapted to its new host. The *Wolbachia* found in somatic muscle tissues of *S. calcitrans*, were morphologically similar to the *Wolbachia* present in *D. melanogaster*.

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