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Biology of interspecies Wolbachia infections

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BIOLOGY OF INTERSPECIES *WOLBACHIA* INFECTIONS

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SUMMARY

Wolbachia are maternally inherited intracellular bacteria known to infect a wide range of arthropods. The artificial transfer by microinjection of the 'popcorn-effect'-inducing *Wolbachia* from *Drosophila melanogaster* larvae to *Stomoxys calcitrans* pupae identified the germ-line tissue as the limiting factor in horizontal transmissions across wider phylogenetic distances. Electron microscopic analysis of adult somatic tissue of *S. calcitrans* documented that this naïve host is fully capable of supporting *Wolbachia*. However, *Wolbachia* does not succeed in achieving sufficient germ-line titres to maintain transovarial transmission beyond five host generations. This leads to the hypothesis that host-symbiont specificity in the case of *Wolbachia* is primarily determined by specific host transport and localisation phenomena surrounding egg development and not by mechanisms involving basic life support by the host cell or by host cell-symbiont communication. Coevolution of *Drosophila* and *Wolbachia* seems to have become species-specific for only very few and specific cellular interactions.

A further consequence of the interaction of *Drosophila* with the popcorn strain of *Wolbachia* has been identified by confocal microscopy. While the ancestral phenotype of the popcorn strain is cytoplasmic incompatibility as evidenced by its capacity to rescue incompatibility, the exclusion of *Wolbachia* from developing sperm cysts explains its failure to induce incompatibility. *Wolbachia's* ability to manipulate reproductive phenotypes of its host seems to be governed by cellular localisation phenomena as well.

The 'popcorn-effect' itself practically halves the adult life span of its *Drosophila* host. This was originally attributed to an unrestricted proliferation of *Wolbachia* in the host cells of the imago. The 'popcorn-effect' was identified as a temperature sensitive trait. A statistical comparison of the quantitative kinetics of *Wolbachia* with the help of the real-time polymerase chain reaction between permissive and non-permissive temperatures does not support the original description of the 'popcorn-effect'. The data suggest heterogeneity of the trait.

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CHAPTER 1.
GENERAL INTRODUCTION

GENERAL INTRODUCTION

Wolbachia are maternally inherited, intracellular bacteria known to infect a wide range of arthropods. Recent surveys indicate that around 16-20% of all insect species may be infected with *Wolbachia* (Werren *et al.*, 1995a). *Wolbachia* are also widespread in filarial nematodes. These bacteria were discovered by Hertig and Wolbach (1924) in the ovaries of the mosquito *Culex pipiens.* In 1936, Hertig formally named them as *Wolbachia pipientis* in honour of his collaborator. Infections with *Wolbachia* have been associated with host reproductive abnormalities such as cytoplasmic incompatibility, feminisation, parthenogenesis, male-killing and the 'popcorn-effect'. It has been named 'Popcorn-effect' because when the bacteria multiply inside the fly's cells and cause a sudden massive degeneration, it resembles the behaviour of popcorn in a microwave (Min and Benzer, 1997).

Interest in *Wolbachia* stems not only from its widespread distribution in arthropods (see Table 1-1), but also from the alterations it causes to the mode of reproduction of its hosts. Both of these lay the foundation for an interest in employment of this bacterium in biological control, as a microbial "natural enemy" or as a driving force for spreading desirable genetic modifications into insect populations (Werren, 1997).

Table 1-1.Catalogue of invertebrate hosts of *Wolbachia* and the phenotypes it induces in its hosts (Bourtzis and Braig, 1999)

Wolbachia host	Common Name	Phenotype
Phylum Nematoda		
Class **Secernentea**		
Order **Spirurida**		
Family **Onchocercidae**	filarial worm, elephantitis worm	enhance larval development, adult fertility
Phylum Arthropoda		
Subphylum **Crustacea**		
Order **Isopoda**		
Family **Armadillidiidae**	woodlice	F
Family **Asellidae**	pillbugs	?
Family **Cylisticidae**	woodlice	?
Family **Ligiidae**	woodlice, beach slater	F
Family **Oniscidae**	sow bugs	F
Family **Philosciidae**	woodlice	?
Family **Porcellionidae**	sow bugs	F, CI
Family **Spaeromatidae**	pillbugs	F
Family **Trichoniscidae**	woodlice	?
Family **Tylidae**	woodlice	?
Subphylum **Chelicerata**		
Class **Arachnida**		
Order **Parasitiformis**		
Family **Phytoseiidae**	predator mite	CI
Family **Tetranychidae**	spider mites	Uni&bi-directional CI, P, hybrid

		breakdown
Subphylum **Tracheata**		
Class **Elliplura**		
Order **Colembolla**		
Family **Isotomodae**	springtails	P
Class **Insecta**		
Order **Thysanura**		
Family **Lepismatidae**	silver fish	?
Order **Odonata**		
Family **Libellulidae**	common skimmers	?
Order **Dermaptera**		
Family **Carcinophoridae**	seaside earwigs, ring-legged earwigs	?
Order **Isoptera**	termites	?
Order **Orthoptera**		
Family **Acrididae**	short-horned grasshoppers	?
Family **Gryllidae**	crickets	CI
Family **Tettigoniidae**	longhorn, grass hoppers, bush crickets, katydids	?
Order **Hemiptera**		
Family **Aleyrodidae**	whiteflies	CI
Family **Anthocoridae**	flower bugs	?
Family **Aphididae**	aphids, plant lice	?
Family **Delphacidae**	planthoppers	CI
Family **Reduviidae**	assasin bugs, ambush bugs, thread-legged bugs	?

Order **Thysanoptera**		
Family **Aelothripidae**	broad-winged thrips, banded thrips	P
Family **Thripidae**	common thrips	?
Order **Coleoptera**		
Family **Bruchidae**	seed and bean beetles	?
Family **Byturidae**	raspberry beetles	?
Family **Chrysomelidae**	leaf beetles	CI
Family **Cleridae**	checkered beetle	?
Family **Coccinellidae**	ladybird beetles	MK
Family **Curculionidae**	snout beetles, true weevils	CI
Family **Dermestidae**	skin beetles, larder beetles	?
Family **Lampyridae**	lightning bugs, fireflies	?
Family **Scolytidae**	bark beetles, engravers, ambrosia beetles	?
Family **Tenebrionidae**	darkling beetles	CI
Order **Hymenoptera**		
Family **Agaonidae**	fig wasps	?
Family **Aphelinidae**	parasitoid wasps	P
Family **Apidae**	parasitoid wasps	?
Family **Braconidae**	parasitoid wasps	?
Family **Cynipidae**	gall wasps	?
Family **Dryinidae**	parasitoid wasps	?
Family **Encyrtidae**	parasitoid wasps	?
Family **Eulophidae**	parasitoid wasps	?
Family **Eucoilidae**	parasitoid wasps	P
Family **Formicidae**	ants	?

Family **Proctotrupoidae**	parasitoid wasps	CI
Family **Pteromalidae**	jewelwasps	P, CI
Family **Sphecidae**	digger wasps	?
Family **Trichogrammatidae**	parasitoid wasps	P
Order **Lepidoptera**		
Family **Gracillariidae**	blotch Leaf miners	?
Family **Noctuidae**	moth	?
Family **Nymphalidae**	Brush-footed butterflies, four-footed butterflies	MK
Family **Papilonidae**	swallowtails, parnassians	?
Family **Pieridae**	White butterflies, yellow butterflies	CI., no CI, F
Family **Pyralidae**	snout moths, grass moths	CI, F, MK
Order **Siphonaptera**		
Family **Pulicidae**	fleas	?
Order **Strepsidera**		
Family **Elenchidae**	twisted-wing parasites	?
Order **Diptera**		
Family **Calliphoridae**	blowflies	?
Family **Culicidae**	mosqutoes	CI
Family **Diopsidae**	stalk-eye flies	CI, enhance male fertility
Family **Drosophilidae**	vinegar flies, pomace flies	Uni&bi-directional CI, mod-res+, 'popcorn-

		effect'
Family **Ephydridae**	shore flies	?
Family **Glossinidae**	tsetse flies	?
Family **Muscidae**	house flies, face flies, horn flies	?
Family **Neriidae**	cactus flies	?
Family **Psychodidae**	sand flies	?
Family **Stratiomyidae**	soldier flies	?
Family **Tephritidae**	fruit flies	CI

CI = cytoplasmic incompatability
F = Feminisation
P = Parthenogenesis
MK = Male killing
? = Unknown

MORPHOLOGY OF *WOLBACHIA*

The morphology of *Wolbachia pipientis* was described by Hertig in 1936. The bacteria are very small and dimorphic: irregular bacilli form (0.5-1.3μm in length) and coccoid form (0.25-0.5μm in diameter). The endosymbiotic vacuole consists of three layers; the outer layer is of host origin (host vacuole membrane), the middle layer is the *Wolbachia* outer membrane and the innermost layer consists of the plasma membrane of the bacteria. Smith (1979) reported that the existence of the vacuole membrane is advantageous for both host cell and microorganism. The microorganism/symbiont may be protected from enzymes and the host cell may exercise some control over the symbiont.

Wolbachia cannot be cultured in a cell-free system (Bourtzis and Braig, 1999). It can be visualised by electron microscopy or light

microscopy combined with a fluorescent DNA stain or conventional stains such as Giemsa, lacmoid or Gimenez. Recently PCR technology has become a tool facilitating the detection of *Wolbachia* (Bourtzis and Braig, 1999).

NAMING OF *WOLBACHIA* STRAINS

Originally *Wolbachia* were named after their host species. For example, *Wolbachia postica* was named after the alfalfa weevil, *Hypera postica.* After the number of new *Wolbachia* strains increased and a molecular characterisation of these strains became possible, another naming system was established which related a strain to its closest sequenced isolate. The strain designation refers to *Wolbachia* followed by the name of the host species and or host strain from which it was collected. For example, *w*Ri is a *Wolbachia* isolate of *Drosophila simulans* collected at Riverside; *w*Haw is a *Wolbachia* isolate of *D. simulans* collected in Hawaii; *w*Cof is a *Wolbachia* isolate of *Drosophila simulans* collected at Coffs Harbour (Australia) and *w*Nou is a *Wolbachia* isolate of *Drosophila simulans* collected in Noumea (New Caledonia). *w*Mau is a *Wolbachia* isolate of *Drosophila mauritiana, w*Alb is a *Wolbachia* isolate of *Aedes albopictus, w*Pip is a *Wolbachia* isolate of *Culex pipiens, w*Mor is a *Wolbachia* isolate of *Glossina morsitans, w*Ori is a *Wolbachia* isolate from *Tagozoides orizicolus* and *w*Aus is *Wolbachia* isolated from *Glossina austeni.*

THE PHYLOGENY OF *WOLBACHIA*

To analyse the relationship between *Wolbachia* strains, various genes have been used. The genes most commonly used are the 16S rDNA gene, the *fts*Z gene (cell division gene), the *gro*EL

gene (bacterial heat shock protein) (Masui *et al.*, 1997) and the *wsp* gene (cell surface protein) (Van Meer *et al.*, 1999). Nowadays, the *wsp* gene is used for the classification of *W. pipientis.* Braig *et al.* (1998) showed that *wsp* gene is evolving at a much faster rate than any other previously reported *Wolbachia* gene and so is the most effective gene for determining phylogenetic relationships between strains of *Wolbachia pipientis* (see Fig. 1-4). This information can be used to divide *Wolbachia* into a number of subgroup that appear to predict the reproductive phenotype of the female *Drosophila* host. Analysis of the 16S rDNA shows *Wolbachia* to be a α proteobacteria, closely related to intracellular bacteria in the Rickettsiaceae (O'Neill *et al.*, 1997; Bourtzis and Braig, 1999) see fig. 1-2 and table 1-2. Analysis of the cell cycle gene *ftsZ* (Holden *et al.*, 1993; Werren *et al.*, 1995b) shows that two main groups of sequences can be distinguished and these have been named group A and group B (Werren *et al.*, 1995b) see Fig.1-3. However, it appears that *Wolbachia* inducing similar effects on hosts are not monophyletic and are present in both groups. These phylogenetic studies have shown that bacterial phylogenies and the phenotypic effect on host are not congruent, predicting horizontal transmission between host species (Fig.1-1).

Fig. 1.1. Relationship between the phylogeny of *Wolbachia pipientis* and the phylogeny of the arthropod hosts.

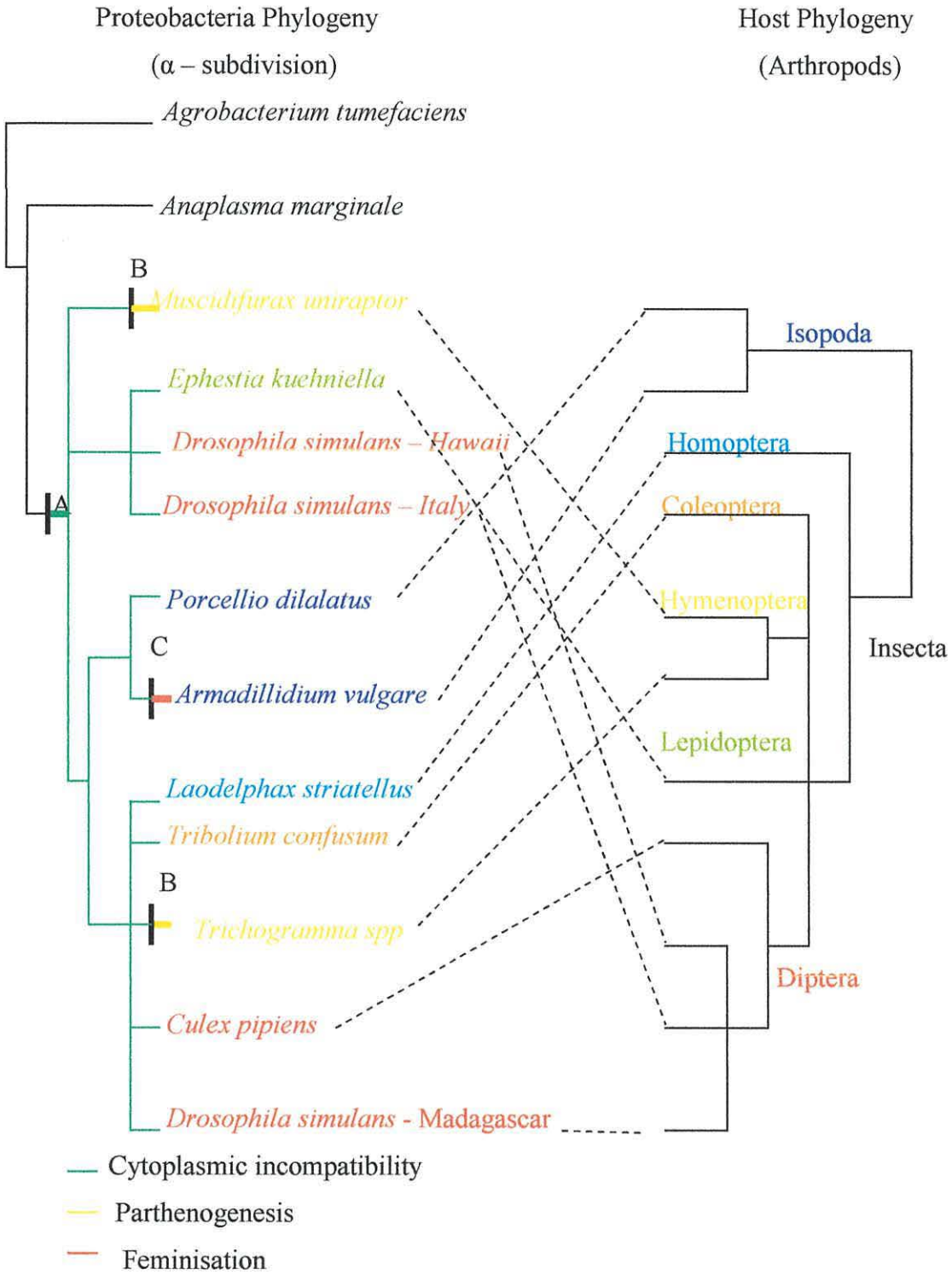


Fig.1.1 shows the phylogeny of selected *Wolbachia pipientis* and the phylogeny of the corresponding arthropod hosts. The bacterial phylogeny is compiled from analyses based on 16S rDNA sequences. The lack of concordance between bacterial and host phylogenies indicates that the arthropod-infecting *Wolbachia* have undergone horizontal transfer among arthropod lineages. Lack of congruence is apparent when associated hosts and bacteria do not form corresponding clades, e.g. associates of *Drosophila* are not closely related. The ability to cause incompatibility (A) appears to have originated in a common ancestor to the *Wolbachia* so far analysed. The ability to induce parthenogenesis (B) originated in lineages leading to the *Trichogramma*-infesting strain and independently in a lineage leading to *Mucidifurax uniraptor.* The ability to induce genetic males to develop as females (C) was derived in a lineage infecting isopods (Moran and Baumann, 1994).

Van Meer and Stouthamer (1999) proposed the following hypotheses for the lack of congruence between the effect on hosts and phylogeny are: (i) *Wolbachia* genes coding for a certain effect (P, CI and F) are located on a plasmid that can jump relatively easily from one *Wolbachia* strain to another; (ii) *Wolbachia* genes inducing P, CI or F can cross over between different strains in double-infected hosts; (iii) *Wolbachia* genes causing P, CI or F are highly congruent and can readily evolve from each other.

Recent study indicated that the sequences reveal 5 major groups (A-E). The A, B and E groups contain the insect, mite and crustacean *Wolbachia* and the C-D groups harbour filarial *Wolbachia* (Werren *et al.*, 1995b; Vanderkerchove *et al.*, 1999).

Fig 1-2. Phylogeny of *Wolbachia* based on 16S rDNA, (O'Neill *et al.*, 1992)

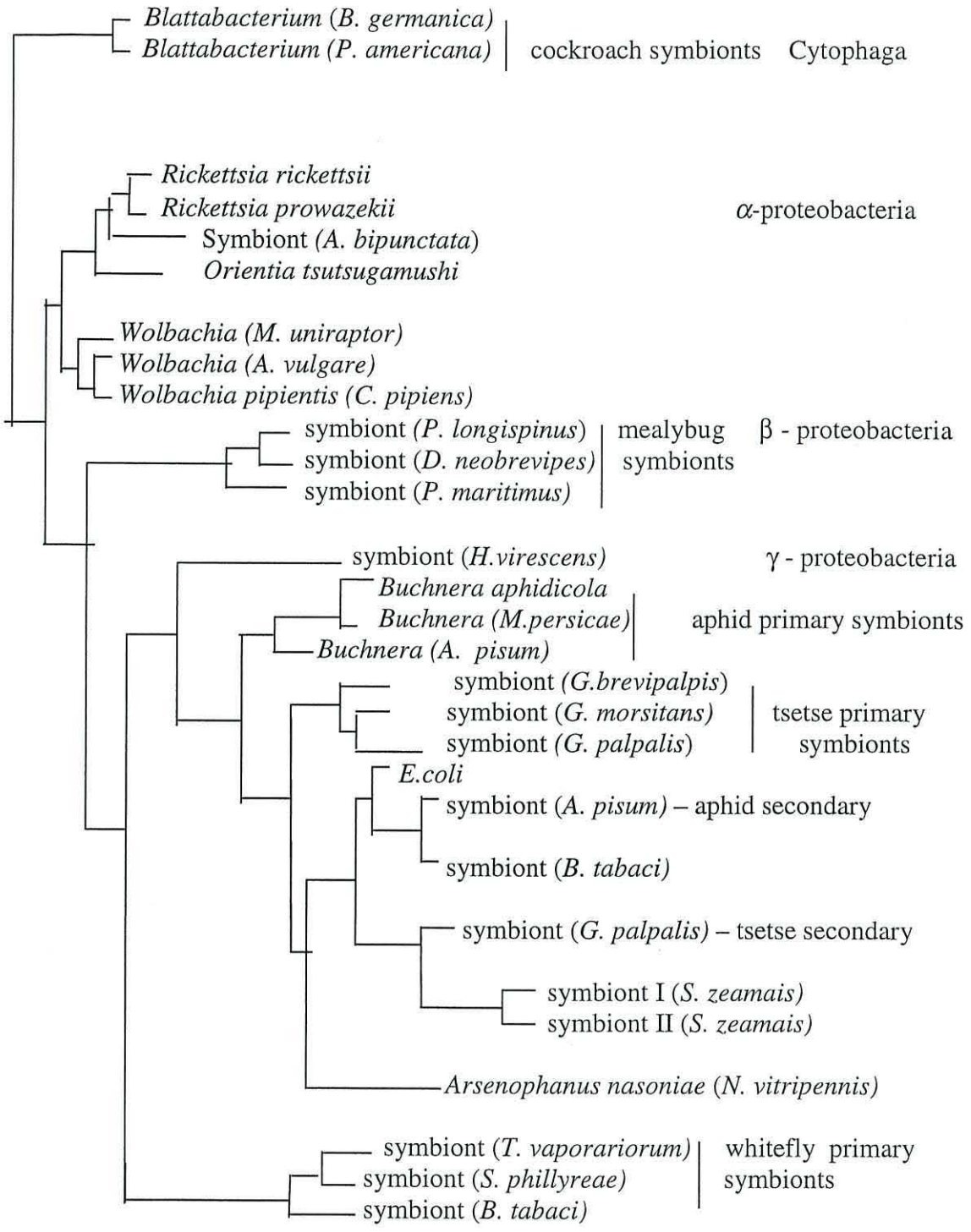


Fig.1-2 Phylogenetic tree derived from maximum parsimony analysis of aligned 16S rDNA sequences. The arthropod host is indicated after the name of the bacterium.

Fig 1-3. Phylogeny of *Wolbachia* based on *fts*Z gene (Holden *et al.*, 1993 and Werren *et al.*, 1995b):

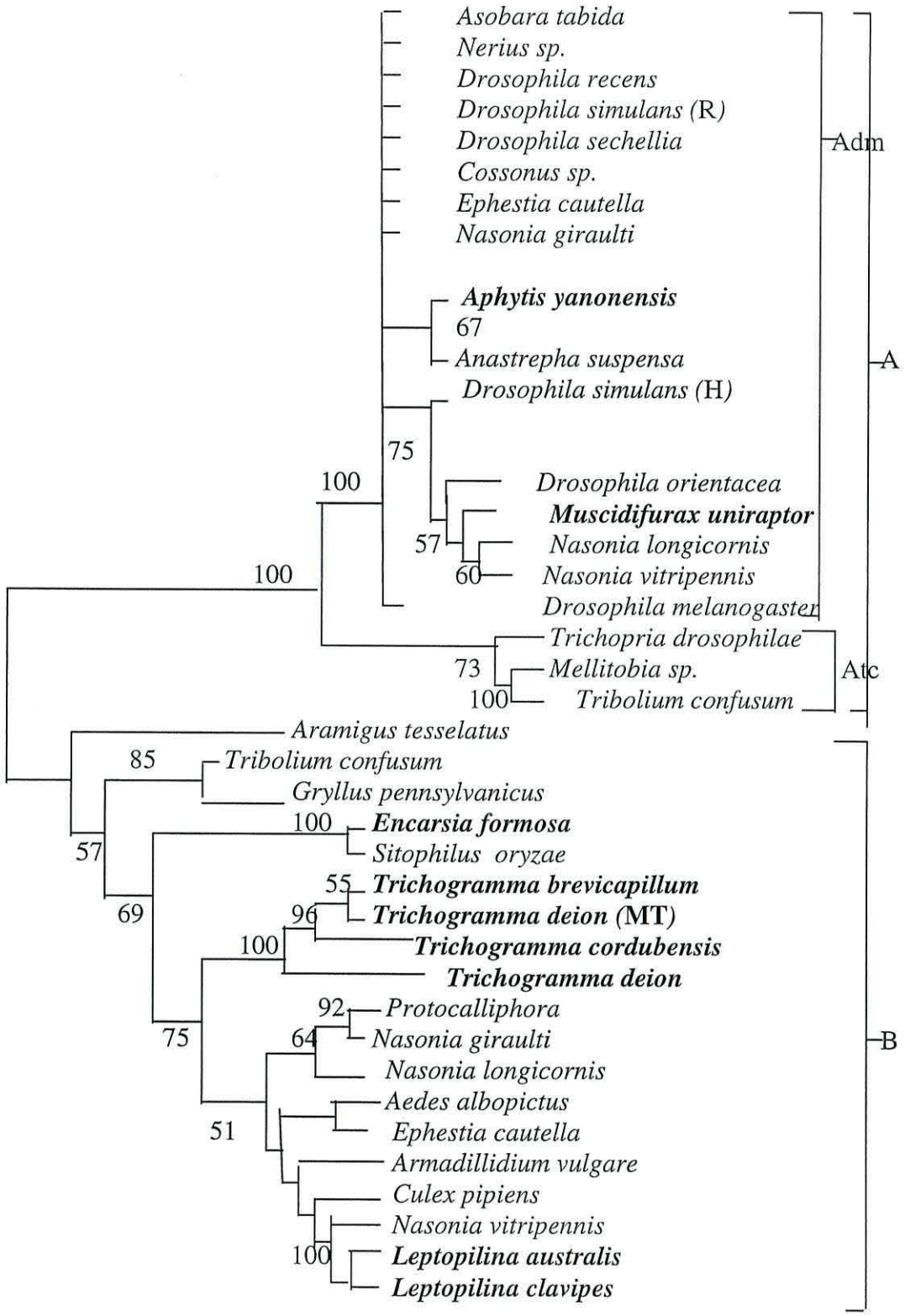


Fig.1-3 Phylogenetic tree of *Wolbachia* based upon sequences of the bacterial *ftsZ* gene. Parthenogenesis-associated bacteria are shown in bold. The tree was generated by neighbour-joining using the *p*-distance including insertions and deletions. Number next to the nodes indicate the number of replicates confirming the node out of 100 (only numbers greater than 50 are shown). The tree clearly shows horizontal transmission of *Wolbachia* between different orders of insects. Closely related *Wolbachia* are found in a parasitic wasp (*Nasonia giraulti*) and its fly host (*Protocalliphora*), suggesting horizontal transfer between parasite and host insect (Werren *et al.*, 1995b)

A = *Wolbachia* group A
Adm= *Drosophila melanogaster*-like *Wolbachia* in group A
Atc = *Tribolium confusum*-like *Wolbachia* in group A
B = *Wolbachia* group B
R = *Drosophila simulans* collected at Riverside
H = *Drosophila simulans* collected at Hawaii
MT = Montana

Fig 1-4. Phylogeny of *Wolbachia* based on the *wsp* gene (Zhou et al., 1997)

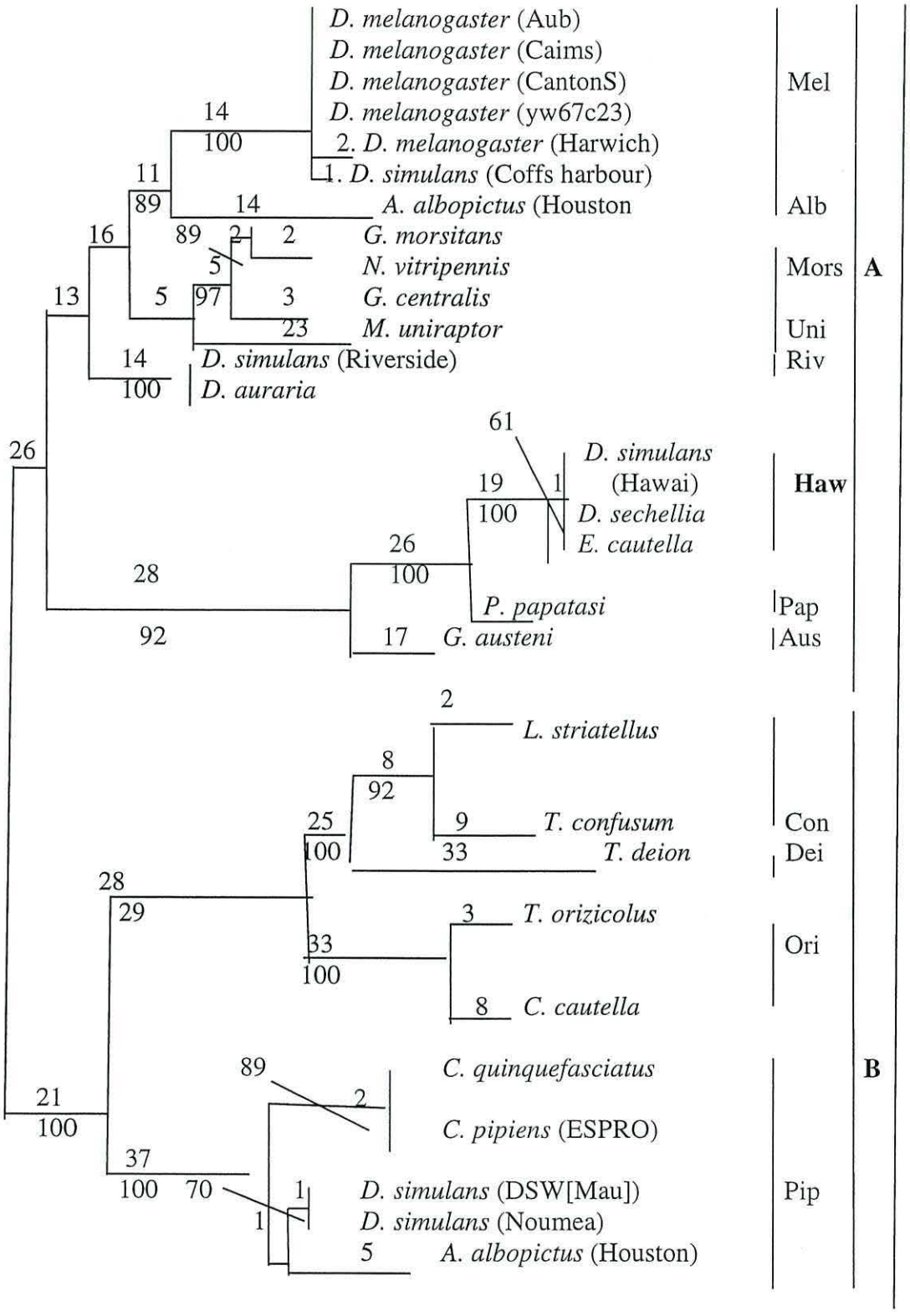


Fig. 1-4 one of four most parsimonious trees generated from a branch and bound search of aligned *wsp* sequences (tree length=472; CI=0.64). Tree shown is midpoint rooted. Branch lengths, as determined from PAUP table

of linkages, are labelled above branches and bootstrap values (500 replicates) are labelled below branches. Bootstrap values less than 50 are not shown. Taxa are labelled as the host from which the *Wolbachia* strain was isolated.

Mel = Melanogaster
Alb = Albopictus
Mors = Morsitans
Uni = Uniraptor
Riv = Riverside
Haw = Hawaii
Pap = Papatasi
Aus = Austeni
Con = Confusum
Dei = Deion
Ori = Orizicolus
Pip = Pipiens
A = *Wolbachia* group A
B = *Wolbachia* group B

Table 1-2. Systematics of Rickettsia (according to the National Centre for Biotechnology Information, the National Library, the National Institute of Health; O'Neill *et al.*, 1997)

Proteobacteria
α - Subdivision
Rickettsiales

Rickettsia group ciliate endosymbionts
Caedibacter
Caedibacter caryophilus
Caedibacter taeniospiralis
(endosymbiont of *Acanthamoeba polyphaga*)
Holospora
Holospora obtusa
Rickettsiaceae
Ehrlichisieae
Anaplasma
Anaplasma centrale
Anaplasma marginale
Anaplasma phagocytophilum group
Anaplasma phagocytophilum (agent of human granulocytic ehrlichiosis)
Cowdria
Cowdria ruminantium (heartwater rickettsia)
Ehrlichia canis group
Ehrlichia canis
Ehrlichia risticii group
Ehrlichia risticii (Potomac horse fever)
Ehrlichia sennetsu
Neorickettsia
Neorickettsia helminthoeca

Rickettsieae
Orientia
Orientia tsutsugamushi
Rickettsia spotted fever group
Rickettsia aeschlimanni
Rickettsia australis
Rickettsia conorii
Rickettsia helvetica
Rickettsia honei

Rickettsia typhus group

Rickettsia canadensis
Rickettsia prowazekii
Rickettsia typhii

Unclassified Rickettsia

Male killing Rickettsia from *Adalia bipunctata*
Male killing Rickettsia from *Adalia decempunctata*

Wolbachieae

Wolbachia pipientis

MODE OF *WOLBACHIA* TRANSMISSION

The principal mode of *Wolbachia* transmission is vertically (maternally through the egg cytoplasm). However, molecular phylogenetic analysis of *Wolbachia* isolates and its arthropod hosts indicate that *Wolbachia* can move horizontally on an evolutionary timescale (see Fig.1-1). Stouthamer (1997) reported the natural horizontal transmission in the parasitoid wasps of the genus *Trichogramma.* Stouthamer (1993), Werren *et al.* (1995a), Schilthuizen and Stouthamer (1997) reported that the *Wolbachia* in *Trichogramma* form a monophyletic group restricted to this host genus. But, within different populations of the same species, different types of *Wolbachia* can be found, and no correlation exists between the phylogeny of the *Wolbachia* and that of the *Trichogramma* species. Noda *et al.* (2001b) showed that a strepsipteran endoparasite of *Laodelphax striatellus* and *Sogatella furcifera* mediates the transmission of *Wolbachia* amongst planthopper species. It has been shown that the strepsipteran endoparasite, harboured *Wolbachia* with gene sequences identical to those of the *Wolbachia* that infect the planthoppers. Therefore,

Wolbachia strain is probably shared among two rice plant hoppers and their strepsipteran endoparasite.

Furthermore, phylogenetic evidence (supported by artificial transfer experiments) suggests that *Wolbachia* have moved horizontally between species in the same family or different families or orders. However, the frequency and mechanism by which this occurs is not clear.

Chang and Wade (1994) carried out the transfer of the cytoplasmic endosymbiont *Wolbachia pipientis* from infected to uninfected flour beetles (*Tribolium confusum*). They showed that around 39% were infected with *Wolbachia.* However, some transfected females failed to transmit *Wolbachia pipientis* to their progeny and that the incompatibility could be lost in the generation following transfection. The capability of transferring *W. pipientis* from infected to uninfected *Tribolium confusum* is a first step toward determining whether or not *W. pipientis* can be used as part of a strategy of biological control of pests. This study shows that microinjection can be used to transfer *W. pipientis* and reproductive incompatibility between an infected and a cured strain of the flour beetle pest *T. confusum.*

Van Meer and Stouthamer (1999) attempted the transfer of *Wolbachia* between different orders. They transferred *Wolbachia* that causes parthenogenesis from the parasitoid wasp *Muscidifurax uniraptor* (Hymenoptera) to *Drosophila simulans* (Diptera). However, neither a permanent infection nor any phenotype could be established

Boyle *et al.* (1993) reported the transfer of *Wolbachia* from *D. simulans* to uninfected *D. melanogaster* and *D. simulans.* The result indicate that *Wolbachia* in *D. simulans* cause strong CI (±95% CI). However, in *D. melanogaster* cytoplasmic incompatibility was strongly reduced (weak).

Braig *et al.* (1994) transferred *Wolbachia* between different families from *Aedes albopictus* (Diptera: Culicidae) to *Drosophila simulans* (Diptera: Drosophilidae). The transinfected *Drosophila* strain became bidirectionally incompatible with two other naturally infected strains of *D. simulans.*

Sinkins *et al.* (1995b) reported the transfer of *Wolbachia* from *Drosophila simulans* strain Hawaii (DSH) to *Drosophila simulans* Riverside (DSR). This resulted in a double infection. Rousset *et al.* (1999) showed the cytoplasmic transfer of *Wolbachia* from one double-infected *Drosophila simulans* strain M4 (double infection [*w*Ha + *w*Ri]) to another double-infected *Drosophila* strain Nou (*w*Ha + *w*No) to result in a triple infection. The total density of *Wolbachia* in the host was increased. Each of the three symbionts was stably transmitted in the presence of the two others and triple-infected males were incompatible with double-infected females. No evidence was obtained for interference between modification effects of the different *Wolbachia* strains in males. Some incompatibility was observed between triple-infected males and females. However, this incompatibility reaction is not a specific property of triple-infected flies since it was also observed in double-infected strain.

Giordano *et al.* (1995) reported *Wolbachia* infection in *Drosophila mauritiana* and *D. sechellia*. However, no phenotype was found in infected *Drosophila mauritiana*. No evidence for incompatibility in crosses between infected and uninfected strains of *D. mauritiana* were found. When *Wolbachia* was transferred from *D. mauritiana* to uninfected *D. simulans*, this infection still failed to cause incompatibility, suggesting that the lack of incompatibility is a consequence of the *Wolbachia* strain rather than of the host nuclear background; *Wolbachia* which infect *D. simulans* expresses very strong CI (± 95% cytoplasmic incompatibility). In another experiment, the transfer of *Wolbachia* from *D. simulans* to uninfected *D. mauritiana* resulted in expression of CI in *D. mauritiana*, indicating that *Wolbachia* can induce CI in *D. mauritiana*.

Using microinjection, Leventhal (1968) reported the transfer of the Sex Ratio (male killing) condition of *D. bifasciata* to uninfected *D. bifasciata, D. melanogaster* and *D. pseudoobscura.* The experiment showed alterations to the sex ratio of the new hosts (*D. melanogaster* and *D. pseudoobscura*).

Using embryonic microinjection techniques, Fujii *et al.* (2001) transferred feminisation-inducing *Wolbachia* from *Ostrinia scapulalis* into *Ephestia kuehniella* (cured with tetracycline). This resulted in *E. kuehniella* producing only female offspring. This was due to male-killing, not feminisation. The reproductive alteration in these lepidopteran species is dependent on the bacterial strain as well as the host's genetic background.

Juchault *et al.* (1974) cited in O'Neill (1997) carried out artificial horizontal transfer from infected *Armadillidium vulgare, Porcellionides pruinosus* and *Chaetophilosa elongata* (Crustacea) into un-infected individuals of their own or other species. Rigaud and Juchault (1995) carried out the transfer of *Wolbachia* from *A. vulgare* ovaries into uninfected *A. nasatum* and from *Chaetophilosa elongata* to uninfected *A. vulgare*. PCR revealed that in *A. nasatum* mothers and their daughters harboured *Wolbachia,* whilst sons were never infected and the sex ratio (feminisation) in *A. vulgare* was unchanged. In this strain, mothers were infected, but none of their offspring. Subsequently they performed the horizontal transfer by simple contact between the blood of infected and un-infected individuals. Un-infected *A. vulgare* males were wounded and were placed in contact with fresh blood of infected *A. vulgare* female. Transmission electron microscopy showed that half of these males (from 15 males) developed a female physiology and phenotype. Table 1-3 lists artificial transmissions of *Wolbachia.*

Table 1-3. Artificial horizontal transmissions of *Wolbachia* between arthropods.

No	Donor	Recipient	Material transferred	Phenotype	Reference
1	*Armadillidium vulgare, Porcellionides. pruinosus, C. elonga ta*	*A. vulgare, P.scaber* and *Oniscus asellus*	ovaries	feminisation	Juchault *et al.* 1994
2	*D. simulans*	*D. simulans* and *D. melanogaster*	cytoplasm	CI in *D. simulans*, weak CI in *D. melanogaster*	Boyle *et al.*, 1993
3	*A. albopictus*	*D. simulans*	purified *Wolbachia* transferred into egg cytoplasm	CI,	Braig *et al.*, 1994
4	*T. confusum*	*T.confusum*	ovaries	CI	Chang and Wade, 1994
5	*D. simulans* Hawaii	*D. simulans* Riverside (DSR)	cytoplasm	CI	Sinkins *et al.*, 1995b
6	*w*Ha+*w*Ri (double-infected strain of *Wolbachia*)	*w*Ha + *w*Nou (double-infected strain)	egg cytoplasm	CI	Rousset *et al.*, 1999

7	*D. mauritiana,* *D. simulans*	uninfected *D. simulans* *D. mauritiana* treated with tetracycline	cytoplasm	CI	Giordano *et al.*, 1995
8	*A. vulgare*	*A.nasatum* (uninfected)	ovaries	Feminisation	Rigaud and Juchault, 1995
9	*T. confusum*	*T.confusum* (uninfected)	cytoplasm	CI	Chang and Wade, 1996
10	*D. simulans*	*D. serrata*	cytoplasm	CI	Clancy and Hoffmann, 1997
11	*Nosema spp.* *G. duebeni* *A. vulgare*	*G. duebeni* *A. vulgare* *G. duebeni*	ovaries	feminisation	Dunn and Rigaud, 1998
12	*T. pretiosum*	*T. dendrolini* pupae	cytoplasm	parthenogenesis (unstable)	Grenier *et al.*, 1998
13	*D. melanogaster*	*D. simulans*	cytoplasm	CI	Poinsot *et al.*, 1998
14	*M. uniraptor*	*D. simulans*	cytoplasm		Van Meer and Stouthamer, 1999

15	*O. scapulalis*	*E. kuehniella*		Change of feminisation to male killing	Fujii, *et al.* 2001

CYTOPLASMIC INCOMPATIBILITY

Cytoplasmic incompatibility in arthropods was reported for the first time by Ghelelovitch (1950) as cited in O'Neill *et al.* (1997) and studied in detail by Laven (1952). Crosses between different strains of mosquitoes failed to produce progeny or produced progeny only when crossed in one direction (i.e. crosses of males from one strain with females from another produce progeny, but not the reciprocal cross). Laven reported that cytoplasmic factors were involved. However, cytoplasmic incompatibility was not associated with the presence of *Wolbachia* until the 1970's (Yen and Barr, 1971, 1973). *Wolbachia* were identified as the causative agent of cytoplasmic incompatibility in *Culex pipiens* by Yen and Barr (1973). They discovered that, when infected male and uninfected female mosquitoes were crossed, *Wolbachia pipientis* caused cytoplasmic incompatibility (CI). Uninfected eggs fertilised by sperm from infected males died. Yen and Barr (1973) also showed that *Wolbachia* infection in *Culex pipiens* was widespread during the early development of the embryo but eventually became restricted to germ cells. In fact, this bacterium is not only found in mosquitoes but also in other insects.

Hoffman *et al.* (1986) were the first to describe cytoplasmic incompatibility in *Drosophila simulans* from California. Using electron microscopy they identified *Wolbachia* as the causative agent of incompatibility in *D. simulans.* Binnington and Hoffman (1989) showed that *Wolbachia* were present in ovaries and testes of naturally infected *D. simulans* but absent from strains that were treated with antibiotics. Hoffman (1988) was the first to describe

partial/ relatively weak incompatibility in *D. melanogaster*. *Wolbachia* were also found in *D. mauritiana* and *D. sechellia* (Giordano *et al.,* 1995; Rousset and Solignac, 1995). Subsequently, it was reported that *Wolbachia* found in *Drosophila mauritiana* did not cause cytoplasmic incompatibility. Werren and Jaenike (1995) found that *D. recens* and *D. orientacea* were infected with *Wolbachia.* Bourtzis *et al.* (1994) found three (*D. auraria, D. ananassae* and *D. sechellia*) out of 30 species of *Drosophila* infected with *Wolbachia.* Whilst the level of incompatibility in *D. sechellia* and *D. auraria* is relatively high, it is weak in *D. ananassae.*

Stanley (1961) and Wade and Stevens (1985) showed that unidirectional cytoplasmic incompatibility was caused by *Wolbachia* in laboratory strains of the flour beetle *Tribolium confusum.* The compatibility can be restored by raising larvae at a higher temperature (37°C) for several days (Stevens, 1989). Subsequently, *Wolbachia*-like organisms were found in the ovaries of *T. confusum* and the bacteria could be eliminated with tetracycline (O'Neill, 1989).

Saul (1961) reported incompatibility in the parasitoid wasp *Nasonia vitripennis.* The incompatibility factor in *Nasonia* is maternally transmitted. Crossing *Nasonia* with laboratory stocks carrying mutant markers resulted in 85% female progeny whereas unfertilised females produce only haploid male offspring. However, when Saul crossed mutant strains with wild type males, one of the strains produced only male progeny and the other produced only 8% females. In contrast, the sex ratio in the

reciprocal cross was normal (50:50). This indicates unidirectional incompatibility. Furthermore, compatibility can be restored by injection of host larvae and pupae with antibiotics (tetracycline).

Breeuwer *et al.* (1992) showed that *Nasonia* naturally could harbour double infections. Werren *et al.* (1995b) found evidence of multiple infections in many other species based on the sequences of the *fts*Z gene. Perrot-Minnot *et al.* (1996) confirmed that some strains of *Nasonia* carry two distinct *Wolbachia* strains whereas others carry only a single strain.

Brower (1976) reported that *Wolbachia* infection in the almond moth, *Cadra cautella*, causes unidirectional incompatibility and the incompatibility factor was shown to be maternally transmitted. Electron microscopy revealed the bacteria in gonadal tissue (Kellen *et al.*, 1981). Compatibility can be restored by rearing larvae in the presence of an antibiotic.

Hsiao and Hsiao (1985) reported *Wolbachia* infection in the alfalfa weevil, *Hypera postica.* Populations from the eastern and western parts of the USA are unidirectionally incompatible. *Wolbachia* were found in the gonads of weevils from western populations, whilst those from eastern populations were not infected. Similarly, Noda (1984) showed cytoplasmic incompatibility between populations of the small brown planthopper (*Laodelphax striatellus*) from the north-eastern and south-western parts of Japan. The incompatibility was unidirectional and partial.

Gotoh and Noda (2000) investigated cytoplasmic incompatibility in mites. Seven species were found to be infected with *Wolbachia: Panonychus mori, Schizotetranychus cercidiphylli, Oligonychus gotohi, Tetranychus kanzawai, T. parakanzawai, T. puerariсola and T. urticae.* After examining the role of *Wolbachia* in the host's reproduction, 5 of the 7 populations of *P. mori* and *Tetranychus spp.* were found to be infected with CI-inducing strains.

Giordano *et al.* (1997) discovered *Wolbachia* that cause unidirectional incompatibility between different populations of the western corn rootworm, *Diabrotica vigifera vigifera*, a pest of maize. After administration of the antibiotic tetracycline, the bacteria were eliminated and compatibility was restored. Similar cases of restored reproductive compatibility were found in the cricket genus *Gryllus* (*G. assimilis, G. integer, G. ovisopis, G. pennsylvanicus* and *G. rubens*). Whilst populations of *G. rubens* and *G. ovisopis* carry the same *Wolbachia* strain, *G. integer* and *G. pennsylvanicus* are infected with two *Wolbachia* strains.

Furthermore, Moret *et al.* (2000) found *Wolbachia* that causes unidirectional cytoplasmic incompatibility in the crustacean *Cylisticus convexus* (*w*Cc). *Wolbachia* commonly causes feminisation in crustaceans and CI is commonly found in insects. An experiment in which *Wolbachia* (*w*Cc) was transferred to the isopod *Armadillidium vulgare*, in which infection with *Wolbachia* normally causes feminisation, showed that *w*Cc phenotype did not change to feminisation. Feminisation was unable to rescue CI induced by *w*Cc. However, the CI-inducing *Wolbachia* were not

transmitted to the next generation (eggs). CI and feminisation are probably due to different mechanisms.

Cytoplasmic incompatibility and the CI level (strong, partial/weak and no effect) are affected by various factors such as *Wolbachia* strain (Giordano *et al.,* 1995; Hoffmann *et al.,* 1996; Bourtzis and O'Neill, 1998; Merçot and Poinsot, 1998), host genetic background (Breeuwer and Werren, 1993 and Bordenstein and Werren, 1998), and host age. Age effects have been correlated with reduced bacterial density. For example, in sperm of older males the bacterial density is lower than in those of younger males (Singh *et al.,* 1976; Subbarao *et al.,* 1977a; Hoffmann *et al.,* 1986). Furthermore, several environmental factors such as temperature (Hoffmann *et al.,* 1990; Clancy and Hoffmann, 1998), nutrition/food quality (Hoffmann *et al.,* 1990; Sinkins *et al.,* 1995a) and larval density may greatly influence the strength of the cytoplasmic incompatibility phenotype. Many authors (Boyle *et al.,* 1993; Bressac and Rousset, 1993; Sinkins *et al.,* 1995b; Clancy and Hoffmann, 1998; Poinsot *et al.,* 1998; Noda *et al.,* 2001a) reported that *Wolbachia* density is associated with the expression and transmission of CI. These factors can interact with each other in complex ways to influence the strength and direction of cytoplasmic incompatibility.

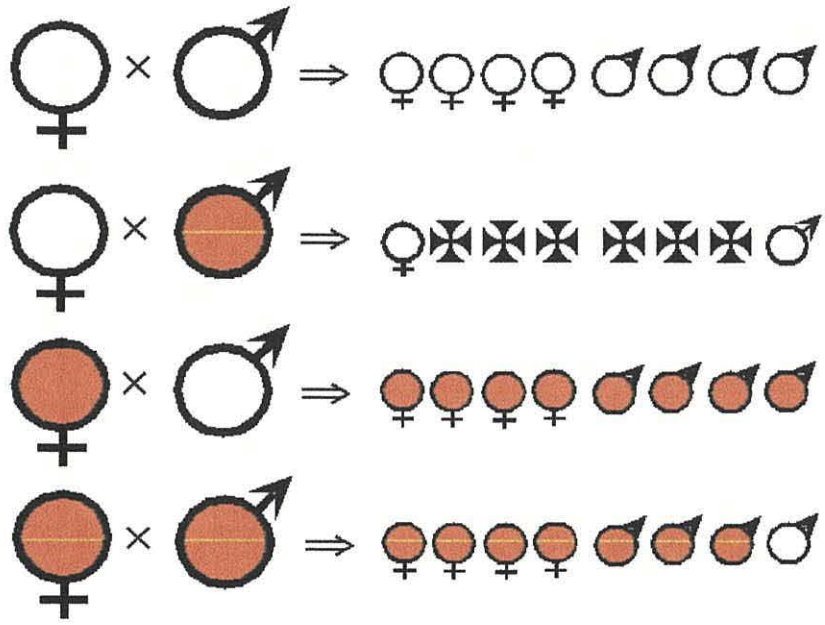
Noda *et al.* (2001b) reported that the number of *Wolbachia* in *Laodelphax striatellus* (Homoptera: Delphacidae) females increases after adult emergence. After microinjection, they also found that CI expression and transmission rates are usually low for several generations until bacterial density increases.

The host genotype affects expression of CI (Giordano *et al.*, 1995). Microinjection of *w*Ri from *D. simulans* to *D. mauritiana* resulted in incompatibility between *w*Mau-infected and the uninfected strains. In *D. mauritiana* there was no evidence for incompatibility in croses between naturally infected and uninfected strains. This indicates that *Wolbachia* induce incompatibility in a *D. mauritiana* nuclear background.

The age of the male has been shown to determine the extent of CI in certain insect species (mosquitoes). Singh *et al.* (1976) found 91% incompatibility in *Culex* when crosses involved males aged 6-7 days and a reduction to 26% incompatibility when crosses involved males aged 16-17 days. Furthermore, Noda *et al.* (2001a) reported that in males of *Laodelphax striatellus, Wolbachia* numbers decreased as adult age increased. And in females there were more *Wolbachia* than in males. Four day-old females of *L. striatellus* possessed 2.3 times higher numbers than the males of the same age.

Cytoplasmic incompatibility can be either unidirectional or bidirectional. Unidirectional CI is expressed when an infected male is crossed with an uninfected female or a female of a different infection status. Bidirectional CI occurs in crosses between infected individuals harbouring different strains of *Wolbachia.*

Fig.1-5 Unidirectional incompatibility of a single infected line.



Crossing types displaying cytoplasmic incompatibility. The DSR strain infected with *Wolbachia pipientis* is represented by the filled red circle. The uninfected strain is represented by the open circle. The death sign indicates unviable progeny. Crossing an infected male and an uninfected female results in 75% progeny to die, crossing an infected female and an uninfected male results in 100% infected progeny, whilst crossing an infected female and male results in 87.5% infected progeny. In the latter cross vertical transmission is not perfect where as in the second cross the penetrance of the phenotype (compatibility) is not perfect (Bourtzis and Braig, 1999).

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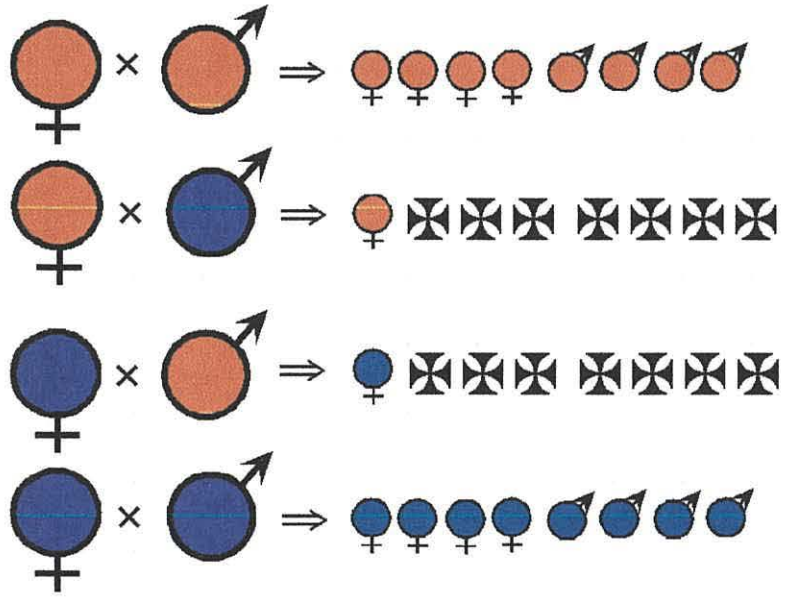
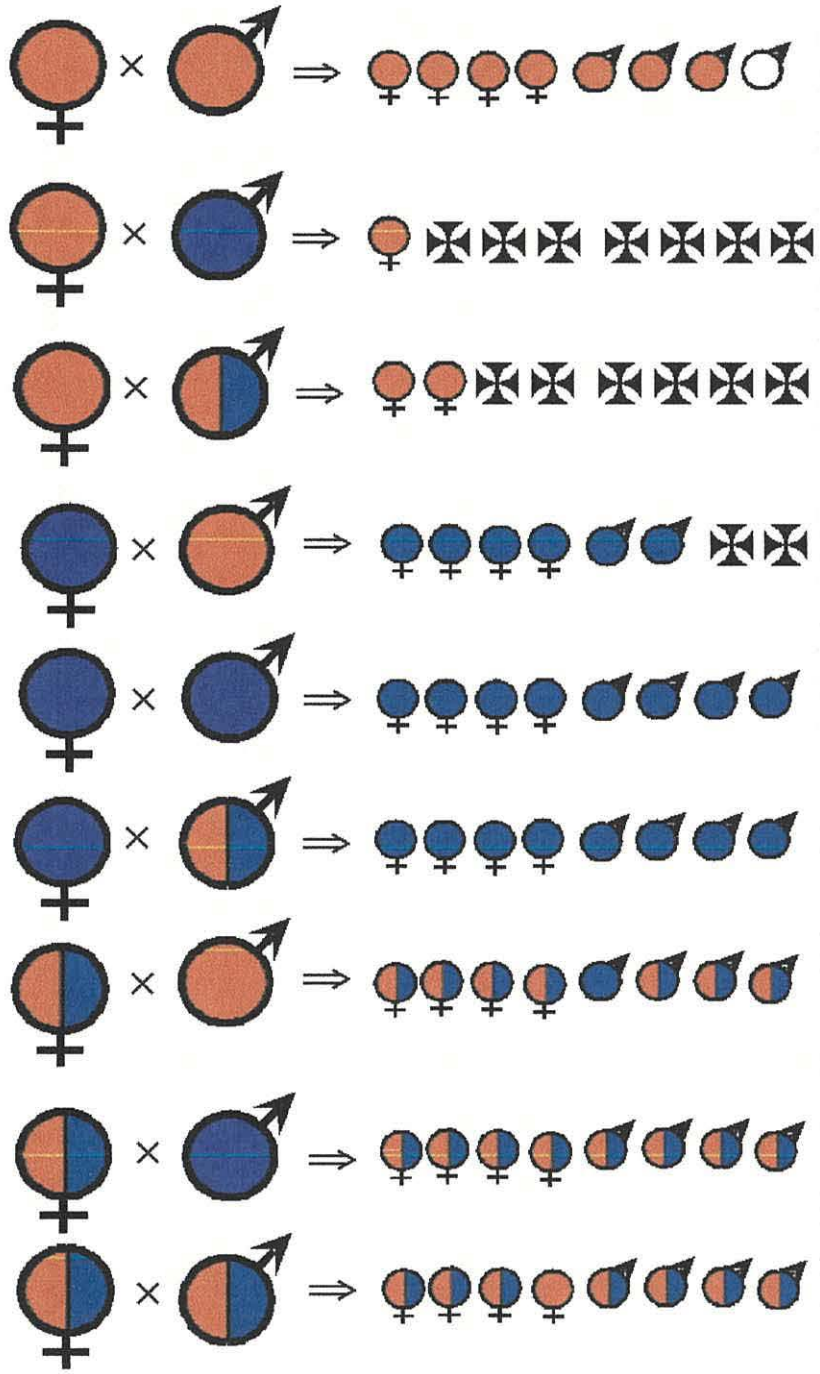
Fig. 1-6 Bidirectional cytoplasmic incompatibility of two single infected lines.



Bidirectional incompatibility of two single infected lines. Cytoplasmic incompatibility occurs in crosses between infected individuals harbouring different strains of *Wolbachia* (red and blue circle). When an infected male (red or blue) is crossed with an infected female of a different strain, 87.5% of the offspring will die. Whilst the cross between an infected female and male of the blue strain results in 100% infected progeny.

Fig. 1-7 Unidirectional incompatibility of a double infected line.



When a double infected male is crossed with a single infected female (red strain), 75% cytoplasmic incompatibility is expressed. Whilst a double infected male is crossed with an infected female (blue strain) 100% infected progeny is obtained.

Mechanism of cytoplasmic incompatibility

The exact mechanism of CI is still not understood. However, based on the genetic and cytologic evidence two hypotheses have been put forward regarding the mechanism of cytoplasmic incompatibility. Either (1) *Wolbachia* in the male produce a product that disrupts sperm processing in the egg unless rescued or (2) *Wolbachia* in the male act as a "sink" of sperm proteins disrupting sperm processing in the egg unless replenished.

1. Modification-rescue hypothesis of cytoplasmic incompatibility
   a. the modification of sperm

      Wolbachia modify maturing sperm. The effect on the male sperm takes place during maturation before *Wolbachia* are excluded from the mature spermatozoon. The modification or imprint is strain specific and additive.

   b. the rescue in the eggs

      The modification is rescued in the fertilised egg cell. Rescue effects occur in *Wolbachia*-infected eggs that allow an imprinted sperm cell to successfully fertilize an infected egg. The rescue is strain specific and additive.

2. The sink hypothesis of cytoplasmic incompatibility:

   Bacteria in the male act as a "sink" to bind with a product necessary for normal processing of the sperm in the egg (Werren, 1997). Karr cited in Werren (1997) claims that a number of host chromatin-binding proteins such as H1 histone have been found to bind to *Wolbachia* within host cells. If the missing sperm proteins are not replenished in the egg cell, the sperm will foul to fertilise the egg and the embryo will die.

However, the biochemical mechanisms of cytoplasmic incompatibility remain unknown, and this clearly is a major area for research.

Werren (1997) describes the modification-rescue (mod-res) mechanism for cytoplasmic incompatibility in genetic terms:

1. mod + res + (designates a strain of *Wolbachia* that can induce cytoplasmic incompatibility by modifying sperm and can rescue these when in the egg)
2. mod – res – (designates a strain of *Wolbachia* that cannot induce or rescue cytoplasmic incompatibility)
3. mod – res + (designates a strain of *Wolbachia* that cannot induce cytoplasmic incompatibility but is capable of rescuing CI)
4. mod + res – (designates a strain of *Wolbachia* capable of inducing cytoplasmic incompatibility but unable to rescue it)

In the modification rescue hypothesis an imprint is put on the immature sperm. The nature of this modification is not known. It might be a modification of the chromosomes themselves or it might be a modification of the chromatin-binding proteins. This strain-specific imprint or modification is rescued in the egg cell in a strain-specific manner. It is assumed in both the immature sperm and the egg cell that *Wolbachia* secretes protein(s) that modifies the paternal chromosome or host protein(s) that are necessary for the proper condensation and decondensation of the paternal chromosomal set and the rescue factor(s) capable of rescuing the sperm. During maturation of the sperm cell

Wolbachia are completely removed and not transmitted to the egg cell (Bourtzis and O'Neill, 1998).

Jost (1971) reported that the mechanism for incompatibility in *Culex pipiens* involves entry of sperm into eggs and induction of two meiotic divisions. However, the sperm pronuclei do not fuse successfully with the female pronuclei; consequently, the developing egg is haploid, although it may show limited development prior to the death of the embryos. Subsequently, Reed and Werren (1995) described CI in the haplodiploid species *Nasonia vitripennis*. They showed that *Wolbachia* disrupts the condensation of chromosomes from the male parent at the first mitotic division of an embryo. As a result these chromosomes become an entangled mass and fragment. However, genetic data indicate that genes from male parents may occasionally be transmitted. Only the female pronucleus forms individual chromosomes in eggs from incompatible crosses.

Callaini *et al.* (1996) studied the mitotic defect of CI in *D. simulans*. Three categories of abnormal development were shown to exist in all embryos produced in *D. simulans* incompatible crosses:

1 Arrested development during early intravitelline mitoses. Embryos that reached later stages of development showed an irregular segmental patterning, anatomical defects and surface areas where microtubules and nuclei were highly disorganised.

2 A small number of embryos reached the syncytical blastoderm stage but then failed to cellularize.

3 Embryos whose development had been arrested shortly before hatching.

Lassy and Karr (1996) describe the defective nature of CI in *D. simulans*. They found that the majority of defects arise very early during the crucial stages of the fusion of pronuclei. An analysis of the earliest observable defects suggest that aberrant chromosome decondensation of the male pronucleus may contribute to lethality. Five categories exist:

1 Sperm defects in the egg
2 Aberrant morphology of the mitotic apparatus. Aberrant mitoses could result in the gradual increase in the number of DNA-containing bodies observed with increasing developmental age. Aberrant mitoses is commonly seen soon after sperm entrance: for example, two spindels form around a chromatin bridge. Spindel or spindel-like structures are commonly observed in incompatible crosses, and are typically shorter and more barrrrel-shaped than a normal spindel.
3 Defects in chromatin structure during mitosis. Such eggs generally display more severe DNA fragmentation.
4 Proliferation of centrosomes in the absence of nuclear division
5 Loss of mitotic synchrony in incompatible crosses.

PARTHENOGENESIS

In some insects parthenogenesis is common. It is reproduction without insemination in which unfertilized eggs develop into

fertile males or females depending on the species. Whilst in some species mating may be obligatory, in many others it is facultative (Gullan and Cranston, 1999). There are several terms used to describe parthenogenesis. *Thelytoky* describes the mode of reproduction in which only daughters are produced from unfertilised eggs. *Deuterotoky* is used to describe the mode of reproduction in which males and females are produced from unfertilised eggs. *Arrhenotoky* describes the mode of reproduction in which males arise from unfertilised eggs and females arise from fertilised eggs. Parthenogenesis often occurs in the Order Hymenoptera, for example, in the parasitoid wasp genera *Trichogramma, Aphytis, Encarsia, Leptopilina* and *Mucidifurax. Wolbachia*-induced parthenogenesis was observed by Stouthamer *et al.* (1990) in *Trichogramma pretiosum*, which always produces females. After administration of antibiotics or rearing at temperatures above 30°C, females revert to production of male progeny. Whilst at 28-30°C, females will produce males, females and gynandromorphs (individuals containing female and male tissue). Treatment with antibiotics is carried out to determine whether parthenogenesis is caused by extrachromosomal factors such as a microorganism. Interestingly and for unknown reasons, in gynandromorphs male tissue is always located in the head and/or abdomen whereas female tissue is found in the rest of the body. This phenomenon might be associated with a gradient of bacterial density. Tetracycline, sulphamethoxazole and rifampicin are effective antibiotics.

Perkins (1905), Smith (I941) and Flander (1945) cited in O'Neill (1997) reported that several thelytokous wasps such as

Ooencyrtus submetallicus occasionally produce males during the summer or when reared at a high temperature above 29.5°C. This phenomenon also occurs in other species such as *Pauridia peregrina, Trichogramma pretiosum, T. oleae, T. cordubensis* and *Ooencyrtus fecundus.* A temperature above 28°C produces males in *Trichogramma* species and a temperature above 30°C produces males in *O. fecundus* and *Pauridia peregrina.*

Schilthuizen and Stouthamer (1997) reported that *Wolbachia* might commonly be transmitted horizontally in *Trichogramma.* Grenier *et al.* (1998) reported the successful horizontal transfer from *Trichogramma pretiosum* to the uninfected species *T. dendrolimi.* After 26 generations it still exhibited thelytoky. This proves that *Wolbachia* induces *thelytoky.* To date it has been reported that there are 30 species of parasitoid wasps infected with parthenogenesis-inducing *Wolbachia.*

Furthermore, Arakaki *et al.* (2001) proved that *Franklinothrips vespiformis* collected in a field in Okinawa in 1997 were all female. Using PCR they showed that this species possesses two kinds of *Wolbachia* belonging to the A and B groups. Weeks and Breeuwer (2000) presented the first report on *Wolbachia*-induced parthenogenesis in *Tetranychidae* mites of the genus *Bryobia.*

Vandekerckhove *et al.* (1999) reported that the parthenogenic *Folsomia candida* (Hexapoda, Collembola) has been shown to be infected by a *Wolbachia* strain belonging to a new group E at the very base of the E-A-B complex.

The cytogenetic mechanism of parthenogenesis caused by *Wolbachia* has been studied intensively in *Trichogramma* species. Stouthamer and Kazmer (1994) working with *Wolbachia*-infected *Trichogramma* describe how, in the first meiosis, the chromosomes fail to segregate so that the nucleus contains two identical sets of chromosomes resulting in a diploidisation of the nucleus. This mechanism is called gamete duplication. This was also found in the parasitoid wasp *Muscidifurax uniraptor* and in the gall wasp *Diplolepis rosae.*

FEMINISATION

Feminisation is the term that describes the process by which genetic males change into functional females. Sex chromosomes make it easy to distinguish between male and female animals. The classical model of sex determination is heterogamy. Males are commonly designated X/Y, whilst females are designated X/X. However, in arthropods the genetic basis of sex determination is highly variable (Rigaud, 1997). In some animals sex is determined by environmental factors. For example, it might be determined by temperature (some reptiles, fish and Crustacea), by photoperiod (some Crustacea) or by crowding (certain Nematoda) (Ginsburger-Vogel and Charniaux-Cotton, 1982, cited in Rigaud, 1997).

Sex determination can also be influenced by intracellular microorganisms. Bacteria causing feminisation have been found in several terrestrial isopod crustaceans, such as the woodlice *Armadillidium vulgare, A. nasatum* (Juchault and Legrand, 1979, cited in O'Neill *et al.,* 1997*), A. album* and *Ligia oceanica*

(Juchault *et al.,* 1974, cited in O'Neill *et al.,* 1997), *Chaetophiloscia elongata* (Juchault *et al.,* 1994 cited in O'Neill *et al.*, 1997), *Porcellionides pruinosus* (Juchault *et al.,* 1997 cited in O'Neill *et al.,* 1997, and Rigaud *et al.*, 1997). Feminisation is also discovered in the amphipod crustaceans *Gammarus duebinii* (Bulnheim and Vavra, 1968, cited in Rigaud, 1997), *Orchestia gammarellus* (Ginsburger-Vogel, 1975, cited in Rigaud, 1997) and *O. mediterranea* and *O. aestuarenis* (Ginsburger-Vogel, 1991, cited in Rigaud, 1997).

The most intensively studied case of feminisation-inducing *Wolbachia* is found in the woodlouse *Armadillidium vulgare.* There are differences in the causative agent for feminisation between isopods and amphipods. Rigaud (1997) reported that, in isopods, only *Wolbachia* cause feminisation. Subsequently, it was reported that feminisation-inducing *Wolbachia* are closely related to the ones causing cytoplasmic incompatibility or parthenogenesis. In *G. duebeni* feminisation is only associated with Microsporidia and, in *O. gammarellus,* with Paramixydia (a group of protists closely related to Microsporidia). Cytoplasmic influence on sex determination has been suspected in the copepod *Tigriopus japonicus* (Igarashi, 1964b) and has also been reported in the decapod crabs *Inachus dorsettensis* and *Leptomithrax longipes* (Smith, 1905, cited in O'Neill *et al.*, 1997) and also in the estuarine isopod *Sphaeroma rugicauda* (Heath and Ratford, 1990; Martin *et al.,* 1994). *Wolbachia* is maternally transmitted by infected pseudo-females producing broods containing more than 90% pseudo-females (genetically male).

Juchault *et al.* (1980) cited in O'Neill (1997) reported that *Wolbachia* in woodlice are thermosensitive; a temperature of 30°C can destroy these bacteria within their host. However, when reared at 30°C, older infected females are unable to revert to males. This is presumably because their gonadal tissue has terminally differentiated. Feminisation can be cured by antibiotics.

In a recent study, Kageyama *et al.* (2002) investigated maternally inherited feminisation in two Asian corn borer species (*Ostrinia furnacalis* and *O. scapulalis).* This was the first report on *Wolbachia*–induced feminisation in insects.

Isopods exhibit male homogamy. Males are ZZ and females are WZ. Because *Wolbachia* is maternally transmitted, the feminising action results in the eventual disappearance of the W chromosome in infected populations so that all individuals are chromosomic males (males = ZZ, and female = ZZ + *Wolbachia*). In fact this outcome is very similar to the effect of symbiont-induced parthenogenesis in *Trichogramma.* The main difference is that feminising microbes transform genotypic males into phenotypic females, whilst parthenogenesis-inducing bacteria transform genotypic males into genotypic parthenogenetic females.

Feminisation might be associated with genetic control of the androgenic gland, the organ responsible for producing the male hormone and bringing about the male phenotype. The existence of *Wolbachia* results in androgenic gland degeneration. In the

absence of the male hormone, the undifferentiated gonads develop into ovaries and show a female phenotype (Juchault *et al.,* 1980, cited in O'Neill *et al.,* 1997). Juchault *et al.* (1994) reported that in *A. vulgare* infected by *Wolbachia,* female genital apertures and oostegites differentiate and androgenic glands (secreting the male hormone) hypertrophy. Whereas asymbiotic females are masculinised by male hormone, females harbouring feminising bacteria (symbiotic females) are not sensitive to the masculinizing effect of this hormone. *Wolbachia* are able to elicit different reproductive phenotypes depending on the host they infect (Bourtzis and O'Neill, 1998). The bacteria will manipulate chromosomal behaviour in insects during the first mitotic division, inducing cytoplasmic incompatibility and parthenogenesis. In terrestrial isopods (*Armadillidium* species) the bacteria will block "male gene" expression thereby inducing feminisation of genetic males.

MALE KILLING

Leventhal (1968) reported that there was a maternally inherited sex ratio/male killing condition in *Drosophila willistoni, D. nebulosa* and *D. equinoxialis* caused by an infectious spirochete. The Sex Ratio condition (SR) is also found in *Drosophila bifasciata.* Leventhal (1968) reported a sex ratio (SR)/male killing condition in *Drosophila bifasciata* as follows: a distinct cytopathology was present in dying males and infectious material was present in the ovaries of females. When ovaries from SR females were injected into normal hosts /uninfected of *D. pseudoobscura* strains from British Colombia and *D. melanogaster*, the progeny expressed the sex ratio condition. This

showed that the sex ratio condition is transmittable in *D. bifasciata.*

The crucial point about SR is that, in the "thermal cure" experiment, incubation in the first 12 hours was shown to produce male progeny. The type of microorganism causing the Sex Ratio condition was obscure for a long time. Hurst *et al.* (1999b) reported male killing in a ladybird and a butterfly caused by maternally inherited *Wolbachia.* However, phylogenetic analysis was unable to ascertain whether the bacteria in the two species were monophyletic or represented independent origins of male killing among the B group *Wolbachia.*

Hurst *et al.* (2000) reported that these bacteria were sensitive to high temperature. Investigation of the effect of temperature on bacterial density in eggs showed that elevated temperature caused a decrease in bacterial density and male offspring were produced.

Microorganisms that cause male killing are found in the genus *Spiroplasma* (members of the Flavobacteria-Bacteriodes group of proteobacteria), the genera *Rickettsia* and *Wolbachia* (members of the α-proteobacteria group) and microsporidia.

Table 1-4. Microorganisms that cause male killing in arthropods (Hurst *et al.*, 1997 cited in O'Neill, 1997).

Host species	Microorganism	Reference
Drosophila willistoni (Diptera)	Spiroplasma, Gp.II (Class Mollicutes)	Hackett *et al.* (1985)
Nasonia vitripennis (Hymenoptera)	*Arsenophonus nasoniae* (Enterobacteriaceae) (γ-Proteobacteria)	Werren *et al.* (1986) Gherna *et al.* (1991)
Adalia bipunctata (Coleoptera) (Cambridge popn.)	*Rickettsia typhi* relative (unnamed) (α-Proteobacteria)	Werren *et al.* (1994)
Adalia bipunctata (Coleoptera) (Russian popns.)	Spiroplasma, Gp.VI	Von der Schulenburg *et al.*. 2000
Harmonia axyridis (Coleoptera)	Spiroplasma, Gp. IV	Hurst *et al.* (1977)
Coleomegilla maculata (Coleoptera)	Sister group to genus Blattabacterium (Class Flavobacteria)	Hurst *et al.* (1996a, 1996b)
Culex halifaxi	*Amblyospora trinus*	Bechnel and Sweeney (1990)
Acraea encedon	*Wolbachia*	Hurst *et al.*, 1999b
Adalia bipunctata	*Wolbachia*	Hurst *et al.*, 1999a,b
Drosophila bifasciata	*Wolbachia*	Hurst *et al.*, 2000

There are two categories causing male mortality; the first category is "late-killers" in which male mortality occurs late on in development (typically late larval instars). The death of the male is associated with rupturing of the cuticle during the release of infective spores. They are less virulent in females, and can be vertically transmitted. The second category is "early-killers" in which male mortality occurs during embryogenesis (or in the first

larval instar). They are maternally inherited/vertically transmitted (Hurst *et al.,* 1997 cited in O'Neill *et al.,* 1997).

Hurst *et al.* (1997) cited in O'Neill *et al.* (1997) reported that late male killers have so far only been recorded in mosquitoes which, when infected with microsporidia parasites, can cause mortality in fourth-instar larvae. 2-40% of mosquitoes are infected with this organism in the wild. The microsporidia cause mortality either in females or males. In some species only the male carries the lethal infection and the females survive to vertically transmit the parasite. In other species both males and females die. For instance, in *Aedes cantator* the effect of microsporidia in the female is variable, sometimes killing them and at other times developing further, transmitting vertically to the next generation (Andreadis, 1991). Horizontal transmission of these microsporidia can be transmitted via two routes. The first route involves infection of a copepod intermediary following release of spores from dead males. The first instar mosquitoes ingest the protists, which then invade the epithelium of the gastric caecum followed by the oenocytes. In such newly infected hosts the microsporidians typically develop in a benign manner in both sexes and are vertically transmitted to the next generation through eggs. It is typically only in this next generation that male-lethal infections arise. The second route involves direct infection of other mosquitoes. Bechnel and Sweeney (1990) reported evidence suggesting that *Amblyospora trinus* might be transmitted by both routes in *Culex halifaxi.* Subsequently, it was reported that in the same species parasites can be transmitted from mosquito to mosquito by cannibalism. Furthermore, Hall (1985)

reported that spores of *Amblyospora* species in *C. salinarius* enter the haemolymph and migrate to the ovary. Whilst Hazard and Anthony (1974) reported that diploid spores of *Thelohania legeri* in *Anopheles quadrimaculatus* remain in the oenocyte which migrates to the ovaries. Microsporidia are then transmitted vertically to the next generation through the eggs. Vertical transmission is not possible in males.

Early male killing is different from late male killing in the import of horizontal transmission. Huger *et al.* (1985) and Skinner (1985) reported a case of a son-killer transmitted horizontally in *Nasonia vitripennis.* This is the only case in which horizontal transmision occurs. Male killing caused by *Wolbachia* is vertical transmitted (O'Neill *et al.*, 1997).

Hurst *et al.* (1997) describe two possible mechanisms for male killing. In the first, bacterial behaviour alters in response to a host cue of sex. The bacteria only produce toxin in the male thereby only causing death in males. In the second, bacterial behaviour is constant but the effect of bacterial infection is dependent upon sex. For instance, interference with the process of dosage compensation or male sex determination might produce sex-specific death. Here, the bacterial behaviour is identical in males and females but its effects are limited to the male.

THE POPCORN EFFECT

Min and Benzer (1997) discovered that *Wolbachia* produced an unusual effect in tissues such as brain, eyes (retina) and muscle of *Drosophila melanogaster.* A sudden massive degeneration of the

fly's cells occurs in response to bacterial multiplication. As this resembles the behaviour of popcorn in a microwave, hence it has been termed the 'popcorn-effect'.

Hertig (1936) reported that *Wolbachia* have been found mainly in the cytoplasm of cells of the reproductive organs (ovaries and testes). However, occasionally *Wolbachia* have been found in the Malpighian tubules, in muscle tissue next to the body cavity and in the nervous tissue (Stouthamer, *et al.,* 1999).

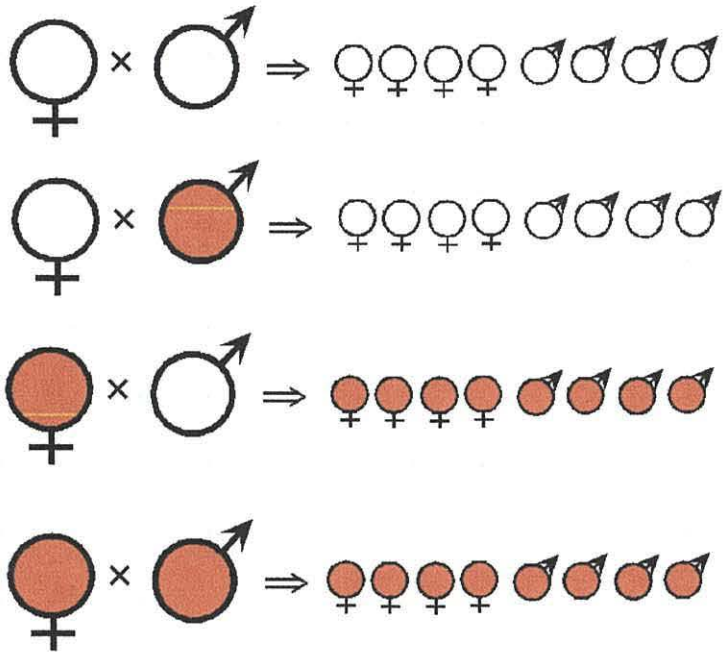
Min & Benzer (1997) found the 'popcorn-effect'-inducing *Wolbachia* serendipitously whilst they were screening for the gene mutations that cause brain degeneration in *Drosophila melanogaster.* They recognised that flies infected with the 'popcorn-effect' had a reduced life span compared with normal flies and predicted the involvement of X-chromosome deficiency. Subsequently they crossed *D. melanogaster* with the white mutant W^{1118} (which has a normal life span) in order to remove the deficiency. In this way they could determine whether the chromosomal deficiency was responsible for the phenotype. In fact, a shortened life span still persisted despite removal of the nuclear background.

In order to determine the cause of early death, *Drosophila* eye and brain tissue was examined by light microscopy using toluidine blue stain on semi-thin plastic sections. Initially young adults showed normal morphology. However, distortions of the brain and retina occurred as age progressed. In further investigations, tissues were examined by electron microscopy. The same results

were found in the brain, eyes (retina), thoracic muscle and ovary where the pathology is similar. 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 the 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.

Min & Benzer (1997) then treated the flies with tetracycline antibiotic to eliminate the *Wolbachia* in order to determine whether the popcorn strain was the cause of the observed reduced life span. The results indicated that flies reverted to wild type/normal life. As *Wolbachia* are maternally inherited, female flies containing the 'popcorn-effect' inducing *Wolbachia* will transmit to males and female progeny. The progeny remained infected. However, transmission did not occur when infected males were crossed with uninfected females.

Fig. 1-8. Transmission of the 'popcorn-effect'. Cross between the female infected with 'popcorn-effect'-inducing *Wolbachia* with the males infected or uninfected results in 100% infected progeny.



WOLBACHIA IN FILARIA

Filarial nematodes (family Onchocercidae) are arthropod-borne parasites of vertebrates, see table 1-5. They are responsible for major human health problems in developing countries. Examples of diseases caused by filarial nematodes in humans are: river blindness (onchocerciasis) caused by *Onchocerca volvulus* and tropical elephantiasis (lymphatic filariasis) caused by *Brugia malayi* and *Wuchereria bancrofti.* Examples of other filarial nematodes found in animals include *Dirofilaria immitis* and *Brugia pahangi*

Table 1-5. Filarial nematodes found in humans and animals, the diseases they cause and the tissue they affect (Taylor and Hoerauf, 1999)

Parasite	Hosts, diseases and tissues affected
1. *Wuchereria bancrofti*	Humans, lymphatic filariasis
2. *Brugia malayi*	Humans, lymphatic filariasis
3. *Brugia pahangi*	Cats, dogs, tigers
4. *Onchocerca volvulus*	Humans, onchocerciasis/river blindness
5. *Onchocerc gibsoni*	Cattle destroying its value as leather
6. *Litomosoides carinii*	Cotton rat, pleural cavity
7. *Litomosoides sigmodontis*	Cotton rat, pleural cavity
8. *Loa loa* (eyeworm)	Humans, loa loa disease
7. *Dirofilaria immitis*	Dogs, cats, foxes and wolves, causing heartworm disease
8. *Dirofilari repens*	Dogs subcutaneous of eye, nose, arm
9. *Dirofilari tenuis*	Racoon
10. *Mansonella ozzardi*	Humans body cavity
11. *Mansonella perstans/ Dipetalonema perstans*	Humans and primates body cavity
12. *Mansonella streptocerca*	Humans and primates (subcutaneous)
13. *Dipetalonema reconditum*	Dogs
14. *Setaria cervi*	Ruminants, equines & pigs (peritonial cavity)
15. *Stephalofilaria stilesi*	Cattle (skin)

Lymphatic filariasis is a disease infecting nearly 120 million people and it occurs primarily in tropical regions of the world (Michael *et al.*, 1996). Symptoms are manifested in limbs, scrotum and other extremities and include lymphadenopathy (swelling of the lymph nodes), acute fever and debilitating chronic oedema (commonly known as elephantiasis). The latter occurs when worms block the lymph vessels. Deposition of connective tissue, cells and fibres contribute to elephantiasis. The

major lesions are restricted to the lymphatics, eye and skin. Elephantiasis is the result of a long-standing infection (Cheng, 1986; Rajan and Gundlapalli, 1997). Cheng (1986) reported that 40 million people are affected by onchocerciasis, of these 30 million are found in tropical Africa and 800,000 in western Guatemala, Colombia and north-eastern Venezuela. Clinical manifestations of onchocerciasis and lymphatic filariasis can differ markedly amongst individuals even within a single regional population.

WHO website (http://www.who.int/inf-fs/en/fact102.html) reported that lymphatic filariasis currently affects 120 million people world-wide. And 40 million of these people have serious disease. A total of 18 million people are infected with onchocerciasis and have dermal microfilariae, of whom 99% are in Africa. Of those infected with the disease, over 6.5 million suffer from severe itching or dermatitis and 270,000 are blind WHO website (http://www.who.int/inf-fs/en/fact095.html).

Control programmes in filariasis have involved chemotherapy with diethylcarbamazine citrate (DEC) and, to a lesser extent, vector control. There is no safe drug able to eradicate filarial infections effectively. In onchocerciasis most lesions develop in response to microfilariae. The drug (DEC) used to kill these microfilariae is notorious for causing local inflammatory reactions and these can damage the host as much and perhaps even more so than the untreated infection itself (Anon., 1987). Suramin is the drug available for killing the adult worms in these infections. Ivermectin, a drug effective in therapy of onchocerciasis has the

capacity to kill the microfilariae of *Wuchereria bancrofti.* It has an advantage over DEC in that only one dose of it is required. However, DEC has severe side effects to recommend it as a save drug of choice. Moreover, by causing release of the antigen from dying parasites, it can cause side effects similar to those of DEC (Michael *et al.,* 1998). No effective form of treatment has been found for other filarial infections such as those caused by *Manzonella perstans* and *M. ozzardi.* Recommended treatment for lymphatic filariasis involves DEC chemotherapy given daily, weekly or monthly. With this treatment some patients will remain microfilaraemic for a few months. Microfilarial counts can be reduced by more than 90% by weekly low dose DEC administration for 18 months (Partono *et al.*, 1984). Administration of DEC-medicated salt in a 0.26% formulation for one week (Sen *et al.*, 1974) or in a 0.1-0.15% formulation (Sharma *et al.*, 1982) for 27 months can also reduce microfilarial counts by more than 90%.

Intracellular bacteria of filarial nematodes were discovered in the 1970s with the introduction of electron microscopy in studies on species such as *Dirofilaria immitis* and *Brugia pahangi.* Ultrastructural evidence indicated that bodies in the hypodermal tissues of larvae were probably Gram-negative microorganisms. The presence of large numbers of these bodies, if appearing in an early embryo, may adversely affect development (McLaren *et al.*, 1975). Subsequently, Sironi *et al.* (1995) identified the intracellular bacterium of *D. immitis* as a *Wolbachia* species. The identification of the *D. immitis* symbiont as *Wolbachia* provided the first evidence for the presence of these bacteria outside the

arthropods. The relationship between *Wolbachia* from different filariae and the relationship between different filariae and arthropods has been studied. Although studies are limited, evidence so far suggests that each species of worm is infected with the same 'strain' of bacteria. The only filarial nematodes, which have consistently been shown (using PCR or immunohistology) to be bacteria free, are the rodent filarial nematode *Acanthocheilonema viteae, Dipetalonema setariosum* and *Onchocerca flexuosa* of red deer, see table 1-6.

Table 1-6. Distribution of *Wolbachia* in filarial species (Taylor and Hoerauf, 1999)

Filarial nematode	EM	IM	PCR	References
Acanthocheilonema viteae	-	ND	-	McLaren *et al.*, 1975; Bandi *et al.*, 1998
Brugia malayi	+	ND	+	Vincent *et al.*, 1975; Kozek *et al.*, 1977; Bandi *et al.*, 1998; Taylor *et al.*, 1999
Brugia pahangi	+	ND	+	Mclaren *et al.*, 1975; Vincent *et al.*, 1975; Bandi *et al.*, 1998; Bandi *et al.*, 1999; Taylor *et al.*, 1999
Dipetalonema setariosum	-	ND	ND	McLaren *et al.*, 1975
Dirofilaria immitis	+	+	+	McLaren *et al.*, 1975; Sironi *et al.*, 1995; Bandi

				et al., 1998; Henkle Dührsen *et al.*, 1998
Dirofilaria repens	+	ND	+	McLaren *et al.*, 1975; Bandi *et al.*, 1998
Litomosoides sigmodontis	+/-	+	+	Franz and Andrew, 1986 Bandi *et al.*, 1998; Hoerauf *et al.*, 1999;
Loa loa	-	ND	ND	McLaren *et al.*, 1975; Franz *et al.*, 1984
Mansonella ozzardi	+	ND	ND	Kozek and Raccurt, 1983
Onchocerca armillata	ND	+c	ND	Taylor and Hoerauf, 1999
Onchocerca fasciata	+	+	ND	Determann *et al.*, 1997; Henkle-Dührsen *et al.*, 1998
Onchocerca flexuosa	-	-	ND	Plenge-Bönig *et al.*, 1995; Henkle-Dührsen *et al.*, 1998;
Onchocerca gibsoni	+	+	+	Franz and Copeman, 1988 Bandi *et al.*, 1998; Henkle-Dührsen *et al.*, 1998;
Onchocerca gutturosa	ND	+c	+	Bandi *et al.*, 1998

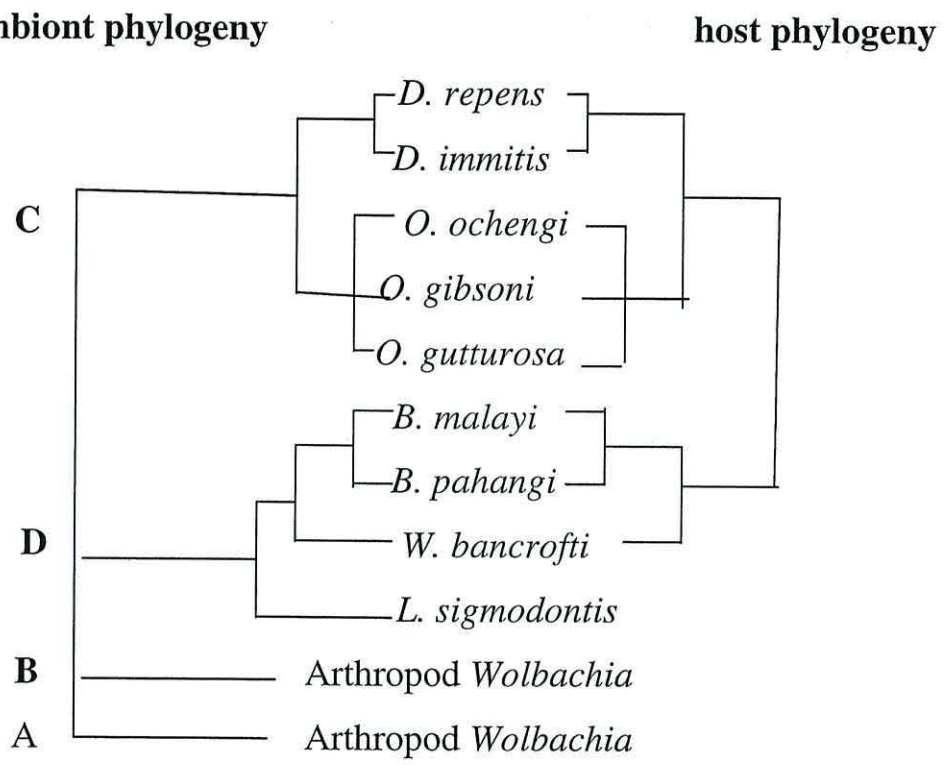
Onchocerca jakutensis	+	+c	ND	Plenge-Bönig *et al.*, 1995
Onchocerca lienalis	ND	ND	+b	Taylor and Hoerauf, 1999
Onchocerca ochengi	+	+	+	Determann *et al.*, 1997, Bandi *et al.*, 1998; Henkle-Dührsen *et al.*, 1998
Onchocerca tarsicola	ND	+c	ND	Taylor (Personal communication)
O nchocerca volvulus	+	+	+	Kozek and Figueroa, 1997; Franz and Büttner, 1983; Henkle-Dührsen *et al.*, 1998;
Wuchereria bancrofti	+	ND	+	Bandi *et al.*, 1998; Taylor *et al.*, 1999

Abbreviations: EM, electron microscopy; IM, Immunohistology; ND, not determined; PCR, polymerase chain reaction/DNA sequencing; +/-, positive and negative; b Taylor and Hoerauf (unpublished); c Büttner *et al.* (unpublished).

Phylogenetic analyses using *fts*Z and 16S rDNA indicated that arthropod and filarial nematode *Wolbachia* can be represented in five main lineages (A, B, C, D, E) (O'Neill *et al.*, 1997; Bourtzis and Braig, 1999 and Vandekerckhove *et al.*, 1999). The A, B and E groups are found in arthropods, group C consists of endosymbionts from the genera *Onchocerca* and *Dirofilaria,* and

group D consists of endosymbionts from the genera *Brugia, Wuchereria* and *Litomosoides* (Bandi *et al.*, 1998).

Figure 1-9 Dendrogram illustrating the coevolution of endosymbiont (*Wolbachia*) and its filarial nematode host (Bandi *et al.*, 1998)



Taylor *et al.* (1999) reported that the distribution and phylogenetic patterns of *Wolbachia* in filarial nematodes indicated that the association is stable and specific, see Fig. 1-9. Although the *Wolbachia* in filariae are very closely related to the *Wolbachia* in arthropods, there is no evidence for horizontal transmission of *Wolbachia* between filarial parasites and the vector insects or between nematodes. Subsequently, it was reported by Taylor and Hoerauf (1999) and Taylor *et al.* (1999) that an endotoxin (lipopolysaccharide/LPS) from *Wolbachia* is a major cause of inflammatory responses induced by the parasite. *Wolbachia* LPS

is also responsible for tumour necrosis factor (TNF)-α released following ivermectin treatment of *B. malayi,* which may be responsible for the adverse reaction to treatment.

In several previous experiments Stouthamer *et al.* (1990), Hurst *et al.* (2000) and Fujii *et al.* (2001) indicated that *Wolbachia* infection in insects can be cured with antibiotics. Tetracycline treatment was attempted by Bosshardt *et al.* (1993) in jirds (*Meriones unguiculatus*) that were infected with *B. pahangi.* Results indicated that tetracycline inhibits the development of third-stage larvae to adult worms and the development of microfilaraemia. Furthermore, Bandi *et al.* (1999) reported that tetracycline treatment is very effective in blocking embryo development in two filarial nematodes, *B. pahangi* and *Dirofilaria immitis.* McCall *et al.* (1999) investigated treatment of *Litomosoides sigmodontis* with tetracycline and found that bacterial density was reduced compared to that of normal worms. Bacterial density was ascertained using immunohistology and electron microscopy. Tetracycline was shown to have no effect on the *Wolbachia*-free nematode *Acanthocheilonema viteae* (Taylor and Hoerauf, 1999). This result suggests that antibiotics have no direct effect on nematodes, although antibiotic treatment leading to the loss of *Wolbachia* is detrimental to those nematodes that harbour it.

Tetracycline has been successful in treating nematode infections in a variety of animals (Bosshardt *et al.,* 1993; Bandi *et al.*, 1999; McCall *et al.,* 1999). It might therefore be possible to use tetracycline therapy in the treatment of human filariasis, see table

1-7. Townson *et al.* (2000) reported that rifampicin, oxytetracycline and chloramphenicol could be used against *Onchocerca gutturosa in vitro* at a concentration of 50.0μM. However, rifampicin is the most active producing an 84% reduction in viability of the parasite (as measured by formasan-based colorimetry). Subsequently, in tests against *O. linealis,* oxytetracycline at 100, 25 or 6.5mg/kg daily for 15 days reduced microfilaria to 56%, 38% and 45% of that recovered from un-treated control respectively. Ultrastructural studies showed that almost all of the endosymbiont bacteria had been cleared from the parasite tissues. The tissues of the adult worms were mainly undamaged but with a granulomatous response of host cell adhering to some specimens. Rao *et al.* (2002) reported the effects of antibiotics (tetracycline, rifampicin, chloramphenicol, azithromycin and doxycycline on *Brugia malayi in vitro* of infective larvae (L3) motility and molting, indicating that all of the antibiotics tested except chloramphenicol decreased L3 motility by 50% or more at 10 days, with minimal concentrations (MECs) of 20-100μg/ml. Tetracycline, rifampicin and chloramphenicol inhibited L3 to L4 molting after 12 days in a concentration and time-dependent manner, with MECs in the range of 1-20μg/ml. Therefore, treatment of the *Wolbachia* bacteria in filariae offers a novel alternative. It would affect both adult worms and microfilariae unlike current chemotherapy, which involves microfilaricidal drugs or adulticidal drugs, all of which have unpleasant side effects.

Treatment of human onchocerciasis with doxycycline results in sterility and inhibits larval development and adult worm viability.

Bacteria were unable to repopulate nematode tissues up to 18 months after depletion, and therefore these effects may be permanent. Table 1-7 lists studies using anti-*Wolbachia* antibiotics in filariasis.

Table 1-7. Antibiotic therapy of *Wolbachia* in filarial nematodes (Taylor and Hoerauf, 2001)

Species	Host/Model	Antibiotic	Parasitological effects	Reference
Acantocheilonema viteae	Jird/Mastomys *In vitro*	Tetracycline	None	Hoerauf *et al.*, 1999; McCall *et al.*, 1999
Brugia malayi	*in vitro*	Tetracycline	Reduced motility, viability and microfilarial release	Smith and Rajan, 2000
		Rifampicin	Inhibits L3 moult	Idem
Brugia pahangi	Jird/ *in vitro*	Tetracycline	Inhibits larval development, embryotoxic, prophylactic	McCall *et al.*, 1999; Smith and Rajan, 2000;
		Rifampicin	Reduced adult viability and microfilarial release, embryotoxic	Bandi *et al.*,1999; Townson *et al.*, 2000
Dirofilaria immitis	Dog/*in vitro*	Doxycycline/ Tetracycline	Embryotoxic, inhibit L3 moult and transovarial transmission	Townson *et al.*, 2000
Litosomoides sigmodontis	Mouse, Cotton rat	Tetracycline	Inhibits larval development	Bandi *et al.*,1999;

			Infertility and stunted growth	Smith and Rajan, 2000
Onchocerca gutturosa	*In vitro*	Tetracycline/ Rifampicin	Reduced motility and viability	Hoerauf *et al.*, 1999
Onchocerca linealis	Mouse	Tetracycline/ Rifampicin	Reduced micrifiladermia	Townson *et al.*, 2000
Onchocerca ochengi	Cow	Oxytetracycline	Embryotoxic, adulticidal	Townson *et al.*, 2000
Onchocerca volvulus	Human	Doxycycline	Embryotoxic, sustained amicrofiladermia following ivermictin treatment	Langworthy *et al.*, 2000 Hoerauf *et al.*,2000; Hoerauf *et al.*, 2001

Stomoxys calcitrans

The order Diptera consists of several families including the Muscidae. Genera of the Muscidae contain biting and non-biting species. Genera of medical importance are *Musca, Stomoxys, Hydrofaea,* and *Haematobia* (Soulsby, 1986; Urquhart *et al.*, 1987). They may be responsible for "fly worry" in livestock and some species are vectors of important bacterial, helminth and protozoan diseases of animals.

The most common species of the genus *Stomoxys* is *S. calcitrans* and it is distributed worldwide. It is commonly known as the stable fly, as it is found in large numbers in and about stables. It is also found as a biting housefly and in areas where agriculture and animal transportation are extensive. The fly's most common breeding habitat is horse manure piled near stables. However,

with the decrease in numbers of horses involved in modern farming, the stable fly has adapted to a wider range of breeding habitats. The bites of this fly are painful. It will bite cattle, other domesticated animals and man. Both males and females are bloodsuckers.

Superficially, *S. calcitrans* resembles the housefly *Musca domestica* and these flies are similar in size, see fig. 1-10. The thorax of *S. calcitrans* is grey with four longitudinal dark stripes (Lapage, 1968; Soulsby, 1986; Urquhart *et al.*, 1987). The abdomen of *S. calcitrans* is shorter and broader than that of *Musca*, in which the dark stripes are equally wide and extend to the posterior border of the scutum. In *S. calcitrans* the lateral pair of dark stripes is narrow and does not reach the end of the scutum. *S. calcitrans* has three dark spots on the second and third abdominal segments, whilst the abdomen of *M. domestica* has a yellowish background colour and a median black longitudinal stripe becoming diffuse on the fourth segment. The proboscis of *S. calcitrans* is longer than that of *Musca* and it projects forward. The mouthparts of *Musca* are adapted for imbibing liquid food (*Musca* are non-biting flies). *S. calcitrans* usually sits head upwards on a vertical surface, whilst houseflies generally face downwards and hold their wings divergent in the resting position.

Figure 1-10. Adult female *Stomoxys calcitrans*.

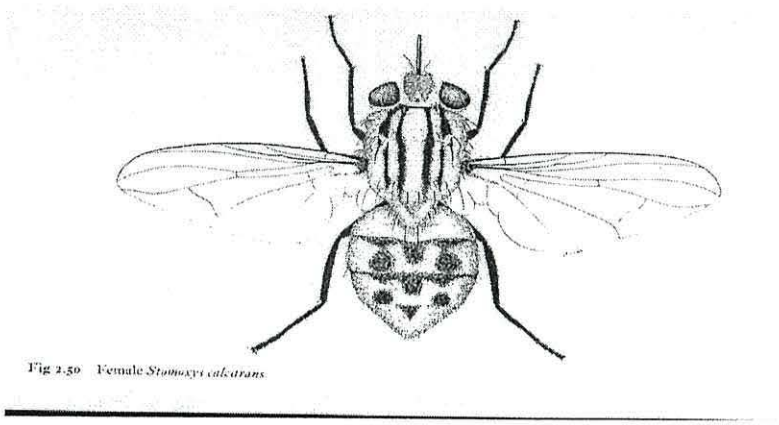


Fig 2.50 Female *Stomoxys calcitrans*

Stable flies prefer fairly strong sunlight and are therefore not found in dark stables or houses. During the heat of the day they move into shaded areas (when the temperature reaches 31°-34°C). The male flies usually rest on prominent objects near their host, waiting for females. The population peaks in late summer and autumn in temperate areas (Urquhart *et al.*, 1987). In winter, adults look for shelter in animal housing and survive low temperatures both as adults and immature stages. At least one vertebrate blood meal is necessary before males can inseminate females. A blood meal is essential for egg production.

The flies bite at any time of day, preferring the legs of cattle (Cameron, 1951). During non-feeding periods the flies commonly rest on the horns of cattle. The adult is able to travel long distances. Bailey *et al.* (1973), cited in Lancaster and Meisch (1986), reported that males and females are capable of flying 29km. However, their average flight distance is generally much less. Flies will travel at least two miles in search of a blood meal. The flies often move between animals in order to continue

their blood meal, requiring approximately three to four minutes, to complete their meal.

Harris *et al.* (1966), cited in Lancaster and Meisch (1989), reported that both female and male flies mate as early as two days after eclosion. The majority are mated by five days after eclosion. One male may inseminate as many as nine females (the average being just over six), whilst females only mate once. Mating normally occurs when the female is 2 to 3 days old (Williams *et al.*, 1949). Before the female can lay eggs she needs several blood meals (Lapage, 1968). 24 hours after the first blood meal, the follicle develops. Egg production peaks after the sixth blood meal but 30-40% of the flies produce eggs by the sixth meal.

The adult females prefer to deposit eggs in manure, cattle and horse dung. Animal faeces alone are apparently not preferable. Lancaster and Meisch (1986) reported that all kinds of decomposing organic material (especially of plant origin) are attractive. The material must be moist to be suitable (Monnig, 1934; Soulsby, 1986). Examples include straw, lawn cuttings, uncovered silage pits, green fodder crops, peanuts litter and beach seaweed deposits. Females may penetrate several inches into the medium to locate an oviposition site (Hafez and Gamal-Eddin, 1959; Griffiths, 1962; Koehler 1978, cited in Lancaster and Meisch, 1986).

A female lays about 25-50 eggs in one batch with a maximum of 632 in a lifetime. Eggs are about 1mm long and are creamy-white in colour. The larva hatches after one to five days, an average of

three days, at a temperature of 21°C (or longer in cold weather) and buries itself in the food, in which the eggs have been laid to avoid desiccation. The larvae actively feed on the vegetable matter and moult twice to complete their growth. They are mature in two to three weeks (eleven days at a higher temperature). At temperatures near freezing the larvae survive by delaying development for up to 120 days. The mature larva is about 10mm long and it is of a creamy-white colour (Williams *et al.*, 1949). The larvae are similar to those of the housefly. They can be distinguished from each other by observation of the posterior stigmal plates; in the housefly larvae these are large, irregularly oval and close together whilst, in *S. calcitrans* they are smaller, round or triangular and much further apart (Underhill, 1920). The fully developed larvae crawl to drier areas on the breeding medium where they pupate. The length of pupae is 6 to 7 mm and they are brown in colour. The puparium is formed by the hardening of the last larval skin. The duration of the pupal stage varies according to temperature and ranges from 6-26 days. In cool weather it may be much longer (Underhill, 1920; Williams *et al.,* 1949). The entire life cycle usually requires a period of 3 to 4 weeks under ordinarily favourable conditions. In tropical climates, breeding is continuous throughout the year whereas in temperate areas flies may overwinter as larvae, pupae or adults.

S. calcitrans is known to act as an intermediate host for parasites such as the nematode *Habronema microstoma* (a stomach worm of equines), the chicken tape worm *Hymenolepis carioca*, and *Setaria labiato-papillosa* (Monnig, 1934; Riley and Johannsen, 1938; Soulsby, 1986). It has been experimentally proven that

flies can act as a mechanical vector of surra caused by *Trypanosoma evansi* and *Trypanosoma equinum* (mal de caderas of equines, cattle, sheep and goats) (Lehane, 1991). The species may also mechanically transmit *T. gambiense* and *T. rhodesiense* (Soulsby, 1986). It has also been implicated in the transmission of other pathogens such as the polio virus, anthrax, fowl pox and equine infectious anaemia (Lehane, 1991).

Improving sanitation and reducing the number of breeding places can reduce the number of flies. Destruction, elimination or treatment of the larval media will restrict population development. Fly numbers can be reduced by regular removal of faeces, moist bedding hay from stables and yards, food waste from feeding troughs and the drying out of fresh cow manure twice weekly. Stored hay or straw should be covered. Addition of an organophosphate or organochlorine insecticide to manure and residual surfaces of resting sites can be carried out.

THE APPLIED BIOLOGY OF *WOLBACHIA*

There are a number of major tropical diseases transmitted by arthropod vectors. These have become a serious problem for public health. For example, in the case of malaria, each year an estimated 500 million cases occur including 1.4-2.6 million deaths (WHO, 1995). The RBM (Roll Back Malaria) website reported that at least 300 million cases of acute illness occur each year and that malaria is the leading cause of deaths in young children. Pregnant women are the main adult risk in most endemic areas of the world. Dengue and dengue haemorrhagic fever (DHF) infect more than 10 million people and there are 20-30 thousand deaths

per year. Lymphatic filariasis infects 120 million people. Other diseases include 'Chagas', onchocerciasis, leishmaniasis and sleeping sickness (WHO, 1995). Furthermore, in agriculture, insect pests are also widespread and are responsible for heavy yield losses in food crops in both developing countries and the developed world. Since DDT was discovered, chemical pesticides have been the main weapons used for the control of insect pests. At the outset DDT was extremely successful for the control of many pests for many years. For instance, in Indonesia in 1965 only 3,500 cases of malaria were reported, down from 500,000 in previous years. However, the widespread use of chemical control for eradication of both disease vectors and agricultural pests led to the development of insecticide resistance. Finding new effective insecticides is a challenging problem. Increasingly, heavy insecticide use to kill resistant pests is too expensive for many developing countries and, moreover, environmental safety has become a major issue. Many scientists have been attempting to find alternative control methods to eradicate serious pests.

The sterile insect technique (SIT), in which the release of large numbers of radiation-sterilized males result in a reduction in the number of viable eggs being laid by the wild females, has been successfully applied in the genetic control of pests. In 1958 the SIT has been the most successful application of genetic control to suppress the screwworm fly (*Cochliomyia hominivorax*) in the USA and Mexico and in Libya in 1987 and the medfly (*Ceratitis capitata*) (Krasfur *et al.,* 1987). The drawbacks with SIT, firstly, it does not work well for insects that mate repeatedly and

secondly, it is only effective if the sterile males can be release into a confined area like a valley where insects do not migrate from far away and do not invade from the surrounding area. Another problem using SIT are cost, the need for considerable resources and the need for a suitable target-insect life history. For example, to suppress tsetse flies, it is expensive to produce huge numbers of sterile males of tsetse flies (*Glossina spp.*), which transmit African trypanosomiasis (causing sleeping sickness in humans and nagana in cattle). These flies have a very low reproduction only producing one or two larvae every nine days, so recovery is relatively slow after population suppression. Mass rearing of tsetse flies is difficult. In addition, both male and female flies take blood meals and therefore transmit trypanosomes. Consequently, any mass-release of male tsetse flies could increase the rate of diseases transmission. Some populations of tsetse are known to harbour *Wolbachia,* and therefore CI could potentially be used in their control or to compliment the SIT programme (O'Neill *et al.,* 1993). When both treatments are employed, there might be a significant reduction in the radiation dosage used to sterilise the males and hence an increase in male competitive ability might be expected. This method (irradiation with CI) has been investigated in *Culex spp.* (Arunacharlam and Curtis, 1985). They propose that in control of the filariasis vector, a release of males cytoplasmic incompatible with a wild pest population, relatively low doses of irradiation should be applied to ensure sterility; male competitiveness might scarcely be affected. Therefore they irradiated *C. pipiens* form *molestus* and *C. quinquefasciatus.* The results showed that crosses between *Culex pipiens* form *molestus* and *Culex quinquefasciatus* confirmed

bidirectional incompatibility. In genetic control operations the risk of release of fertile females could be avoided by irradiation, but complete sterilisation of *C. pipiens* form *molestus* females required a higher dose (7Kr) than did *C. quinquefasciatus* females (6Kr). The unirradiated *molestus* males competed equally with unirradiated *quinquefasciatus* males, but the irradiated *molestus* males showed significantly reduced competitiveness.

There are several ways in which endosymbionts could be utilised to suppress an insect population of pest status. For example, *Wolbachia* induces cytoplasmic incompatibility when an infected male is crossed with an uninfected female and as a result offspring are not produced. In order to control vectors of disease it is necessary to release vast quantities of infected males. These will render the females with which they mate sterile. Laven (1967) used cytoplasmic incompatibility in order to introduce sterility in wild mosquito populations (in Burma). It was an attempt to control a vector of filariasis, *Culex pipiens/C. quinquefasciatus*. Released males were incomptible with the target population. This result is promising but no wide scale control attempts have been made (Sinkins *et al.*, 1997 cited in O'Neill *et al.*, 1997). This early attempt focused on the use of cytoplasmic incompatibility to introduce sterility into wild populations of mosquitoes. The challenge for such releases would be the separation of infected males from females. *Wolbachia* is a good tool for pest control because it causes a quick spreading infection. Turelli and Hoffman (1991) reported that it was possible for the rate of spread of *Wolbachia* infection in a

Drosophila simulans population (California) to reach approximately 100 km/year.

There are three advantages of using *Wolbachia*-induced parthenogenesis in parasitoids of pests. Firstly, the female parasitoid insect will only produce female offsprings, therefore an increasing number of pest pupae will be infected. Secondly, it is possible to incur only half the cost of a mass-rearing programme, and thirdly, all female parasitoid wasps may be able to depress the insect pest population to a lower level than that of uninfected parasitoid wasps

Interest in using *Wolbachia* as a tool for pest control has revolved around genetical manipulation of the pests rather than their eradication. For instance, *Wolbachia* induce cytoplasmic incompatibility. Cytoplasmic incompatibility resulting from *Wolbachia* infections occurs when uninfected females mate with infected males. The offspring of this cross do not develop due to disruptive events in early karyogamy (O'Neill and Karr 1990; Callaini et al. 1996). Infected females are able to successfully mate with both infected and uninfected males. Thus, *Wolbachia*-infected females have a reproductive advantage and *Wolbachia* can spread rapidly through a population. *Wolbachia* in *D. simulans* is capable of spreading at a rate of 100km per year (Turelli and Hoffmann, 1991). Two ways have been proposed by which the intracellular symbiont *Wolbachia* could be used to spread desirable genes into wild populations. First, *Wolbachia* infections could be used to drive genes located on any maternally inherited expression vector into a wild type population. *Wolbachia* and the genes of interest would share a common mode

of inheritance and as such would form a non-genetic linkage (Beard *et al.* 1993). Second, if the *Wolbachia* genes which cause CI could be isolated and expressed from an insect chromosome, then population genetic modelling suggests that they would drive themselves, as well as linked chromosomal genes, into a population (Sinkins *et al.*1997 cited in O'Neill *et al.*1997).

Genetical manipulation of the insect population can reduce its ability to transmit the pathogen to humans. Genetic control strategies consist of the creation and release of transgenic insects (Crampton *et al.*1994; Gwadz 1994; Collins and Paskewitz 1995). These strategies aim to replace natural vector populations with transgenic insects unable to transmit disease, thereby effectively 'immunising' the vector rather than the human population. Three interconnected research objectives must be achieved before attempting the release of transgenic vectors of a disease control strategy. Firstly, the genes which encode traits that render the vector refractory to a particular pathogen must be identified. Secondly, methods to introduce and express these genes in insects in a stable, heritable fashion must be developed. Thirdly, a means for spreading these genes to high frequency in natural vector populations must be accomplished.

Male killing could also be used in pest control but this is less efficient. Although the survivorship of females is increased if they suffer reduced competition with their male siblings during their early growth, there is no increase in the number of females in a brood.

The 'popcorn-effect'-inducing *Wolbachia*, which causes a reduction in insect life span, might be effective in vector control because the microorganism/parasites need a long time to develop in their host. For example, adult *Anopheles gambiae,* which transmits *Plasmodium spp.* (causing malaria), has a life expectancy of 15 days (Jones and Shidrawi, 1969, cited in Anderson and May, 1991) whilst *Plasmodium* has an incubation period of 11 days (Baker, 1966, cited in Anderson and May, 1991). Similarly, adult *Aedes trivittatus* has a life expectancy of about 25 days in the laboratory (Christensen, 1978, cited in Anderson and May, 1991) whilst the parasite it transmits (*Dirofilaria immitis*) has an incubation period of 16 days at 22.5°C (Christensen and Hollander, 1978, cited in Anderson and May, 1991). They will only be infectious and transmitted after the host has taken at least three to four blood meals. If an insect vector of disease was infected with the 'popcorn-effect'-inducing *Wolbachia,* the insect will experience early and sudden death through its adult life. Therefore, the 'popcorn-effect'-inducing *Wolbachia* may be effective in the control of disease carried by vectors as the vector might be killed prior to disease transmission.

Such transfer experiments have been carried out successfully with CI-inducing *Wolbachia* among several *Drosophila spp.,* both intra- species (Rousset and De Stordeur, 1994) and interspecies (Boyle *et al.,* 1993; Clancy and Hoffmann, 1997) and also interspecies in *Tribolium confusum* (Chang and Wade, 1994). In addition, interfamily transfers of *Wolbachia* have also been realized. Braig *et al.* (1994) transferred *Wolbachia* from the mosquito *Aedes albopictus* to the fly *Drosophila simulans,* and

this revealed that the transinfected *Drosophila* strain became bidirectional incompatible with other naturally-infected strains. *Wolbachia* causing feminisation have also been transferred inter- and intraspecies (Juchault *et al.,* 1994; Rigaud and Juchault 1995; Fujii *et al.*, 2001). In addition, *Wolbachia* causing parthenogenesis has also been transferred (Grenier *et al.,* 1998). The successful transfer of *Wolbachia* by microinjection from infected to uninfected host has been established with Cytoplasmic incompatibility, Parthenogenesis and Feminisation. However, there is no recent study of the artificial transfer of the 'popcorn-effect'. Therefore, microinjection was carried out to transfer *Wolbachia* intrafamily from *Drosophila melanogaster* to *Stomoxys calcitrans.* PCR (polymerase chain reaction) analysis and transmission electron microscopy was used to examine whether 'popcorn-effect'-inducing *Wolbachia* can be transferred to the new host. Besides using transmission electron microscopy to investigate *Wolbachia* bacteria, confocal microscopy has the advantage of being able to follow the fate of cells and the movement of *Wolbachia* in a three dimensional frame/structure, especially the destiny of *Wolbachia* in sperm cysts/bundles of *D. simulans,* and *D. melanogaster.*

The 'popcorn-effect' *Wolbachia* that are observed in the tissue of *Drosophila melanogaster* (Min and Benzer, 1997) are present in low numbers during development through the embryonic, larval and pupal stages. However, as soon as the flies emerge as adults, the bacteria start to multiply rapidly. Until recently, the numbers of bacteria have not been determined. To evaluate the density of bacteria, nuclei and mitochondria in the egg, larval and adult fly, quantitative (realtime) PCR was carried out. Specific primer for

wsp (*Wolbachia* surface protein), 16 S and Na^+ pump α subunit, respectively for the detection of *Wolbachia,* mitochondria and nuclei were designed with the primer select software provided by DNA Star Program.

Furthermore, Hoffmann *et al.* (1990) and Clancy and Hoffmann, (1998) reported that cytoplasmic incompatibility levels (strong, weak or no effect) are affected by various factors including environmental factor such as temperature. These factors may also affect to the popcorn strain. To investigate whether temperature influences 'popcorn-effect'-inducing *Wolbachia*, *Wolbachia* treated with tetracycline and untreated were reared at two different temperatures (20°C and 29°C). The temperature of 20°C was chosen because this temperature is a close approximation of the optimal physiological temperatures of *Drosophila melanogaster*, 29°C was the temperature originally used by Min and Benzer to show the 'popcorn-effect'.

CHAPTER 2

HORIZONTAL TRANSFER OF THE "POPCORN-EFFECT" STRAIN OF *WOLBACHIA* FROM *DROSOPHILA MELANOGASTER* TO *STOMOXYS CALCITRANS*

2.1 INTRODUCTION

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.,* 1992a; Breeuwer and Jacobs 1996; Werren, 1997 and Grenier *et al.,* 1998). These endosymbionts, belonging to the alpha subdivision of the Proteobacteria, infect different tissues of arthropod species. After the development of molecular techniques and the availability of polymerase chain reaction (PCR) technology by the end of the 1980s, we now know that *Wolbachia* is widespread in arthropods and infects 16-20% of all insect species (Werren *et al.,* 1995a).

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.,* 1992), male killing in *Drosophila bifasciata* and the butterfly *Acraea encedon* (Hurst *et al.,* 1999b and 2000), and the 'popcorn-effect' in *Drosophila melanogaster* in which an early death of the insect occurs. *Wolbachia* is included in the family Rickettsiaceae, but *Wolbachia* has not yet been reported to infect mammals (Min and Benzer, 1997).

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.,* 1992a; Werren *et al.,* 1995a). Comparative molecular phylogenies of 20 parthenogenetic *Trichogramma* populations and their symbiotic *Wolbachia* suggest the occurrence of occasional horizontal transmission (Schilthuizen and Stouthamer, 1997). This is a good indication that individual *Wolbachia* species /strain are not restricted to a single host.

Experimental transfers have been performed, in which *Wolbachia* has been transferred by microinjection (within and between species, between family, and between orders (Boyle *et al.,* 1993; Braig *et al.,* 1994; Chang and Wade, 1994; Juchault *et al.,* 1994; Karr, 1994; Rousset and De Stordeur, 1994; Rigaud and Juchault, 1995; Sinkins *et al.,* 1995b; 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). 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 *Drosophila simulans*, conferring complete CI on the adults. Grenier *et al.* (1998) successfully transferred the symbiont within the Trichogrammatidae.

The successful transfer of *Wolbachia* by microinjection from an infected host to an uninfected host has been established with CI-inducing *Wolbachia*, P-inducing *Wolbachia,* F-inducing *Wolbachia* and *Wolbachia* without any known phenotype (Boyle *et al.,* 1993; Braig *et al.,* 1994; Chang and Wade, 1994; Juchault *et al.,* 1994; Rousset and De Stordeur, 1994; Rigaud *et al.,* 1995; Sinkins *et al.,*

1995b; Clancy and Hoffmann, 1997; Poinsot *et al.,* 1998; Dunn and Rigaud, 1998; Grenier *et al.,* 1998; Van Meer and Stouthamer, 1999; Fujii *et al.,* 2001). However, there is no study to date of horizontal transfer of the 'popcorn-effect'.

2. 2 Aim

The purpose of the experiment described here was to transfer the popcorn-effect inducing *Wolbachia* from *Drosophila melanogaster* by microinjection into uninfected *Stomoxys calcitrans* pupae and to investigate whether the 'popcorn-effect' phenotype is specific to *Drosophila* or whether it can be transmitted to another species. *Wolbachia* containing haemolymph from *Wolbachia* infected *Drosophila* third/fourth instar larvae was transferred through microinjection into pupae 5 days old of the blood sucking stable fly, *Stomoxys calcitrans.* There was no previous record of *Wolbachia* being present in *Stomoxys calcitrans.* PCR analysis (Fig. 2-5 below) and electron microscopy (Chapter 3) were used to prove that there was no *Wolbachia* in *Stomoxys calcitrans.* The pupal instar lasts for 9 to 10 days at 25°C. 5 days will be in the middle of the pupal period. If the pupae are too old, they will have a barrier between haemolymph and germ line which might be unpenetrable for *Wolbachia.* However, if too young, they have a very up-regulated immune system, and will destroy the bacteria (H. Braig, personal communication). PCR analysis and electron microscopy will be used to prove the successful transmission of *Wolbachia* to *S. calcitrans*

2.3 MATERIALS AND METHODS

Microinjection experiments

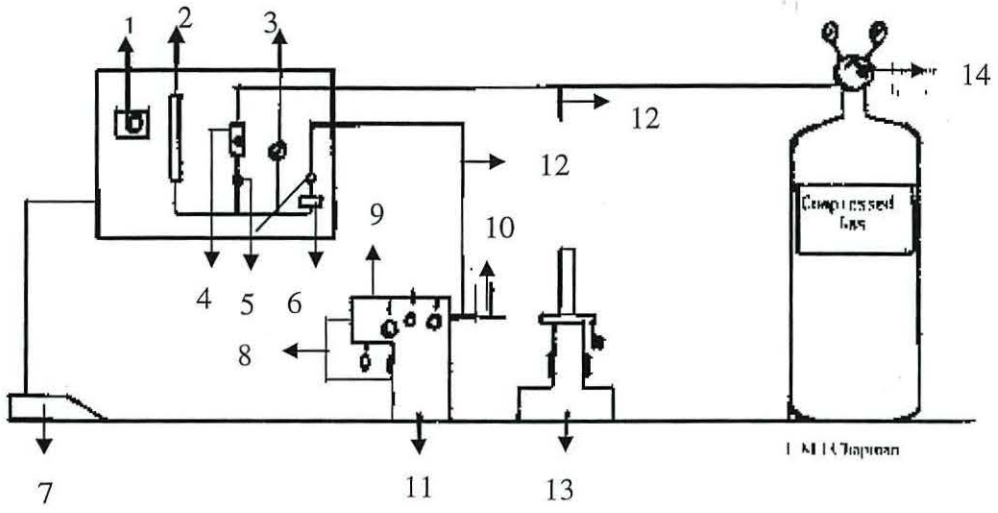
Needles of appropriate size are very important for microinjection. They are made from capillary tubes with an inner filament (1.0mm x 0.58mm x 20cm). The needles were pulled using a specialised vertical needle puller (Harvard Apparatus). The shape of the micro-capillary tube was determined by: varying the pulling force by adjustment of the solenoid (60 volts), varying the pulling distance by adjustment of the contact rod, by choosing various turns, of the heating filament (2.5 turns) and by varying the melting temperature by adjustment of the heating filament (60 volts).

The microcapillary needles produced were usually closed or with an insufficient hole. It was therefore necessary to grind them down in 70% ethanol using a diamond grinder (Narishige Model EG-40) at a speed of 30 revolutions per second and under a light microscope. Following this procedure the size of the microcapillary's hole was checked to be within a 3-11μm range using an inverted light microscope fitted with a sub-ocular graticule (with a micrometer scale, calibrated against a stage micrometer graticule).

Two pieces of microinjection equipment were set up:

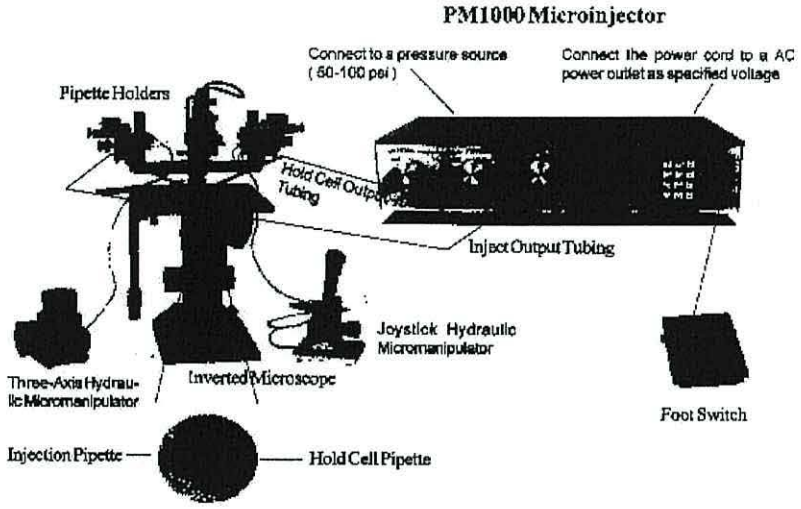
1. Prof. Lehane's microinjection apparatus
2. Cell micro-injector PM1000

Fig.2-1 Prof. Lehane's microinjection equipment.



1. Switch
2. Vacuum pump
3. Pressure gauge
4. Valve
5. Bleeds
6. Inert valve
7. Control pedal
8. Micro control
9. Macro control
10. Needle
11. Needle control
12. Tubes filled with compressed gas
13. Light microscope
14. Pressure increase valve

Fig. 2-2 The cell microinjector PM 1000



Microinjection System Connection

Microinjection experiment using Prof. Lehane's equipment

Before using this equipment it was necessary to adjust the injection pressure to 5psi (pounds per square inch) and the valve pressure to 150psi. Following this the microcapillary needle, held with the "needle holder", was lowered onto the slide on the stage of a light microscope. A *Drosophila 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 *Stomoxys calcitrans* pupa was placed in front of the needle and punctured with the use of the macro and micro controls. The *Drosophila melanogaster* haemolymph in the needle flowed into the pupa when the control pedal was pressed. The micro-capillary 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).

Microinjection using cell micro-injector PM 1000

Before using this equipment it was necessary to adjust the timer, balance pressure control, injection pressure control, vent pressure and filling pressure control.

In this experiment an injection pressure of 6.8psi was used. Balance pressure was 1.6 psi and vent pressure was 30psi. To adjust the time, press "ready" (timer on/off), then press "set timer" followed by "hold" 2 times so that 0.2 sec appears on the LCD display. To adjust the balance pressure, do the same as before, then adjust the balance until the LCD display indicates "B 1.6ps". To adjust the injection pressure, do the same as before, then followed by adjustment of the "inject" pressure until the LCD display indicates "I 6.8psi", then press "ready" again. With these settings in place, microinjection is performed as above.

Rearing, collection and killing of flies

After microinjection the pupae were put in a plastic cup container (10.5cm diameter, 4cm high) containing moist cotton wool.

Humidity was maintained by regular addition of water. The plastic cups were housed in net cages (26cm length, 26cm width and 26cm high) at 25°C. The number of pupae in every plastic cup container was depending 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 (10cm diameter and 4cm height) containing larval feeding medium. This consisted of the following:

1. Wood shavings	1.2l
2. Milk powder	0.5l
3. Powdered yeast	5g
4. Bran	1.2l
5. Molasses chaff	1.5l
6. Malt extracts	1 serving spoon (53ml water equivalent)
7. Water	2.5l

The larval feeding was done in a larval tank with size (31cm length, 20cm width and 19cm high). The medium placed in a plastic cup container was about 3 serving spoons. 28 days after emergence adult flies were killed and the females tested for *Wolbachia* infection. 28 days after emergence is the longest possible time before risking loosing any flies due to natural mortality. The flies were not killed sooner in order to allow the bacteria to grow in the new host. 28 days gave the female flies the possibility to lay eggs so the line

would continue to the next generation. The eggs in the plastic cup were reared to obtain the second generation. When the second generation was 28 days old the adult female flies were killed and tested for *Wolbachia* infection. As *Wolbachia* are maternally inherited, the bacteria would be transmitted to the progeny just through the females not males.

DNA EXTRACTION

The procedure using DNeasy tissue kit (Qiagen) was as follows: the abdomen of an adult female was cut and put in a labelled 1.5ml microcentrifuge tube. 180µl of STE (100 mM Sodium chloride, 10mM Tris hydrochloride and 1 mM EDTA, at pH 8.0) was added to the sample and the sample was ground using a disposable microtube pestle. 20µl of >12 mAU proteinase K and 200µl of buffer AL (lysis buffer) was added to each sample. This was then mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. 200µl of 100% ethanol was subsequently added to each sample and again the sample was vortex in order to denature the proteinase K. The mixture was then pipetted into separate DNeasy ion exchange spin columns sitting in 2ml collection tubes and centrifuged at 8000rpm for 1 minute. The flow-through and collection tubes were then discarded. The DNeasy spin columns were placed in new 2ml collection tubes and 500µl of buffer AW 1 (wash buffer 1) was added to each in order to "wash" out unwanted ethanol from the samples. Again, the flow-through and collection tubes were discarded. The DNeasy spin columns were placed, as before, in new 2ml collection tubes and

500µl of buffer AW 2 (wash buffer 2) was added. This time, the samples were centrifuged at 16,000 rpm for three minutes to dry the DNeasy membrane. The flow-through and collection tubes were again discarded. The DNeasy columns were placed in clean 1.5ml microcentrifuge tubes and 200µl of buffer AE (elution buffer) was pipetted directly onto the membrane. The samples were then centrifuged for one minute at 8000 rpm to elute the DNA. The DNeasy spin columns were then discarded and an elute was frozen (-20°C) for future use.

POLYMERASE CHAIN REACTION

The extracted DNA from the adult *Stomoxys calcitrans* female was analysed using the Polymerase Chain Reaction (PCR) to determine evidence of *Wolbachia* infection. The following procedure was adopted.

Manufacture instruction (Promega).

A master mix was made consisting of the following substances:

13.5µl of H_2O (UHQ)

2µl of 10x polymerase buffer (Promega)

2µl of 25mM $MgCl_2$

0.5µl of (20µM) primer F

0.5µl of (20µM) primer R

0.5µl of (10mM) dNTP (deoxynucleotide triphosphate)

1µl of 1U/µl *Taq* polymerase

It was put into a 1.5ml micro-centrifuge tube.

Two 0.5ml micro-centrifuge tubes were labelled. To each was added the sample (1µl) and master mix (19µl). One master mix was used to detect *Wolbachia* (using *wsp* [*Wolbachia* surface protein] gene). The forward primer is *w*81F: 5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′ and the reverse primer is *w*391R: 5′-AAA AAT TAA ACG CTA CTC CA-3′ the length is 610 bp (Braig *et al.*, 1998); as a control to detect mitochondria (using the forward primers 12SbiF: 5′-AAG AGC GAC GGG CGA TGT GT-3′ and reverse primer 12SaiR: 5′- AAA CTA GGA TTA GAT ACC CTA TTA T-3′). The micro-centrifuge 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 for an infinite amount of time. The cycling took approximately 3-4 hours in total. 3µl of loading buffer (15% Ficol, 0.5% bromophenol blue and 0.5% xylene cyanol) was then added to the sample and mixed thoroughly using a pipette. 17µl of the sample and 12µl of a 1 kilo base ladder (Promega) were then pipette into the wells of a prepared 1% electrophoresis gel. 200ml of gel was made as follows: 2g of agarose powder was added to 200ml of 1 X TBE (54g Tris base, 27.5ml Borax and 20ml 0.5M EDTA, pH8 for 1litre of 5X TBE, and the molarities were 0.045M Tris-borate and 0.001M EDTA. For 1X TBE: 200ml TBE 5X in 1000ml water). The powder was dissolved by heating at medium high in a microwave for 3 minutes and then allowed to cool. 7µl ethidium bromide was added to the new gel or 2µl to an old gel (Ethidium bromide was made of

1g of Ethidium bromide to 100ml of water, dissolved with a stirrer, then put in brown bottle). The gel was poured into an electrophoresis gel tray (Life Technology) and allowed to set in 1X TBE (Tris-borax EDTA). The gel was run at a voltage of 130V for 1 hour at room temperature. The gel was removed and, using ultra-violet light, a photograph of the gel was taken to determine the presence and size of the amplified DNA.

2.4 RESULTS

Table 2-1 shows that the percentage of emerged *Stomoxys calcitrans* adults varied in every injected group from 0% to approximately 71%. The average number of adult flies that emerged was approximately 36%.

From a total of 682 emerged adults, 236 were females and of these, seven females tested positive (approximately 3%) for *Wolbachia* infection. An additional ten females (*) had been found to be positive for infection in PCR analysis but, when the PCR was repeated, the bands had disappeared suggesting a false result. Based on this, positive results were always checked by a repeat of the PCR analysis in order to eliminate false results.

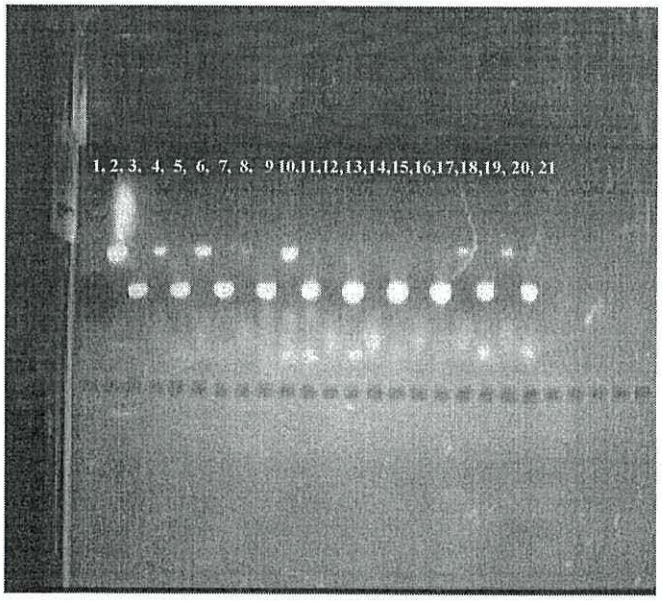
Table.2-1. The numbers of *Stomoxys calcitrans* pupae that were injected with haemolymph from *D. melanogaster* larvae harbouring *Wolbachia* and the number of adult female flies that emerged and the females that tested positive for *Wolbachia* infection

Group Injected	Number of injected pupae	emerged adults					*Wolbachia*	
		F	M	L	Σ	%	-	+
1	187	25	27		52	27.8	25	-
2	69	3	1		4	0.5	3	-
3	173	4	7		11	6.3	4	-
4	48	-	-		0	0	0	-
5	30	2	-		2	6.6	2	-
6	184	32	27	1	60	32.6	32	-
7	199	57	76	8	141	70.9	57	-
8	119	19	39	2	60	50.4	19	-
9	87	31	21	3	55	63.2	31	-
10	169	13	59	3	75	45.5	13	10*
11	109	2	17	-	19	17.4	2	-
12	109	14	53	-	67	61.4	10	4
13	109	11	29	-	40	36.7	11	-
14	116	19	46	8	73	62.9	17	2
15	37	4	19	-	23	62.2	3	1
Total	1745	236	421	25	682		216	7

Note: F, Females; M, Males; L, Lost; *) see text

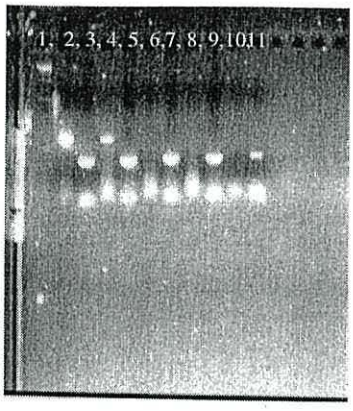
Figure 2-3 and 2-4 show gel electrophoreses of injected female *S. calcitrans* testing positive for *Wolbachia* infection. The first lane was one kilobase ladder, second lane was *Wolbachia* positive control from *Drosophila melanogaster* infected with 'popcorn-effect'. Therefore for the next lanes, which have the same size of band as the *D. Melanogaster* positive control, indicate that *Stomoxys calcitrans* were *Wolbachia* positive: lanes 4, 6, 8, 10, 18 and 20 in fig. 2-3 and lane 4 in Fig. 2-4. Mitochondrial primers (12SbiF and 12SaiR) were used for checking whether the condition for PCR were OK in every sample.

Fig. 2-3 Gel electrophoresis of *S. calcitrans* testing positive for *Wolbachia* infection.



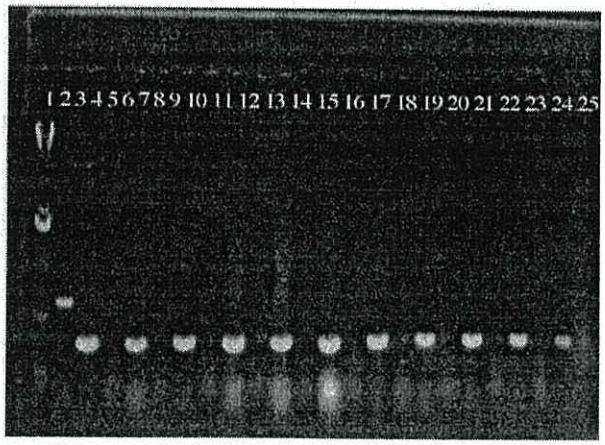
Lane 1. One kilobase ladder
Lane 2. Positive control for *Wolbachia* from *Drosophila melanogaster*
Lane 3. Positive control for mitochondria from *Drosophila melanogaster*
Lane 4, 6, 8, 10, 18 and 20. *Wolbachia* positive results from *Stomoxys calcitrans*
Lane 5, 7, 9, 11, 13, 15, 17, 19 and 21. Mitochondria from *Stomoxys calcitrans*
Lane 12, 14, 16. *Wolbachia* negative results from *Stomoxys calcitrans*.

Fig. 2-4 Gel electrophoresis of *S. calcitans* testing positive for *Wolbachia* infection.



Lane 1. One kilobase ladder
Lane 2. Positive control for *Wolbachia* from *Drosophila melanogaster*
Lane 3. Positive control for mitochondria from *Drosophila melanogaster*
Lane 4. *Wolbachia* positive result from *Stomoxys calcitrans*
Lane 5, 7, 9, and 11. Mitochondria from *Stomoxys calcitrans*
Lane 6, 8, and 10. *Wolbachia* negative from *Stomoxys calcitrans*

Fig. 2-5 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 band indicated negative of *Wolbachia*

2.5. DISCUSSION

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. Therefore, the success of transmission for the bacteria are influenced by several factors, including bacterial density and host genotypic (Breeuwer and Werren, 1993; Bourtzis *et al.,* 1996; Grenier *et al.,* 1998). 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 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, however, permanent, inheritable infections might not establish. 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 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. 2-3 and lane 4 in Fig. 2-4 approach the intensity of the *Wolbachia* signal of the infected donor, 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 strong 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 *Drosophila simulans* (Diptera) without establishing a stable infection. *Stomoxys 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 stably maintained. However, investigation by electron microscopy showed that the bacteria were present in the tissues of infected *S. calcitrans* such as thoracic muscle and brain. This indicates that *Wolbachia* develops in the tissues of the new host. I therefore assume 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.,* 1995b) provides evidence that, in some cases, this bacterium has successfully transfer over large phylogenetic distances in its interspecific movement. It remains unknown what permits particular inter-family transfers to occur successfully other than time.

CHAPTER 3

ELECTRON AND CONFOCAL MICROSCOPY OF *WOLBACHIA SP.* IN TISSUES OF *STOMOXYS CALCITRANS, DROSOPHILA SIMULANS* AND *DROSOPHILA MELANOGASTER*

3.1 INTRODUCTION

Wolbachia pipientis is an obligate intracytoplasmic, transovarially transmitted symbiont of arthropods. Infection of the ovaries and the testes with the Rickettsia *Wolbachia* has been implicated in cases such as cytoplasmic incompatibility, parthenogenesis, feminisation and male killing (Hertig, 1936; O'Neill *et al.,* 1992; Breeuwer *et al.,* 1992; Rousset *et al.,* 1992a, b; Stouthamer, 1993).

Due to the small dimensions of *Wolbachia pipientis*, the identification using light microscopy has usually been considered unreliable and most investigations have been based on electron microscopy.

The ultrastructure of microorganisms of the genus *Wolbachia* have been looked at in reproductive organs of mosquitoes especially of the genera *Culex* and *Aedes,* but also of *Drosophila simulans* and *Tribolium confusum* (Hertig, 1936; Wright *et al.,* 1978; Wright and Barr, 1980; Larsson, 1983; O'Neill, 1989; Binnington and Hoffmann, 1989; Louis and Nigro, 1989; Kambhampati *et al.,* 1992 and Popov *et al.,* 1998).

Hertig (1936) described the characteristics of *Wolbachia,* was exhibiting an enlarged form, usually 0.5-1.3μm in diameter, containing individuals which appear as rings, curved or bent rods, or compact aggregations consisting of various numbers and proportions of rod and coccoid form.

Yen and Barr (1971, 1973) and Yen (1975) implicated *Wolbachia pipientis* as the agent of cytoplasmic incompatibility in *Culex pipiens.* It is called cytoplasmic because the factors responsible for the incompatibility are transmitted from one generation to the next through the maternal cytoplasm. They reported that these organisms were found in the follicules of the ovaries and in the eggs, they are especially abundant near the microphyle. Subsequently, they described these organisms as intracellular, cytoplasmic, pleomorphic cocco bacilli ranging in size from about 0.25μm in diameter and 1.5μm in length to large coccoid forms over 1 micron in diameter, elongate or spherical surrounded by two membranes, an outer cell wall and an inner plasma membrane, and in addition, surrounded by a third membrane of host origin. Yen (1975) reported that in the gonads cells are filled with the organism but exhibit no obvious pathologic characteristics. The nuclei were normal and cytoplasmic organelles such as the mitochondria were not disrupted or displaced. As in the female, the *Wolbachia* in the male are found only in the cytoplasm. When spermatocytes develop into spermatids, the cytoplasm is shed. The microorganisms are removed with cytoplasm and the mature sperm contain no *Wolbachia.*

Wright *et al.* (1978) and Wright and Barr (1980) described the *Wolbachia* in *Culex pipiens* as coccoid (diameter ≤ 1.0μm) to bacilliform (0.25 X 1.5μm), with an outer cell wall, inner plasma membrane and a cytoplasm of ribosomes and filamentous deoxyribonucleic acid strands and always surrounded by a third membrane of host origin. Furthermore, they described

Wolbachia in *Aedes scutellaris* as follows: they were pleomorphic and vary in shape from spherical to elongate. The diameters of them varied from 0.4μm to 1.1μm. Those diameters at the small end of the range 0.4μm-0.7μm are of elongate form. The length of elongate forms measured up to 2μm. Furthermore, they reported that *Wolbachia* is surrounded by a single host vacuole membrane. At all times *Wolbachia* carried an outer cell membrane separated from an inner plasma membrane by a periplasmic space. The most prominent feature in *Wolbachia* cells were ribosomes which ranged from 12 nm to 15nm in diameter, whilst mosquito ribosomes vary in diameter from 21 nm to 25nm. *Wolbachia* are like most intracellular symbionts. They are enclosed by a host vacuole. Smith (1979) suggested that both host and symbiont derive some benefit from a vacuole membrane, the symbiont may be protected from enzymes and the host cell may exercise control over the symbiont. Late stages of cell division were represented as two cells connected by an isthmus of cytoplasm.

Larrsson (1983) described the Rickettsia-like microorganisms in *Culex pipiens* and *C. torrentium* from Sweden resembling *Wolbachia* pleomorphic, slightly bent or straight rods or of coccoidal shape. The diameter of the rounded forms varied between 0.13μm and 0.76μm, usually 0.3-0.5μm. The bacterial membrane was about 14nm thick and consisted of two unit membranes separated by an electron-translucent space.

Louis and Nigro (1989) described the ultrastructure of *Wolbachia* in the ovarial cells of *Drosophila simulans* as follows: *Wolbachia* were roundish or less frequently rod shaped, diameter was about 0.5μm. Their cytoplasm consisted of ribosomes, slightly smaller than those of the cell cytoplasm and nucleic acid fibril. They were surrounded by three membranes. The first membrane represented the plasma membrane of the prokaryote. The second membrane, irregularly distant from the plasma membrane, was often difficult to distinguish. It represented the outer part of the cell wall of the microorganism. The third membrane was closely related to the cytoplasm of the host cell.

O'Neill (1989) described Rickettsia-like organism (RLO) as symbionts found in the ovaries of *Tribolium confusum* as follows: they were highly pleomorphic with a mean length of 0.57μm and a width of 0.28μm. The microorganisms were enveloped by three membranes, the inner membrane being difficult to detect in most instances. Of these membranes the outermost was often seen to be detached from the other two and is likely to be of host origin. The internal structure consisted of cytoplasm and a network of nucleic acid filaments.

Kambhampati *et al.* (1992) identified the *Wolbachia* in *Aedes albopictus* as follows: they have a three-fold boundary consisting of plasma membrane, cell wall and vacuole membrane of the host. The average length of *Wolbachia* had a minimum of 0.54μm and a maximum of 1.03μm.

Popov *et al.* (1998) identified *Wolbachia* in *A. albopictus* as small round (0.4μm) or oval (0.5 X 1.0μm), the cell wall membrane as smooth, rather than wavy and a narrow periplasmic space was seen in some locations along the circumference of the cell. Some cells were observed in a process of binary fission and the vacuolar membrane followed the contours of the dividing cells.

Byers and Wilkes (1970) reported Rickettsia-like microorganisms in the reproductive organs of *Dahlbominus fuscipennis* that have no cytopathic effect nor do they have any noticeable effect on the reproduction or fitness of the host. The microorganisms were seen in thin sections as pleomorphic circular or rod-shaped profiles. The diameter was 0.2μm to 0.5μm and the length was up to 1.7μm. Each microorganism is surrounded by at least three membranes. The innermost two, the cell wall and the underlying plasma membrane, are clearly the limiting membranes of the microorganism. The cell wall is usually clearly defined and slightly rippled; the plasma membrane is frequently obscured by the underlying constituents and thus less clearly defined. The third membrane layer lines the vacuoles within which the microorganisms lie.

To verify the presence of *Wolbachia* in individuals of *Stomoxys calcitrans* which were infected by microinjection of haemolymph from *Drosophila melanogaster* infected with the popcorn strain of *Wolbachia,* the tissues from *S. calcitrans* testing positive for *Wolbachia* by PCR were 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 presence of *Wolbachia* in non-reproductive tissues has been largely ignored. Western blots indicate *Wolbachia* infections in somatic tissues (head, muscle, midgut, malpighian tubulus, haemolymph and wing) in *D. simulans* (Dobson *et al.,* 1999). Min and Benzer (1997) using electron microscopy discovered *Wolbachia* in somatic tissues (eye brains and muscle) of *D. melanogaster.* To verify the presence of *Wolbachia* in somatic tissue of *D. simulans,* muscle tissue and reproductive organs of *D. simulans* and *D. melanogaster,* which invariably contain *Wolbachia,* were examined using electron microscopy.

Drosophila testes are highly elongated structures making detail observations and visualisation of the fate and the movement of *Wolbachia* difficult. Therefore, 3-D projections of optical images of confocal microscopy were used to examine *Wolbachia* during individualisation from different species, *Drosophila simulans* Riverside and *D. melanogaster* infected with 'popcorn-effect'. Confocal microscopy had been used to examine *Wolbachia* distribution during sperm development in *D. simulans* (Clark and Karr, 2002; Clark *et al.*, 2003). They reported that *Wolbachia* were observed within spermatocytes of DSR, DSH and C167 *Drosophila simulans* lines. Likewise, few or no infected spermatocytes were observed in adult males of *D. simulans* Queensland, Kilimanjoro and Noumea and males of *D. mauritiana.* However, no studies have previously examined *Wolbachia* in *D. melanogaster* infected with 'popcorn-effect'.

2.2 AIM

To investigate the possibility of infecting *Stomoxys calcitrans* with the popcorn strain of *Wolbachia* using electron microscopy.

To investigate by electron microscopy the presence of *Wolbachia* in somatic and reproductive tissues of *D. simulans* in order to provide more information on its persistence in somatic tissue and to obtain details about the structure of the bacteria and their relationship to the host cell.

To investigate by electron microscopy the presence of *Wolbachia* in somatic and reproductive tissues of *D. melanogaster.*

To investigate by confocal microscopy the fate and movement of *Wolbachia* in testes from different species (*Drosophila simulans* and *D. melanogaster* infected with 'popcorn-effect').

2.3 MATERIALS AND METHODS

Transmission electron microscopy

Observation of the tissues infected by *Wolbachia* was done by transmission electron microscopy. The tissues (muscle, testes, ovaries, brain and eyes) were taken from flies dissected in PBS (phosphate buffer saline) and put in Karnofsky's fixative consisting of 0.4% paraformaldehyde and 0.16% glutaraldehyde in sodium cacodylate, 10% w/v sucrose, pH 7.2 (5ml 0.4M buffer [sodium cacodylate] plus 2ml of 2% paraformaldehyde, 0.8ml of 2% glutaraldehyde and 1g sucrose with addition of distilled water

to 10ml so that the final concentration of fixative is 2%) for 1 hour, then washed twice using buffer (5ml 0.4M sodium cacodylate plus 1g sucrose and 5ml water filled up to 10ml to make a final concentration of 0.2M). 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 (0.1g uranyl acetate in 5ml water) and kept in the fridge (4°C) overnight. After fixation the material was dehydrated using ethanol starting at 50%, then 70%, 95% and repeated twice for 100% (15 minutes for each). The tissues were then treated with 100% propylene oxide for 15 minutes and transferred to Spurrs resin and 100% propylene oxide (1:1) and slowly rotated in a rotor overnight. They were then put in Spurrs resin (100%) in an oven at 60°C overnight in order to firmly embed the samples in the resin. The resin used was standard embedding medium formulated as follows:

ERL 4206	10.0g
DER 736	6.0g
NSA	26.0g
S – 1	0.4g

Harder or softer blocks can be obtained by varying the quantity of DER 736 used to 4-8g. An increase in DER 736 gives softer blocks (Arthur, 1969). A glass knife is used to shape and cut the embedded sample in the piramitome (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.

Uranyl acetate can be used as a saturated solution in 50% alcohol brought to a pH of 4-5 with NaOH, or as a 2% w/v aqueous solution without adjustment of the pH. I used the latter, made up by mixing 0.1g uranyl acetate in 5ml distilled water. Uranyl acetate was used for staining after dehydration.

Lead citrate (Reynolds, 1963) was made by adding 1.33g $Pb(NO_3)_2$ and 1.76g $Na_3(C_6H_5O_7)2H_2O$ (sodium citrate) to 30ml water. This was shaken well for one minute and allowed to stand for 30 minutes with occasional shaking. 8ml 1M NaOH was added to dilute the suspension to 50 ml. Parafilm was put in a petridish, tissue paper was put on the edge of the parafilm. A drop of the lead solution was placed on the parafilm. The grids (pioloform coated nickel 200 mesh grids) were put dull side down on the lead droplet and were allowed to stand for 5-7 minutes. The grids were removed using forceps, washed with water and dried. They were then place in the grid box.

Scanning electron microscopy

Testes and ovaries were fixed in Karnofsky's fixative (as above), dehydrated in a grade series of alcohol, 25%, 50%, 75%, 100%, respectively, then put in acetone (100%) + alcohol (100%) (1:1), subsequently transferred to 100% acetone each for 1 hour, then dried in a critical point drier (Polaron E 5000). Mounting on stub with selotape glue (made by placing a 15cm long piece of sellotape in 10ml chloroform) and sputter-coated with gold (Polaron E 5000). Viewed at 10kV with Hitachi S 520 Scanning Electron Microscope.

Confocal imaging

The following procedure were followed: Testis of *D. melanogaster* infected with 'popcorn-effect' *Wolbachia*, *D. simulans* Riverside and *D. simulans* Riverside treated with tetracycline, 2 days old, were fixed in 3.7% formaldehyde in TBST (50mM TRIS, 150mM NaCl, 0.1% Tween and 0.05% NaN_3 pH 7.5) for 15 minutes and washed in TBST for two days at room temperature. Tissues were then stained with SYTOX Green nucleic acid stain (Molecular Probes) for overnight. *Wolbachia* were then visualized using a Zeiss LSM 510 confocal microscope with a Kr/Argon laser (488 nm) with 63 x lens and a pinhole of 212μm (Clark and Karr, 2002).

3.4 RESULTS

Transmission and scanning electron microscopy

Using electron microscopy it was shown that *Wolbachia* were presence in the eye and muscle tissues of *Stomoxys calcitrans*. *Wolbachia* were also found in the muscle and testes of *D. simulans*, and in the eye, brain, muscle, ovaries and testes of *D. melanogaster*. Pictures below, shows several numbers of bacteria found in different kind of tissues of *S. calcitrans, D, simulans* and *D. melanogaster*.

Stomoxys calcitrans

Fig. 3-1. Brain tissue of *Stomoxys calcitrans* (x 47,000).
Red arrow: *Wolbachia.*

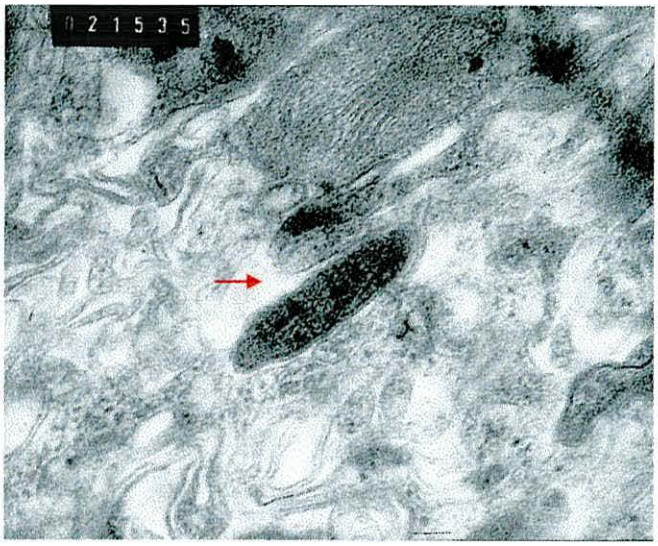


Fig. 3-1 shows electron dense *Wolbachia* found in the brain of *Stomoxys calcitrans.* It appears as a rod, enveloped by two membranes, a host membrane and bacterial membranes. The diameter of *Wolbachia* is 0.16μm and the length 0.66μm.

Fig. 3-2. Brain tissue of *Stomoxys calcitrans* (x 24,000).
Red arrow: *Wolbachia*

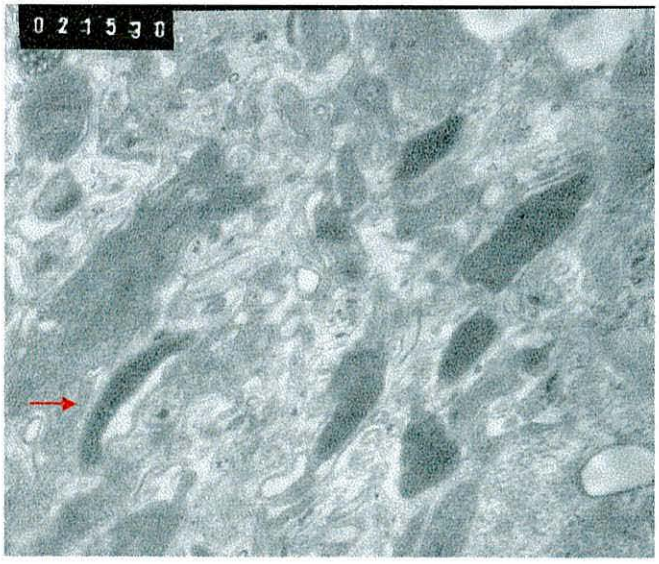


Fig. 3-2 shows a rod shaped electron dense *Wolbachia* found in the brain of *Stomoxys calcitrans* enveloped by host and bacterial membranes. The diameter of *Wolbachia* is 0.15μm and the length 1.1μm.

Fig.3-3. *S. calcitrans* muscle tissue (x 51,000). Red arrow: *Wolbachia.*

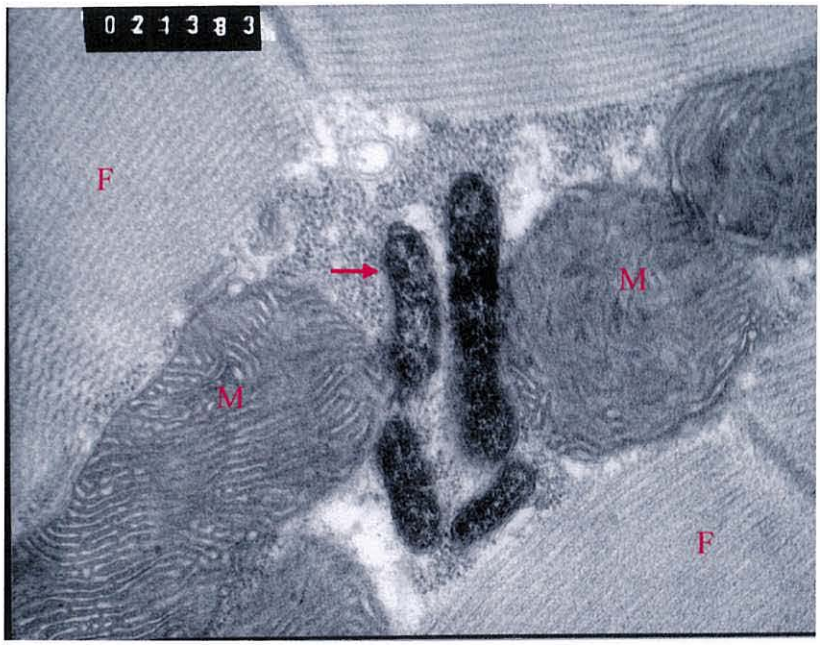


Fig. 3-3 shows four rods shaped electron dense *Wolbachia* within *Stomoxys calcitrans* muscle tissue. The diameter of *Wolbachia* ranges from 0.07-0.14μm and the length ranges from 0.26-0.7 μm. They are surrounded by mitochondria (M) and F (fibril of muscle).

Fig.3-4. *S. calcitrans* muscle tissue (x 19,000). Red arrow: *Wolbachia.*

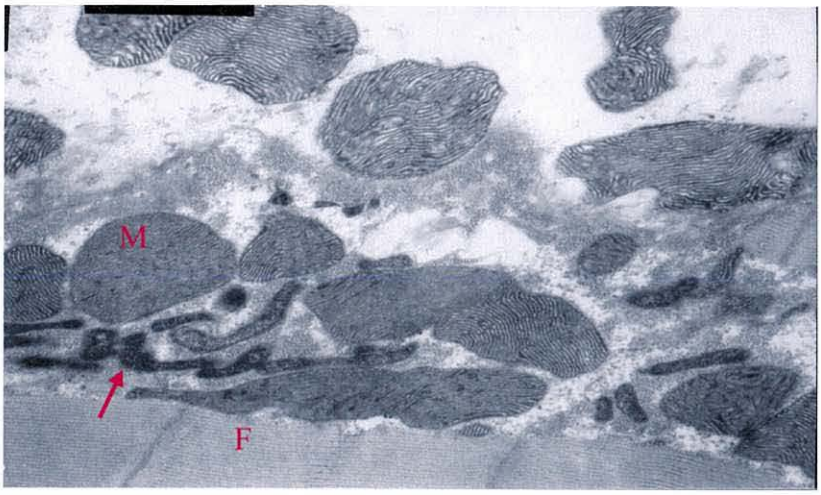


Fig. 3-4 shows electron dense *Wolbachia* within *Stomoxys calcitrans* muscle tissue. The cells were slightly bent rods or straight rods. Enveloped by bacterial membrane, the diameter of *Wolbachia* is 0.1μm and the length ranges from 0.5-0.75μm. They are surrounded by mitochondria (M) and F (fibril of muscle).

Fig. 3-5. *S. calcitrans* muscle tissue (x 23,000).
Red arrow: *Wolbachia.*

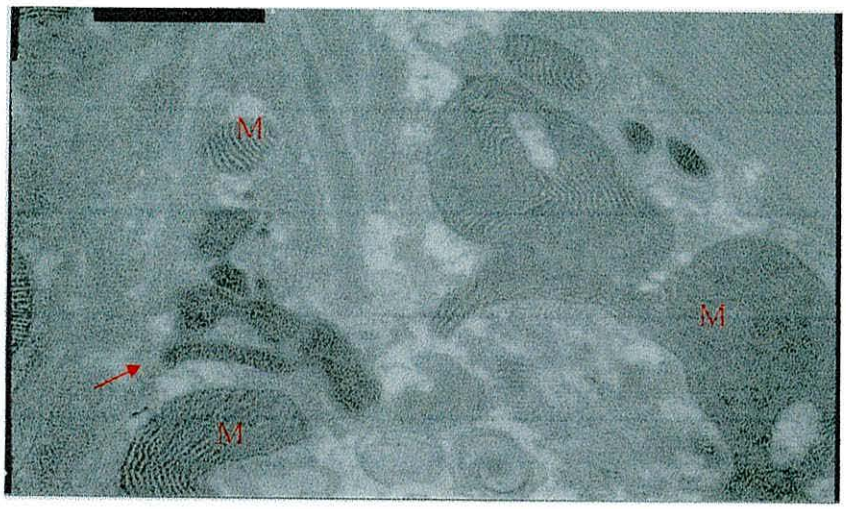


Fig. 3-5 shows rod shape and oval structure of electron dense *Wolbachia* within *Stomoxys calcitrans* muscle tissue (x 23,000). The bacteria are enveloped by bacterial membranes. Diameter of *Wolbachia* ranges from 0.13-0.2μm and the length ranges from 0.5-0.75μm. They are surrounded by mitochondria (M).

Fig. 3-6. *S. calcitrans* muscle tissue (x 37,000).
Red arrow: *Wolbachia.*

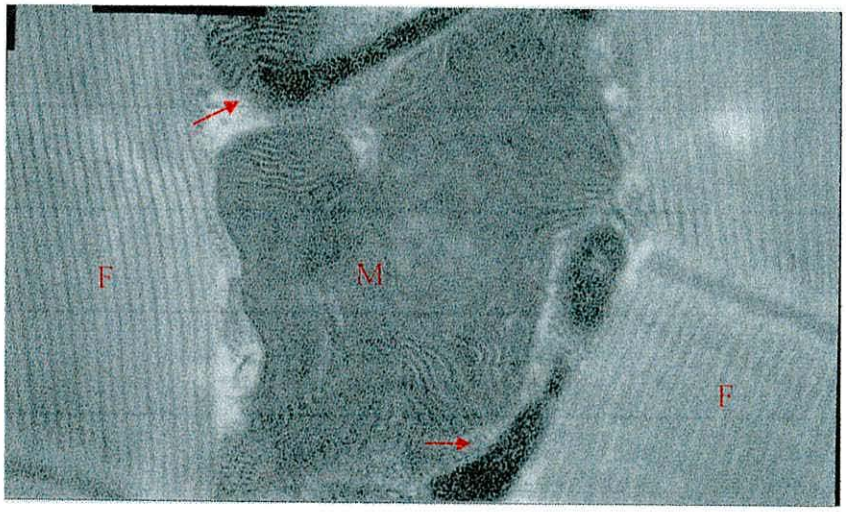


Fig. 3-6 shows rod shape, electron dense *Wolbachia* within *Stomoxys calcitrans* muscle tissue. The bacteria are enveloped by bacterial membranes. Diameter of *Wolbachia* ranges from 0.11-0.16μm μm and the length ranges from 0.4-0.8μm. They are surrounded by mitochondria (M) and fibril of muscle.

Fig. 3-7. *S. calcitrans* muscle tissue (x 41,000).
Red arrow: *Wolbachia*

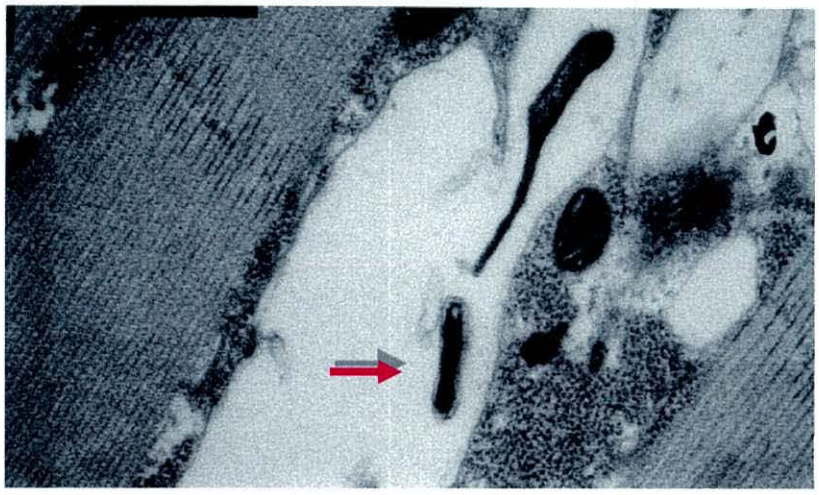


Fig. 3-7 shows a rod electron dense of *Wolbachia* within *Stomoxys calcitrans* muscle tissue. Enveloped by two membranes, a host membrane and bacterial membranes, between host and bacterial membrane there is a vacuolar space. The diameter of *Wolbachia* was 0.06μm and the length was 0.4μm.

Fig. 3-8. Muscle of uninfected *Stomoxys calcitrans* before injection (x 20,000).

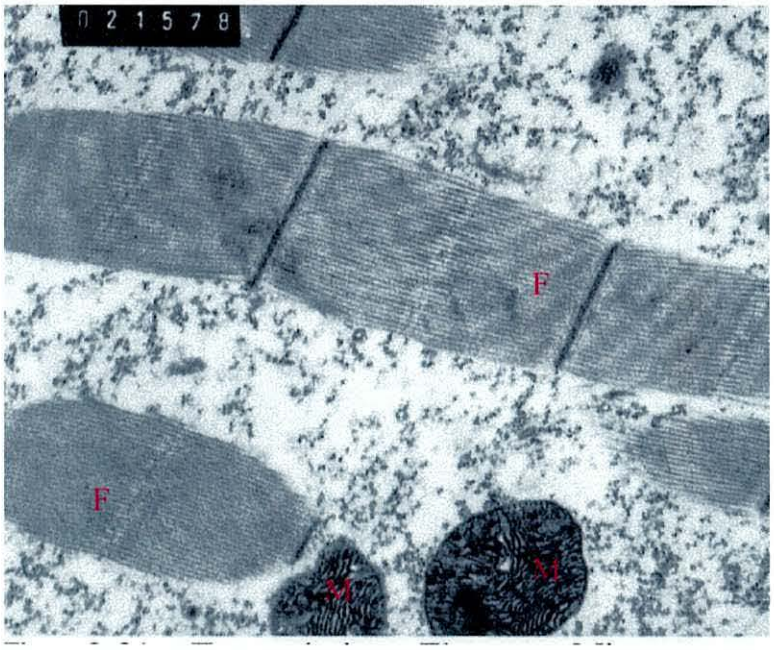


Fig. 3-8 shows muscle of uninfected *Stomoxys calcitrans*. There is no *Wolbachia* in the tissue. M (mitochondria) and (F) fibril of muscle.

Fig. 3-9. Muscle with tracheol (red arrow) of uninfected *Stomoxys calcitrans* before injection (x 20,000).

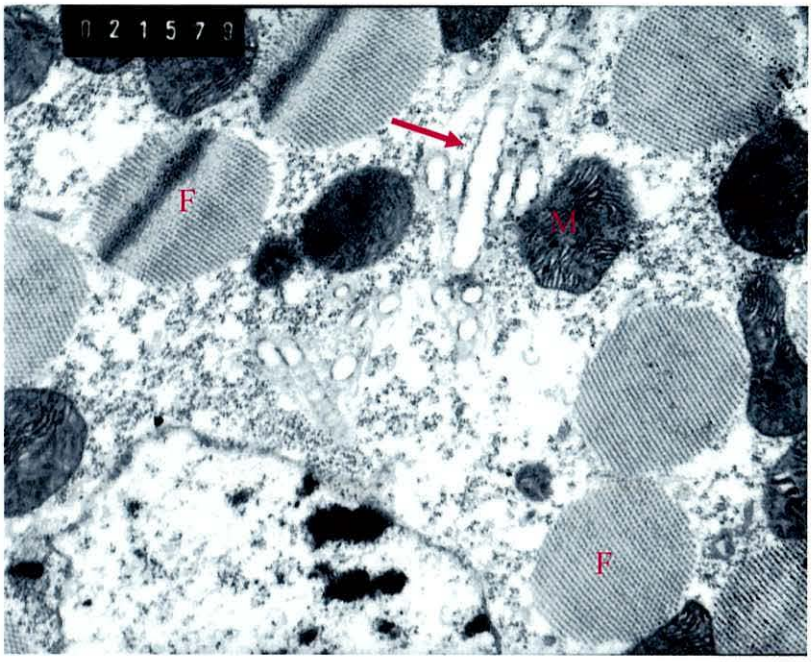


Fig. 3-9 shows muscle with tracheol (red arrow) of uninfected *Stomoxys calcitrans.* M (mitochondria) and F (fibril of muscle).

Fig. 3-10. Muscle of uninfected *Stomoxys calcitrans* before injection (x 20,000).

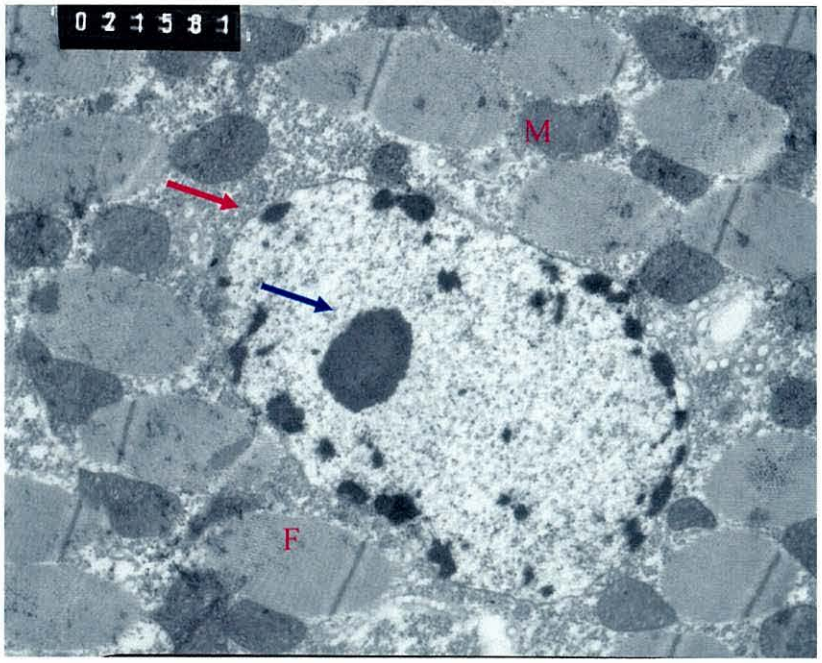


Fig. 3-10 shows muscle of uninfected *Stomoxys calcitrans* . Red arrow is nucleus and blue arrow is nucleolus. M (mitochondria) and F (fibril of muscle).

Fig. 3-11. Muscle of uninfected *Stomoxys calcitrans* before injection (x 20,000).

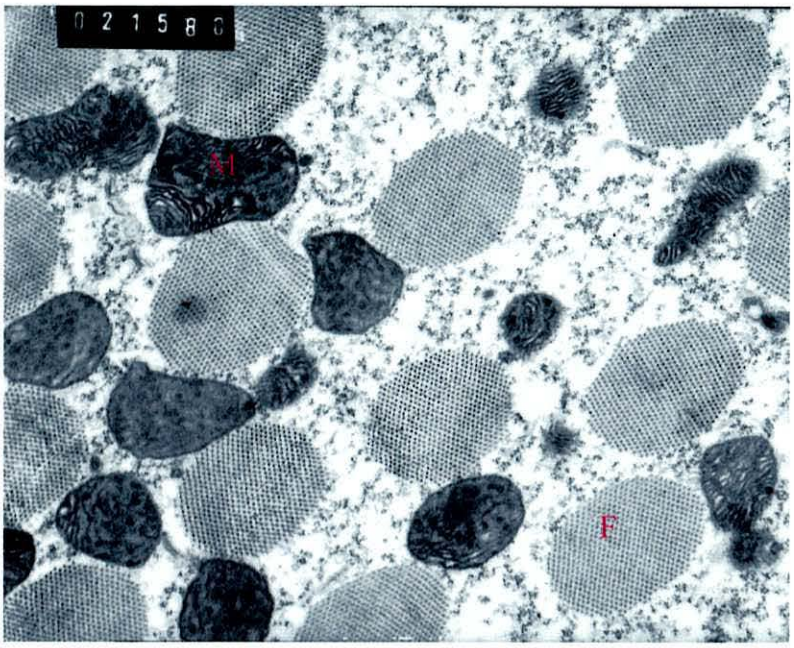


Fig. 3-11 shows muscle of uninfected *Stomoxys calcitrans*. M (mitochondria) and F (fibril of muscle).

Fig .3-12. Eye of uninfected *Stomoxys calcitrans* before injection (x 20,000).

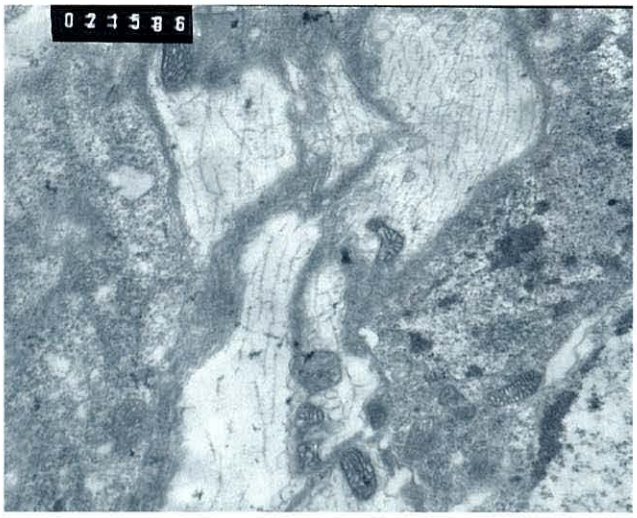


Fig. 3-12 shows eye of uninfected *Stomoxys calcitrans*

Figs. 3-13, 14 and 15. Brain of uninfected *Stomoxys calcitrans* before injection (x 20,000)

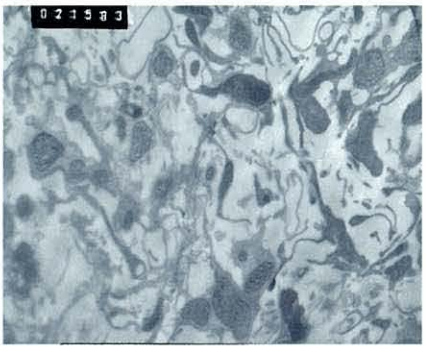


Fig. 3-13

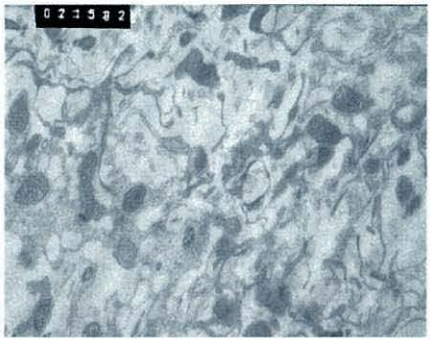


Fig. 3-14

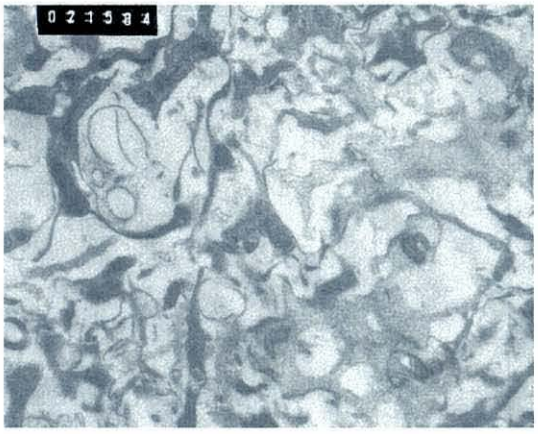


Fig. 3-15

Fig. 3-13, 14 and 15 shows brain of uninfected *Stomoxys calcitrans*

Drosophila simulans

Fig. 3-16. *D. simulans* (DSR) testis (x 42,000).
Red arrow: *Wolbachia.*

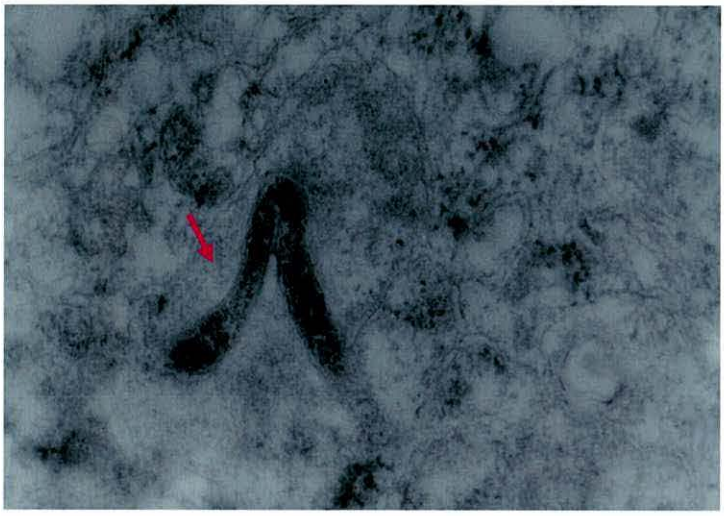


Fig. 3-16 shows two tightly-connected, electron dense *Wolbachia* in *D. simulans* testis, sharing a host membrane and appear to have been undergoing fission. The diameter of *Wolbachia* ranges from 0.14 and 0.19μm and the length was 0.8 and 0.9μm.

Fig. 3-17. *D. simulans* (DSR) muscle (x 23,000). Red arrow: *Wolbachia.*

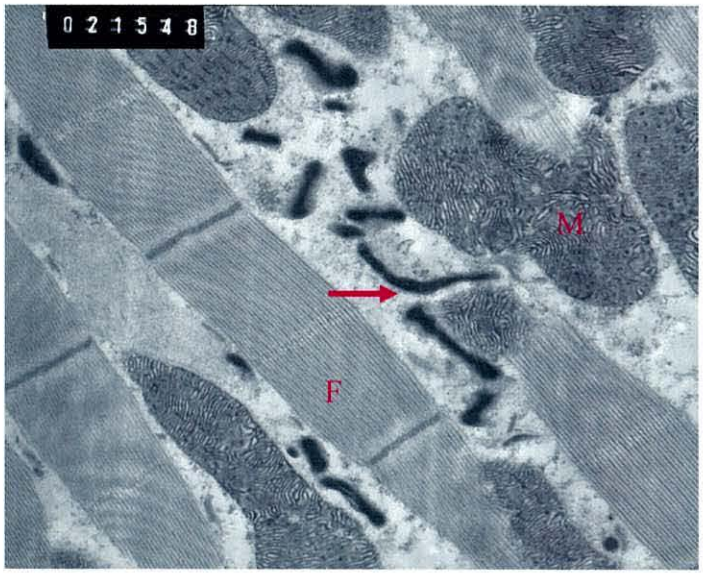


Fig. 3-17 shows *D. simulans* (DSR) muscle tissue infected with electron dense *Wolbachia* surrounded by mitochondria (M) and F (fibril of muscle). The cells are oval or shaped as bent rods. The diameter of *Wolbachia* ranges from 0.07-0.1μm and the length ranges from 0.1-0.17μm. These bacteria are enveloped by host and bacterial membranes.

Fig. 3-18. *D. simulans* (DSR) muscle. Red arrow, this could be four *Wolbachia* joined together by an isthmus (x 53,000). Enveloped by host (1) and bacterial (2) membranes.

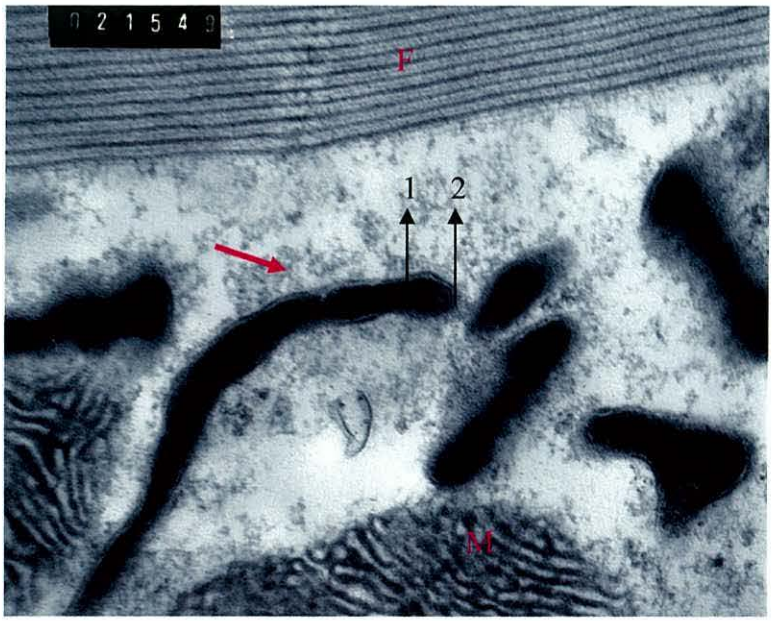


Fig. 3-18 shows *D. simulans* (DSR) muscle tissue infected with electron dense *Wolbachia.* These could be four *Wolbachia* joined together or the bacteria have been undergoing division and one still connected by an isthmus of cytoplasm. The diameter of *Wolbachia* is 0.12μm and the length ranges from 0.3-0.5μm. The cells are oval, bent rods, straight rods or elongate in form. These bacteria are enveloped by host and bacterial membranes.
M (Mitochondria) and F (fibril of muscle).

Fig. 3-19. *D. simulans* (DSR) testis (x 42,000). Red arrow: *Wolbachia.*

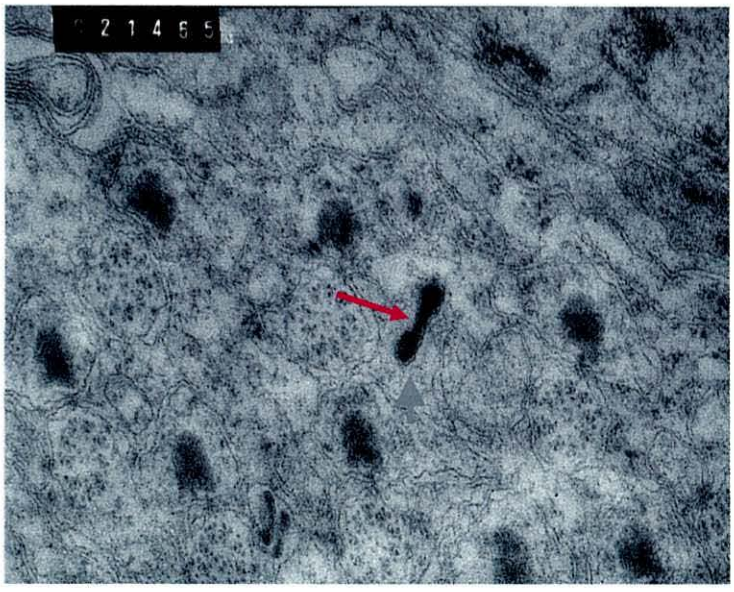


Fig. 3-19 show *D. simulans* (DSR) testis tissue depicting a single rod shaped *Wolbachia.* The diameter of *Wolbachia* is 0.06μm and the length is 0.28μm.

Drosophila melanogaster

Fig. 3-20. *D. melanogaster* (W^{1118}) ovary (x 49,000). Arrows: see text

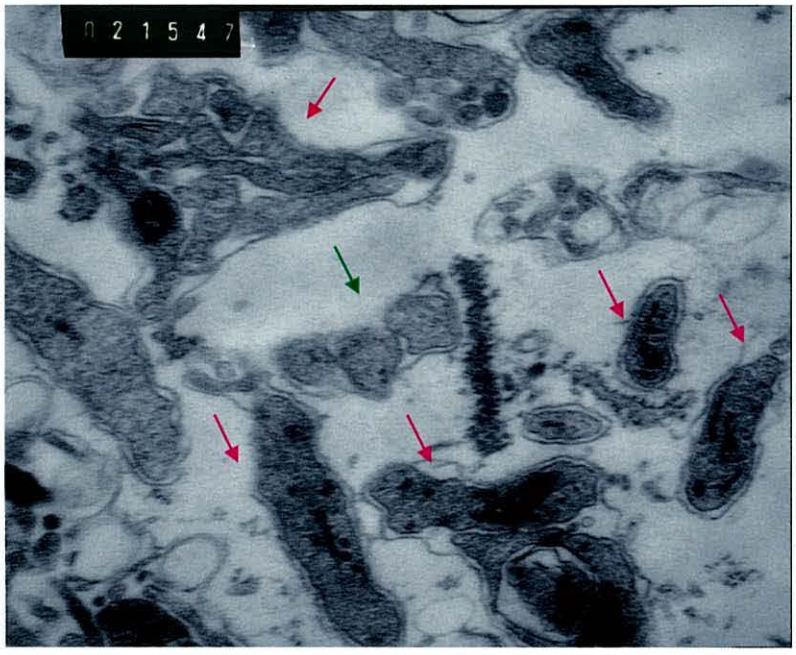


Fig. 3-20 shows an unidentified membrane-bound structure surrounded by host membrane in *D. melanogaster* (w^{1118}) ovary. Red arrow, this could be a group (11 organisms) of rod shape, electron light *Wolbachia.* The green arrow points to round structure and presumably this is a transection of *Wolbachia.* The pink arrow points to electron dense *Wolbachia* sharing a vacuolar membrane. The diameter of *Wolbachia* ranges from 0.1-0.17μm and the length ranges from 0.28-0.69μm.

Fig. 3-21. *D. melanogaster* (W^{1118}) ovary (x 33,000). Red arrow: *Wolbachia.*

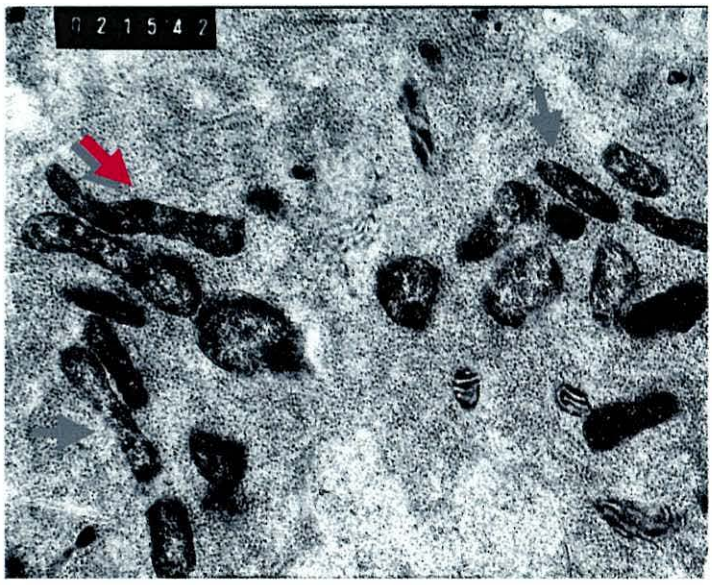


Fig. 3-21 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) ovary. The cells are oval (twice the diameter of rod shaped) and rod shaped (with bulbous ending) or rod shaped without bulbous ending. The diameter of *Wolbachia* ranges from 0.08-0.2μm and the length ranges from 0.33-0.87μm. The outer and inner bacterial membranes are difficult to distinguish.

Fig. 3-22. *D. melanogaster* (W^{1118}) ovary (x 51,000). Red arrow: *Wolbachia.*

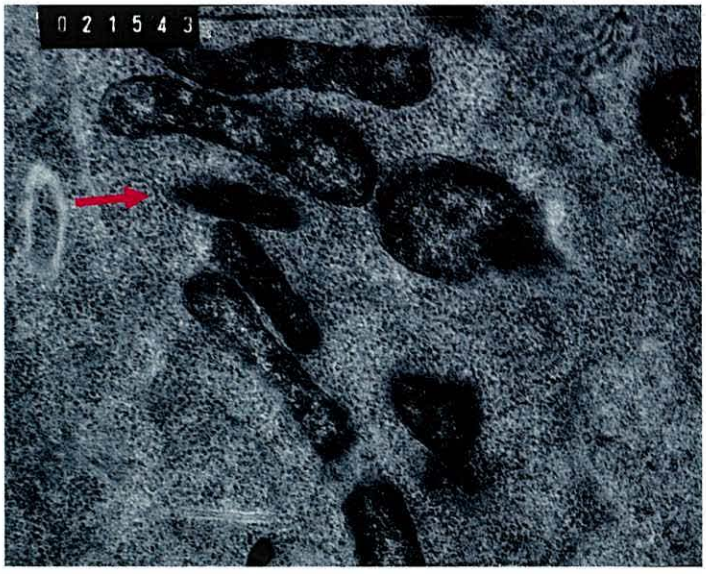


Fig. 3-22 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) ovary. The cells are oval (twice the diameter of rod shape) and rod shaped (with bulbous ending). The diameter of *Wolbachia* ranges from 0.1-0.19μm and the length ranges from 0.6-0.75μm. Enveloped by host and bacterial membranes.

Fig. 3-23. *D. melanogaster* (W^{1118}) ovary (x 51,000). Red arrow: *Wolbachia.*

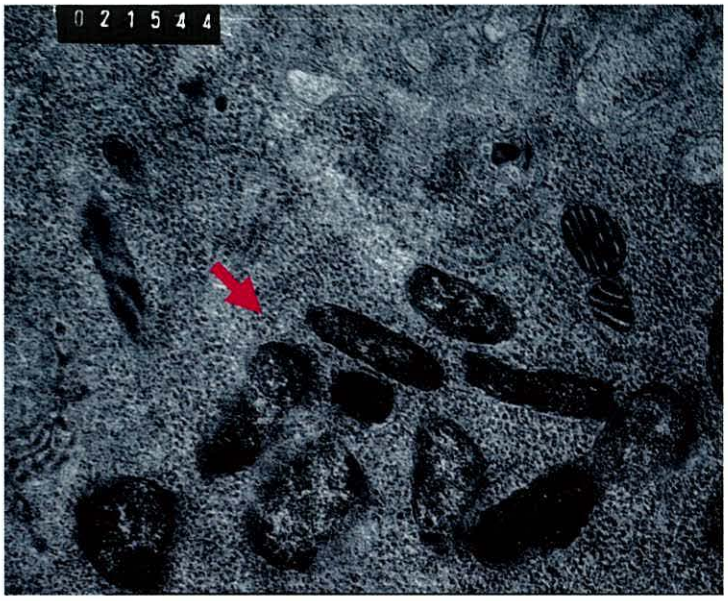


Fig. 3-23 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) ovary. The cells are oval and rod shaped. The diameter of *Wolbachia* is 0.1μm and the length is 0.4-0.75μm. These are enveloped by host and bacterial membranes.

Fig. 3-24. *Wolbachia* in the *D. melanogaster* (W^{1118}) eye (x 21,000). Red arrow: *Wolbachia.*

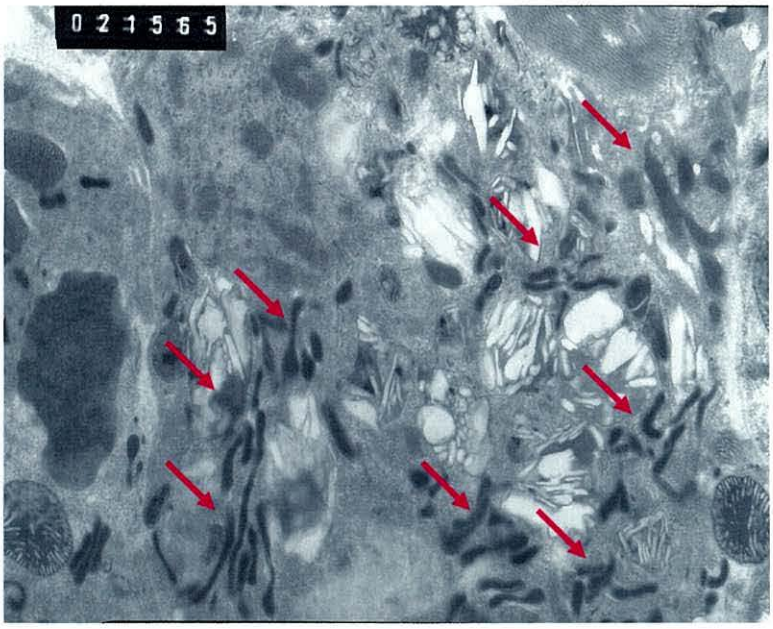


Fig. 3-24 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) eye. The cells are slightly bent, straight rods or of oval shape. The diameter of *Wolbachia* ranges from 0.08-0.12μm and the length ranges from 0.3-0.56μm. These bacterial are enveloped by host and bacterial membranes

Fig. 3-25. *D. melanogaster* (W^{1118}) eye (x 21,000). Red arrow: *Wolbachia.*

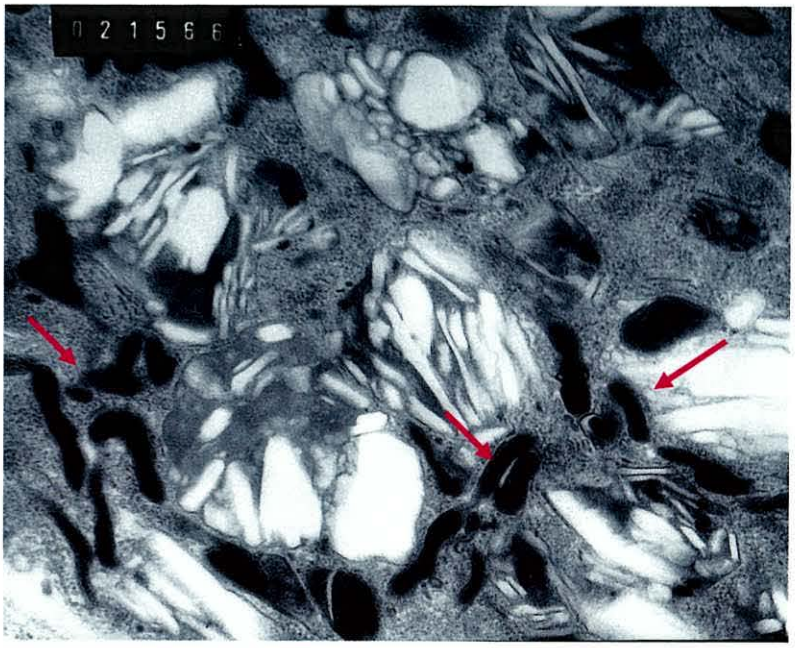


Fig. 3-25 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) eye. The cells are slightly bent, straight rods or oval shaped. The diameter of *Wolbachia* ranges from 0.08-0.1μm and the length ranges from 0.28-0.5μm. These bacteria are enveloped by host and bacterial membranes.

Fig. 3-26. *D. melanogaster* (W^{1118}) eye (x 44,000). Red arrow: *Wolbachia.*

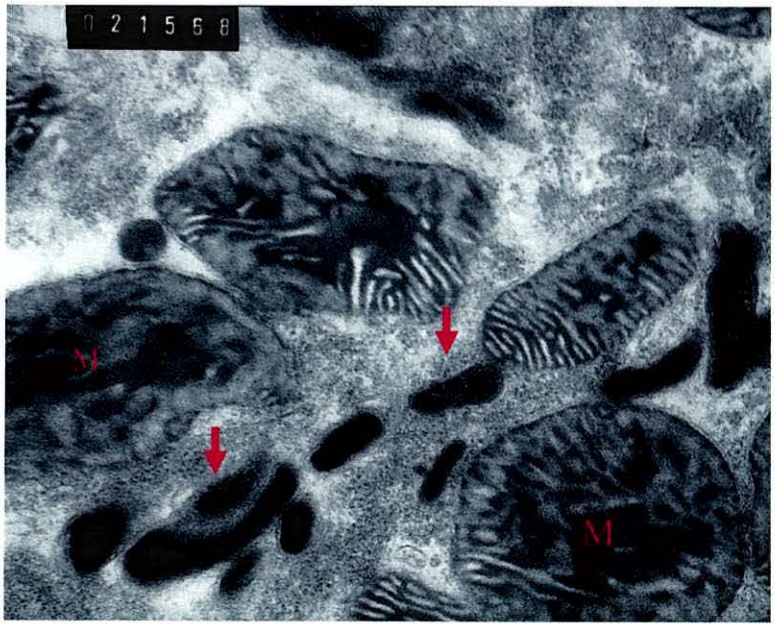


Fig. 3-26 shows electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) eye. The cells are slightly bent, straight rods or oval. The diameter of *Wolbachia*

ranges from 0.06-0.15μm and the length ranges from 0.2-0.6μm. They are surrounded by mitochondria (M). These bacterial are enveloped by host and bacterial membranes.

Fig. 3-27. *D. melanogaster* (W^{1118}) eye (x 51,000).
There are two membranes, surrounding *Wolbachia.* 1 (host membrane) and 2 (bacterial membrane). Red arrow: *Wolbachia.*

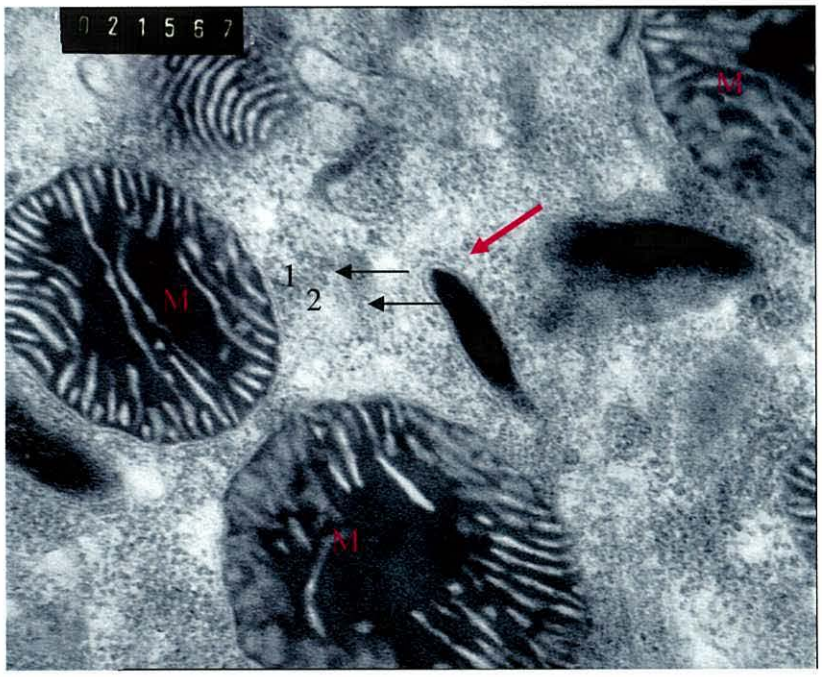


Fig. 3-27 shows a single electron dense rod of *Wolbachia* enveloped by host membrane and bacterial membranes in *D. melanogaster* (w^{1118}) eye. There is vacuolar space between host and bacterial membrane. The diameter of *Wolbachia* is 0.08μm and the length is 0.33μm. They are surrounded by mitochondria (M).

Fig. 3-28. *D. melanogaster* (W^{1118}) eye tissue (x 51,000). Enveloped by host (1) and bacterial (2) membranes. Red arrow: *Wolbachia.*

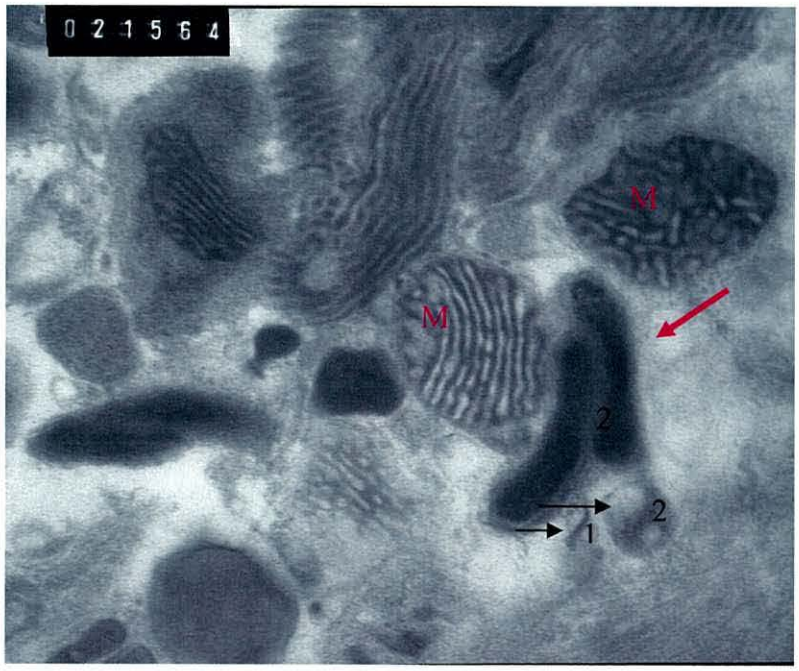


Fig. 3-28 shows two tightly-connected electron dense *Wolbachia* in *D. melanogaster* (w^{1118}) eye, sharing a vacuolar membrane and appear to have been undergoing fission. The diameter of *Wolbachia* ranges from 0.1-0.15μm and the length ranges from 0.4-0.43μm. They are surrounded by mitochondria (M). The bacteria are enveloped by host (1) and bacterial membranes (2).

Fig. 3-29. *D. melanogaster* (W^{1118}) brain tissue (x 51,000). Red arrow: *Wolbachia.*

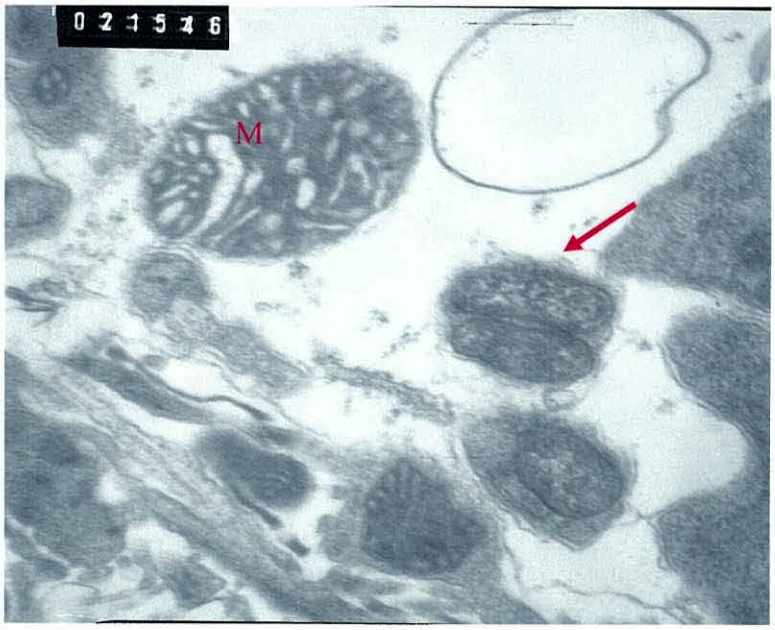


Fig. 3-29 shows two tightly-connected, electron dense *Wolbachia* in the *D. melanogaster* (w^{1118}) brain sharing an irregular host membrane and appear to have been undergoing fission. The diameter of *Wolbachia* is 0.15μm and the length ranges from 0.4-0.43μm. M (mitochondria).

Fig. 3-30. *Wolbachia* in *D. melanogaster* (W^{1118}) brain tissue (x 11,000). Red arrow: *Wolbachia*

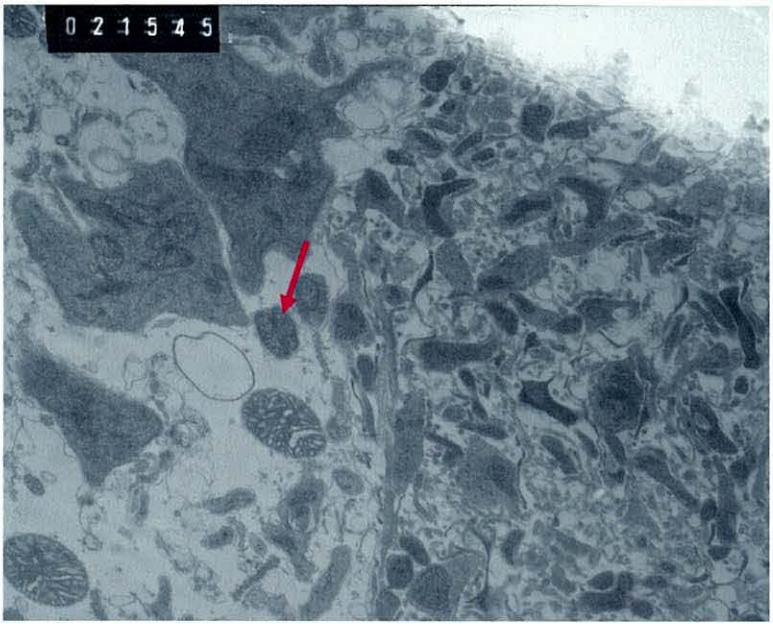


Fig. 3-30 shows two tightly-connected *Wolbachia* in *D. melanogaster* (w^{1118}) brain sharing an irregular host membrane and appear to have been undergoing fission.

Fig. 3-31. Testis of *D. melanogaster* (W^{1118}) developing of spermatids (x 3,500). Red arrow: *Wolbachia.*

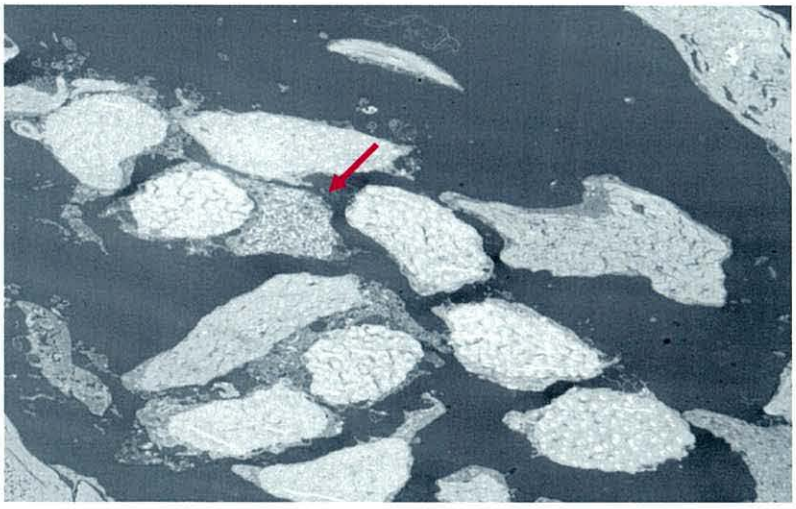


Fig. 3-31 shows the removal electron dense of *Wolbachia* and cytoplasm from the developing spermatid of the testis *D. melanogaster* (w^{1118}).

Fig. 3-32. 'Waste basket' pictured on negative film (x 16,000)

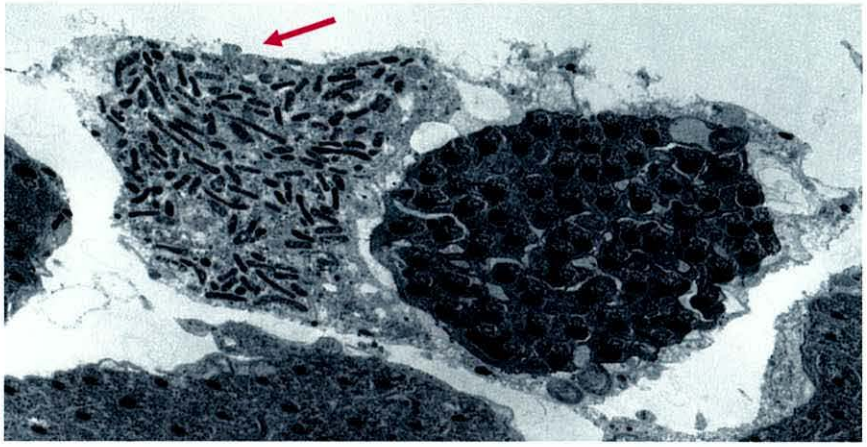


Fig. 3-32 shows the removal of electron dense *Wolbachia* and cytoplasm from the developing spermatid of the testis of *D. melanogaster* (w^{1118}) on negative film. The structure receiving the cytoplasm and *Wolbachia* is called a waste basket. The *Wolbachia* cells are oval, rod shaped or elongate.

Fig. 3-33. 'Waste basket' pictured on positive film (x 16,000)

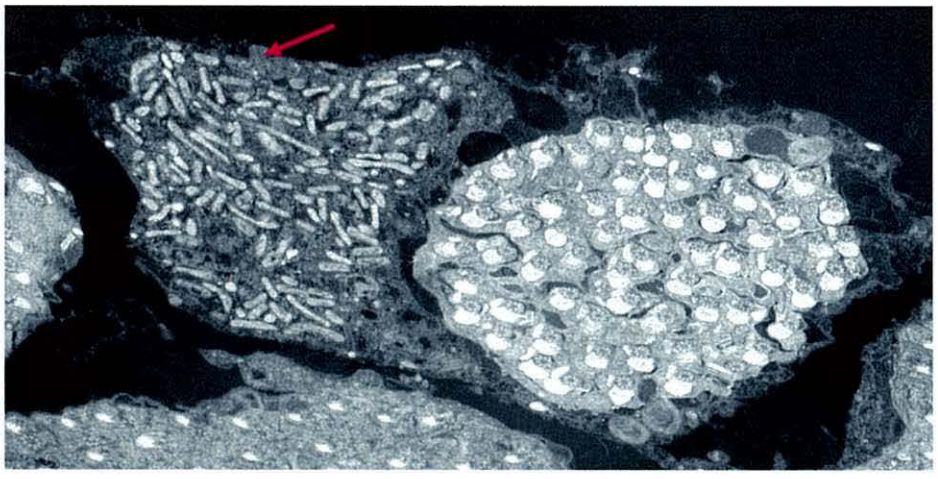


Fig. 3-33 shows the same picture as Fig. 3-32 but now on positive film.

Fig. 3-34. 'Waste basket' being filled with electron dense *Wolbachia* (x 16,000).

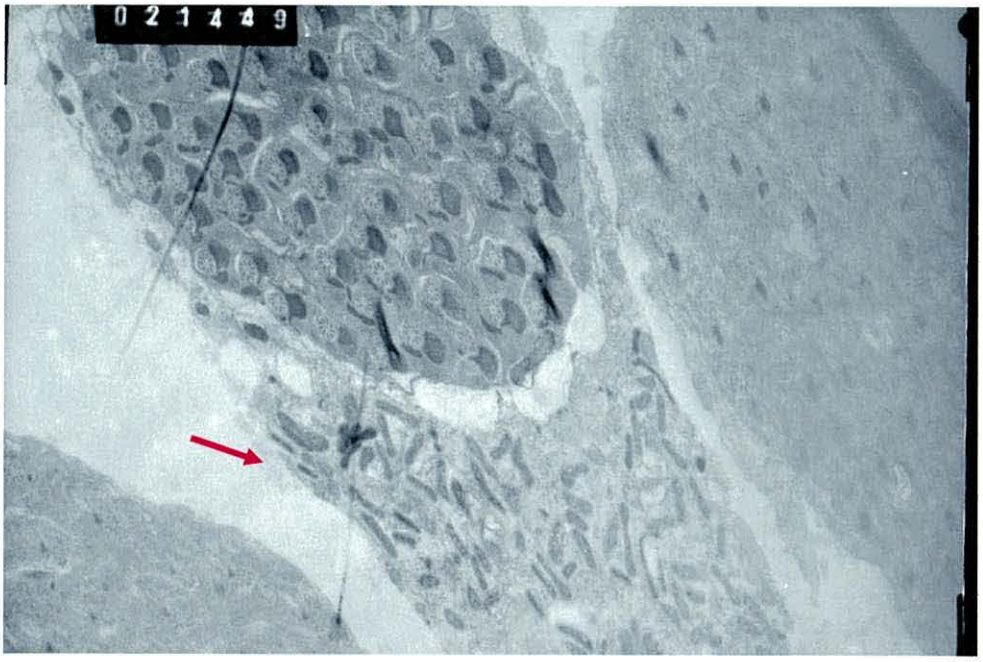


Fig. 3-34 shows a similar structure as Fig. 3-32

Fig. 3-35. High magnification electron dense of *Wolbachia* in waste basket showing the host membrane (x 40,000).

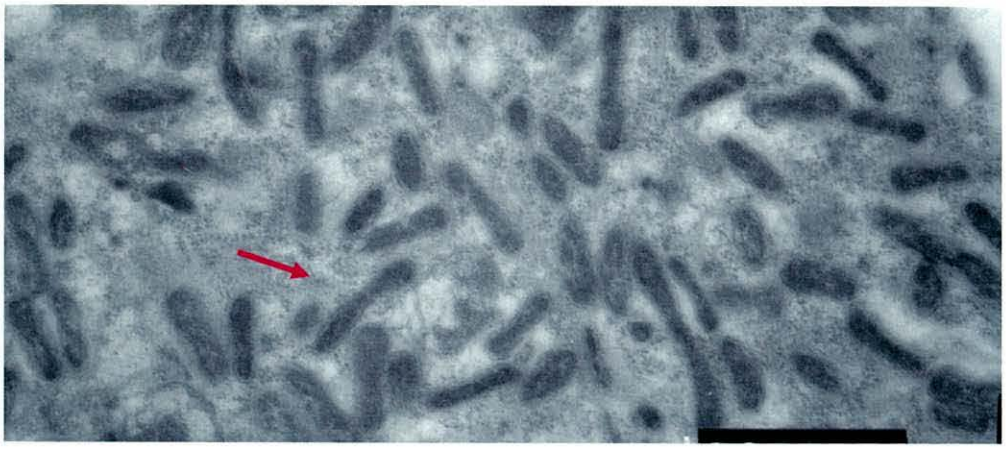


Fig. 3-35 shows a high magnification image of *Wolbachia* emphasising the enormous numbers of electron dense *Wolbachia* in the waste basket of the testis of *D. melanogaster* (w^{1118}). The cells are oval, rod shaped or elongate. The diameter of *Wolbachia* ranges from 0.04-0.07 μm and the length ranges from 0.24-0.33μm.

Fig. 3-36. Using higher magnification (x 96,000) two membranes (host membrane and bacteria membranes). Red arrow: *Wolbachia.*

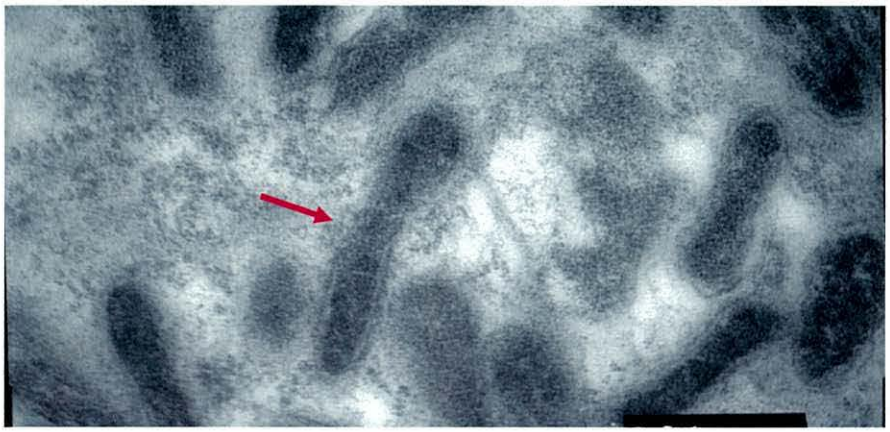


Fig. 3-36 stresses that even in the waste basket every single *Wolbachia* cell is still enveloped by a host membrane. The diameter of *Wolbachia* ranges from 0.07.08μm and the length ranges from 0.28-0.38μm.

Fig. 3-37. *Drosophila melanogaster* (W^{1118}) muscle (x 36,000). Red arrow: *Wolbachia.*

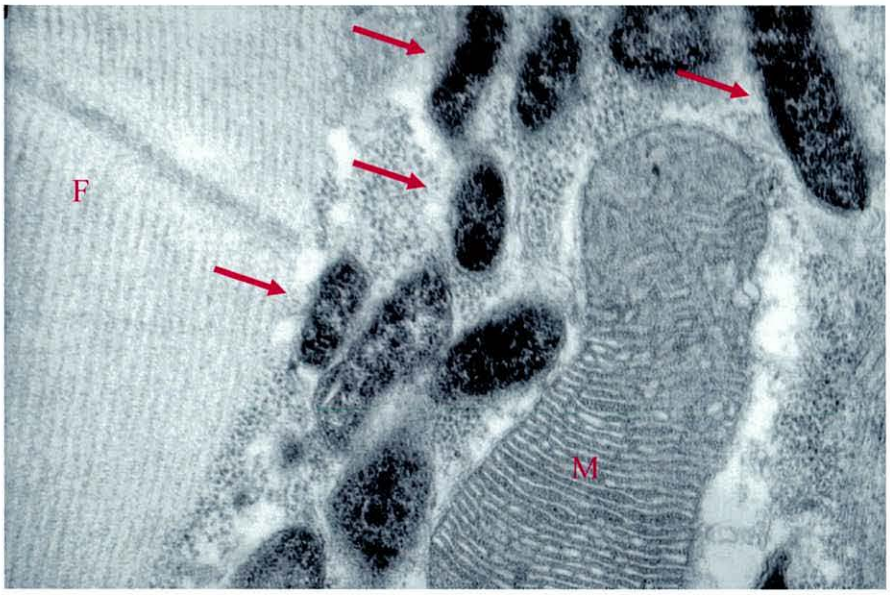


Fig. 3-37 shows *D. melanogaster* (w^{1118}) muscle tissue infected with electron dense *Wolbachia.* The cells are oval or rod shaped, surrounded by mitochondria (M). The diameter of *Wolbachia* ranges from 0.15-0.4μm and the length ranges from 0.3-1.2μm. These bacteria are enveloped by host membranes. M (mitochondria) and F (fibril of muscle).

Fig. 3-38. *Drosophila melanogaster* (W^{1118}) muscle (x 36,000). There is vacuole space (VS) between host membrane (1) and bacterial membrane (2). Red arrow: *Wolbachia.*

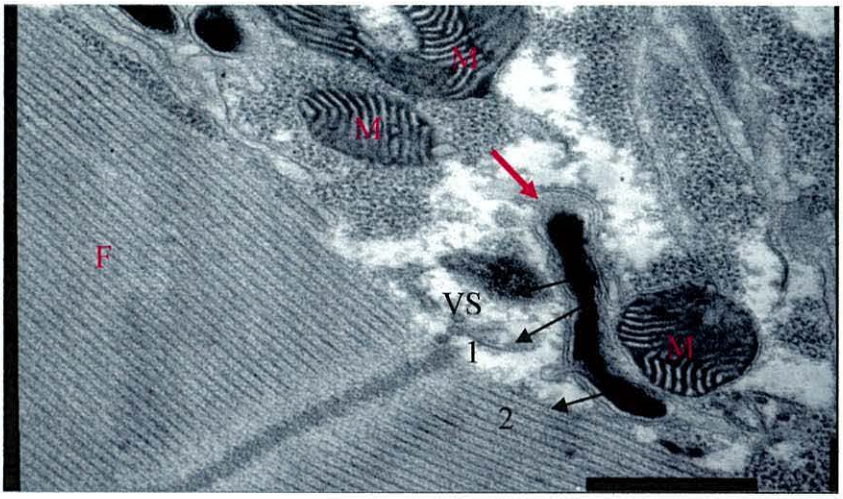


Fig. 3-38 shows *D. melanogaster* (w^{1118}) muscle tissue infected with electron dense *Wolbachia* enveloped by a double membrane, which could be part of the endoplasmic reticulum or part of the vacuolar host membrane and the third membrane is the bacterial membrane. There is a vacuolar space between the vacuole membrane and the cytoplasmic membrane. The cell is bent it probably consists of three bacteria in the process of cell division, which are connected by an isthmus of cytoplasm. The diameter of *Wolbachia* is 0.12μm and the length is 0.8μm. M (Mitochondria) and F (fibril of muscle).

Fig. 3-39. Muscle of uninfected *Drosophila melanogaster* (W^{1118}) (x 36,000).

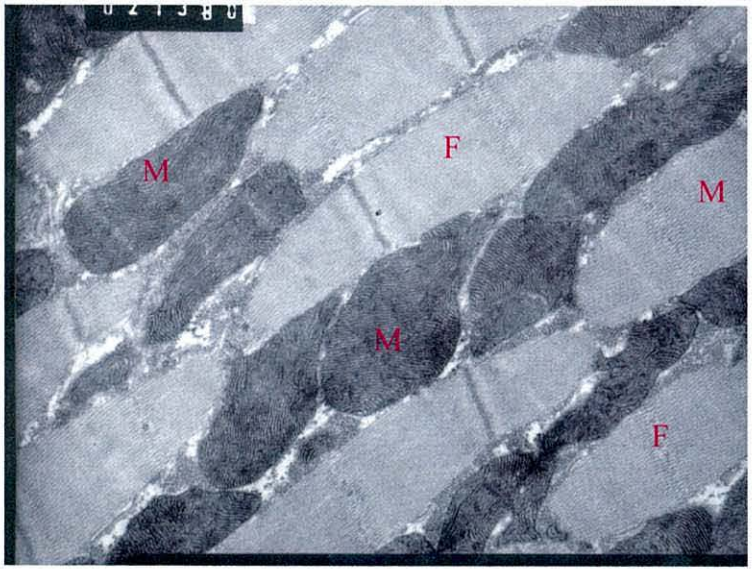


Fig. 3-39 shows muscle of uninfected *Drosophila melanogaster* (W^{1118}) Mitochondria (M) and fibril of muscle (F).

Fig. 3-40. Scanning electron micrograph of testis of *D. melanogaster* (W^{1118}).

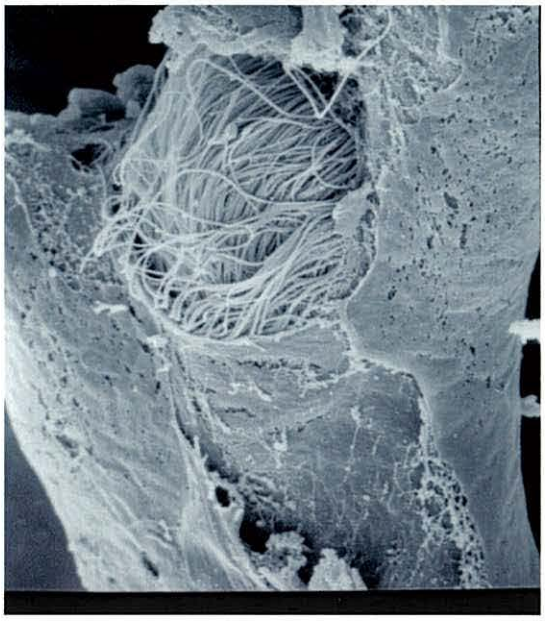


Fig. 3-40 shows scanning electron micrograph of testis of *D. melanogaster* (W^{1118}).

Fig. 3-41. Scanning electron micrograph of sperm cells in testis of *D. melanogaster* (W^{1118}).

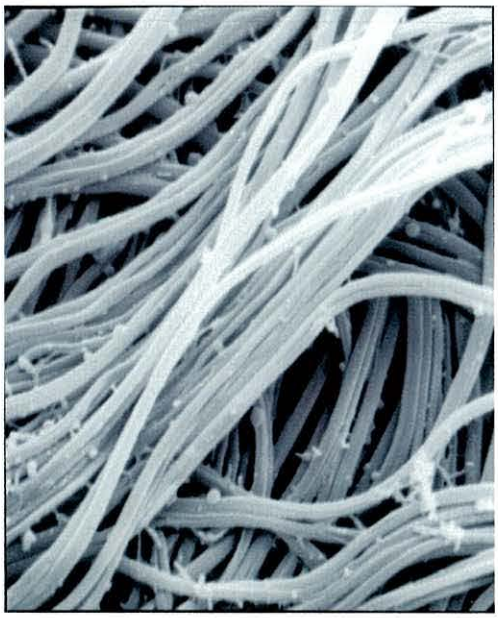


Fig. 3-41 shows scanning electron micrograph of sperm cells in testis of *D. melanogaster* (W^{1118}).

Fig. 3-42. Transmission electron micrograph of sperm cells in testis of *D. melanogaster* (W^{1118}).

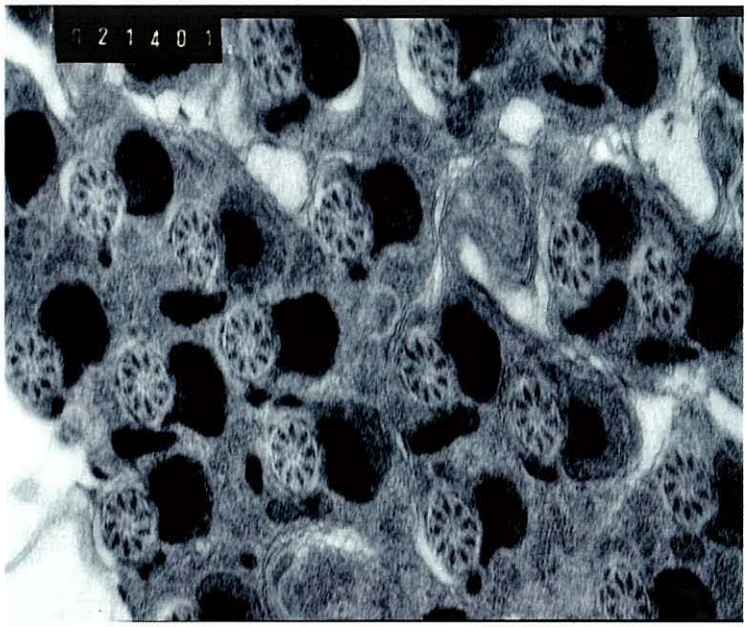


Fig. 3-42 shows transmission electron micrograph of sperm cells in testis of *D. melanogaster* (W^{1118}).

Fig. 3-43. Scanning electron micrograph of ovary of *D. melanogaster* (W^{1118})

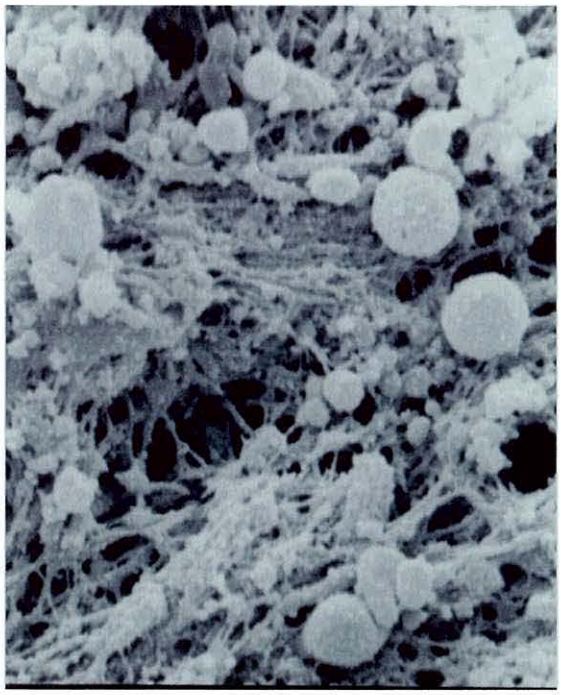


Fig. 3-43 shows scanning electron micrograph of ovary of *D. melanogaster* (W^{1118}).

Confocal microscopy

Large numbers of *Wolbachia* were visible in sperm cysts in the testes of *D. simulans* infected with the Riverside strain of *Wolbachia*. *Wolbachia* were absent in the tetracycline treated control strain. However, the sperm cysts in testes of *D. melanogaster* infected with the popcorn-strain of *Wolbachia* did not show *Wolbachia* either. The pictures below show confocal images of *Wolbachia* and sperm cyst.

Testis *Drosophila simulans*

Fig. 3-44, 45, 46, 47, 48, 49, 50 and 51 show *Wolbachia* being removed from sperm cells during individualisation in *D. simulans*. Red arrows indicate the sperm cysts.

Fig. 3-44 Sperm cysts in *D. simulans* Riverside

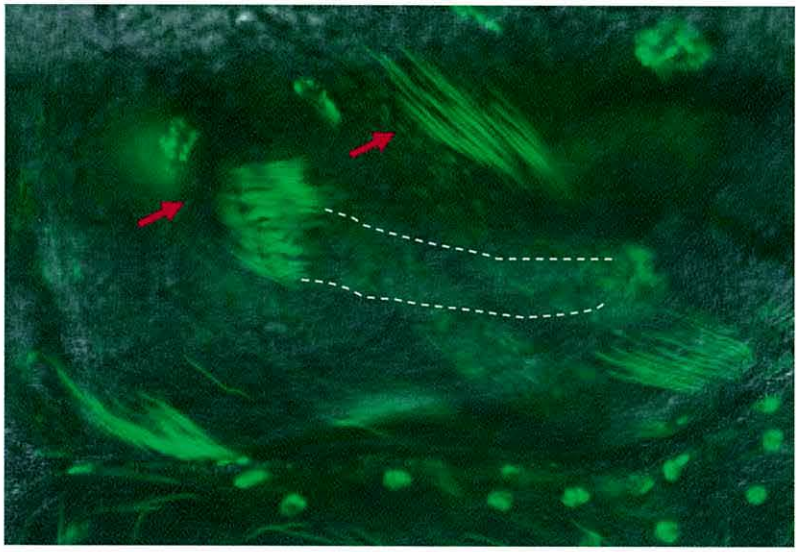


Fig. 3-45 Sperm cysts in *D. simulans* Riverside

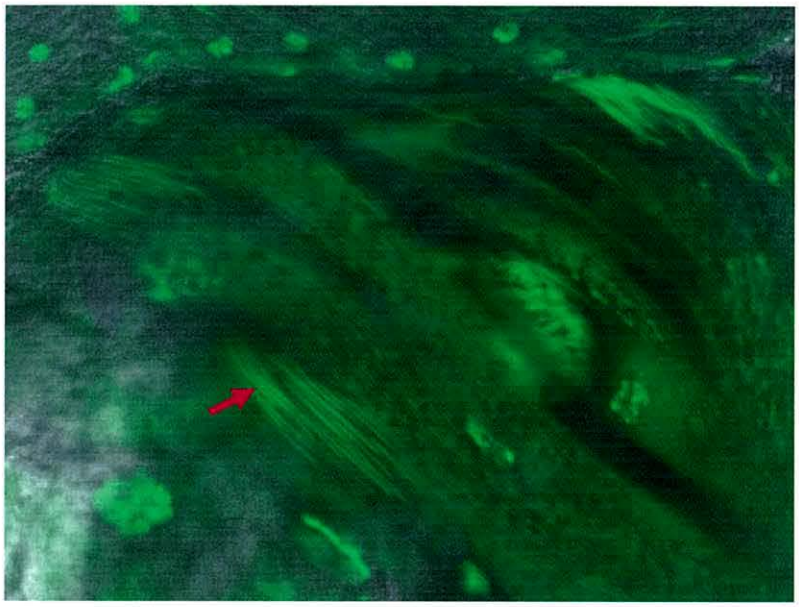


Fig. 3-46 Sperm cysts in *D. simulans* Riverside

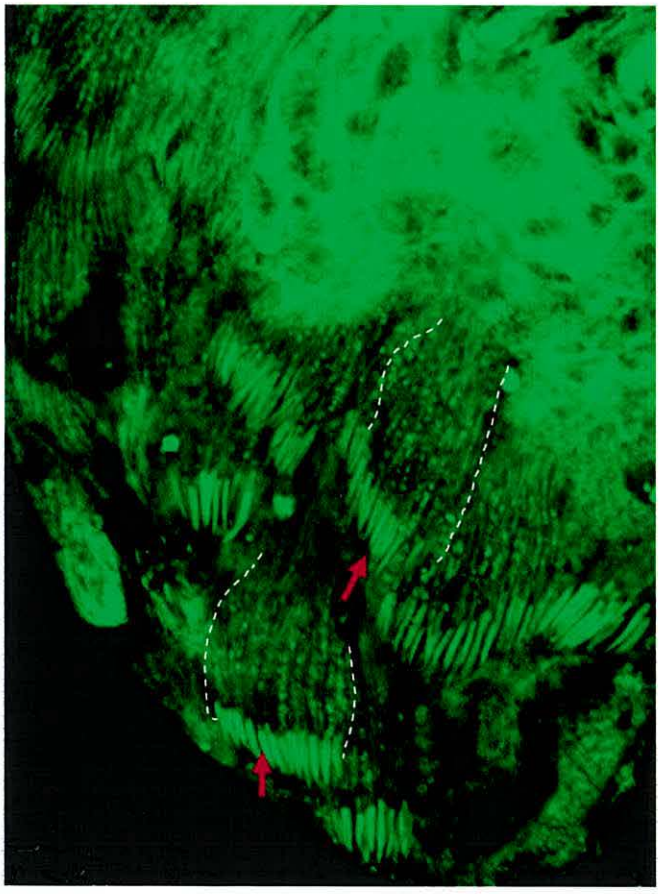


Fig. 3-47 Sperm cysts in *D. simulans* Riverside

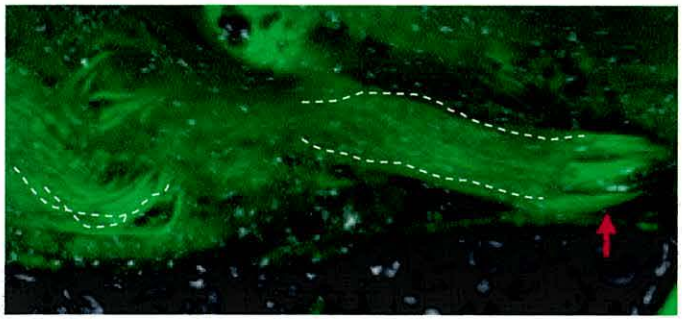
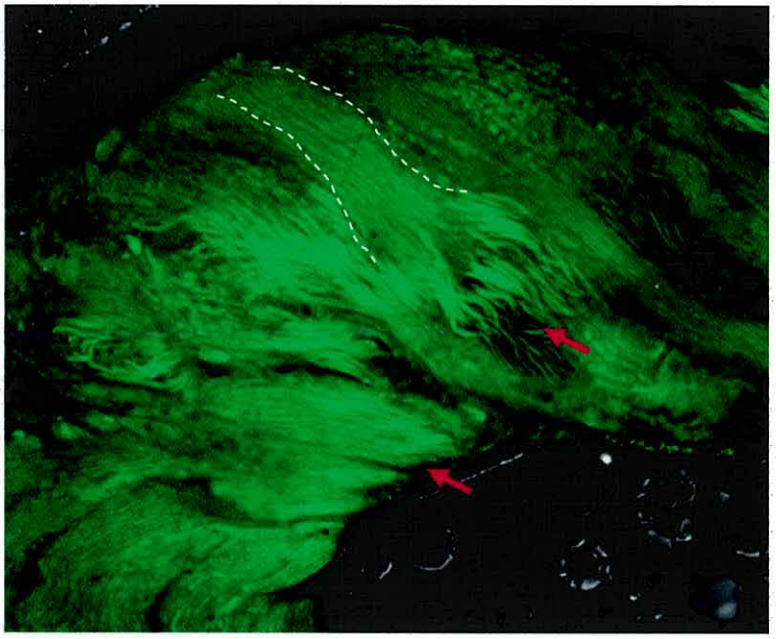


Fig. 3-48 Sperm cysts in *D. simulans* Riverside



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3-49 Sperm cysts in *D. simulans* Riverside

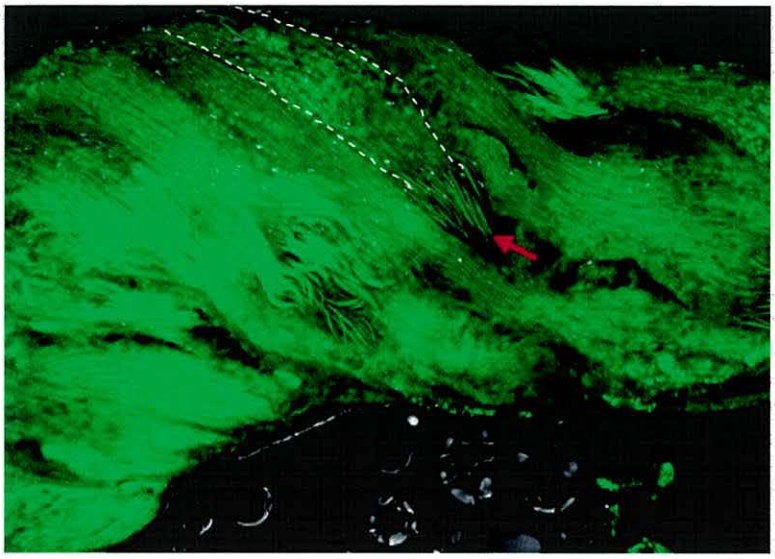


Fig. 3-50 Sperm cysts in *D. simulans* Riverside

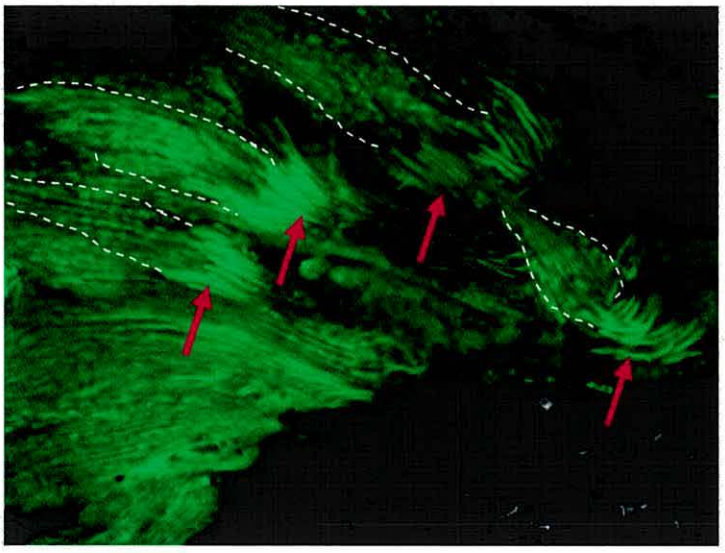


Fig. 3-51 Sperm cysts in *D. simulans* Riverside

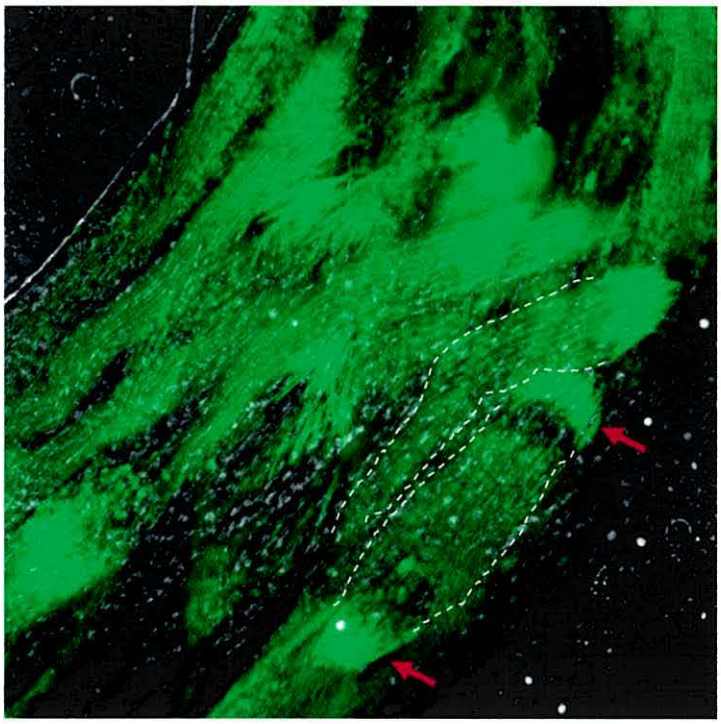
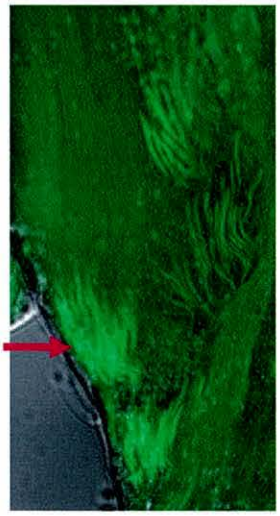


Fig.3-52 Sperm cysts of *D. simulans* Riverside lacking *Wolbachia*



Testis *Drosophila melanogaster*

Fig. 3-53, 54, 55, 56, and 57 show there is no *Wolbachia* in the sperm cells of *D. melanogaster* during individualisation. Red arrows indicate the sperm cycts.

Fig. 3-53 Sperm cysts of *D. melanogaster* popcorn

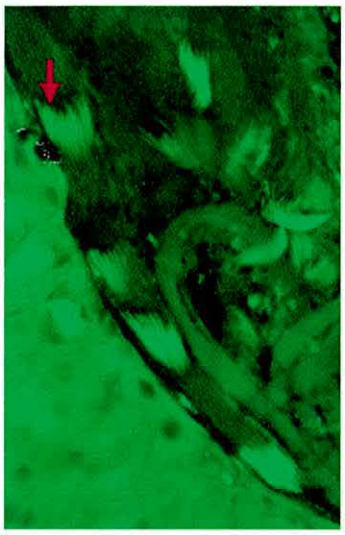


Fig. 3-54 Sperm cysts of *D. melanogaster* popcorn

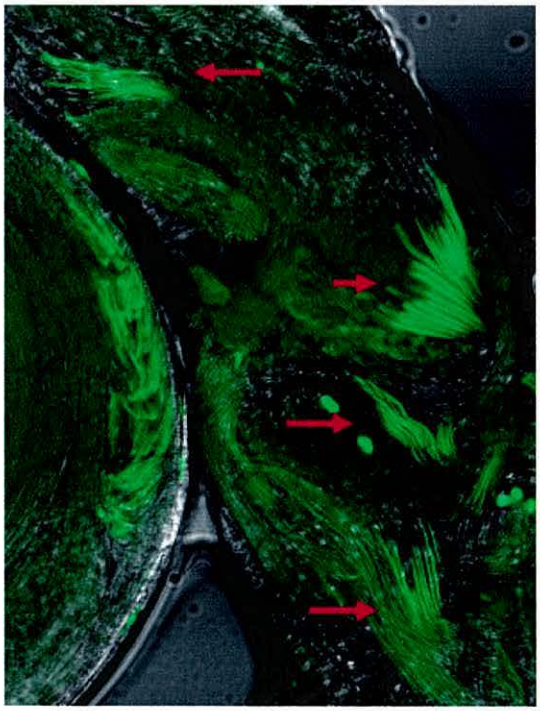


Fig. 3-55 Sperm cysts of *D. melanogaster* popcorn

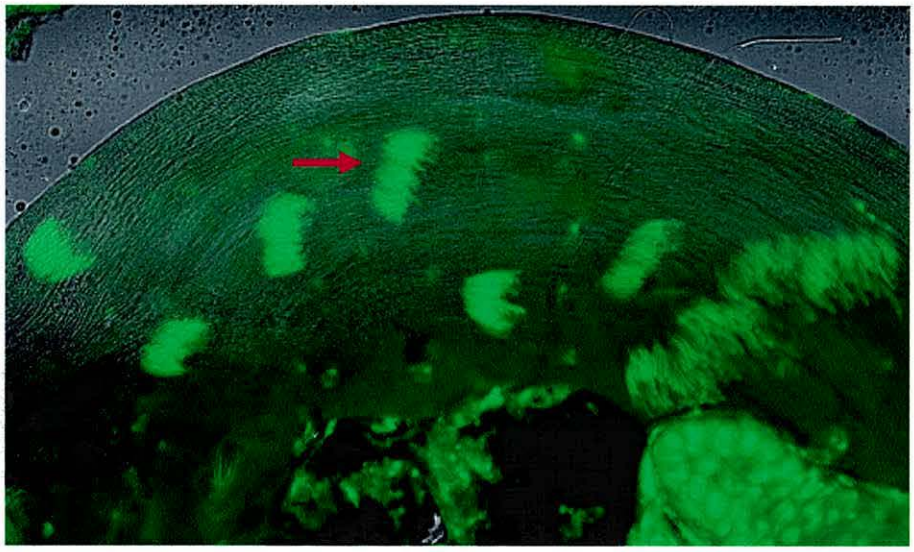


Fig. 3-56 Sperm cysts of *D. melanogaster* popcorn

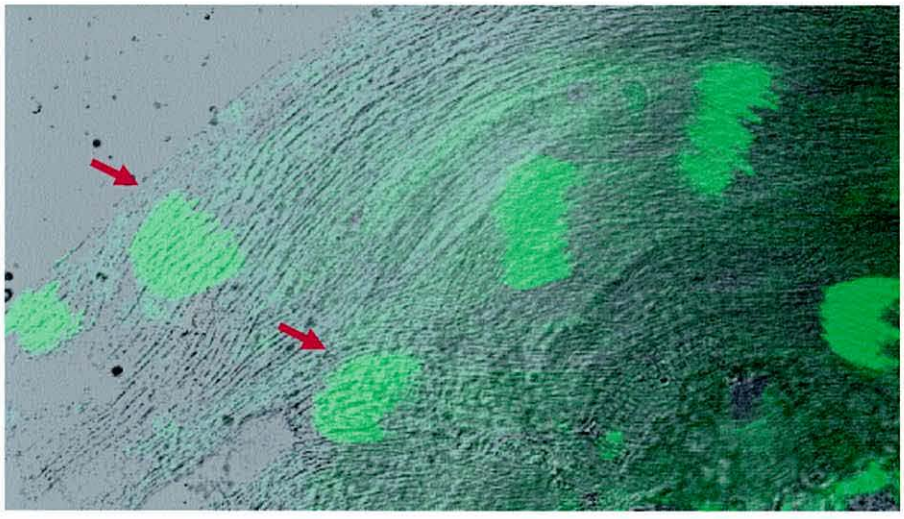
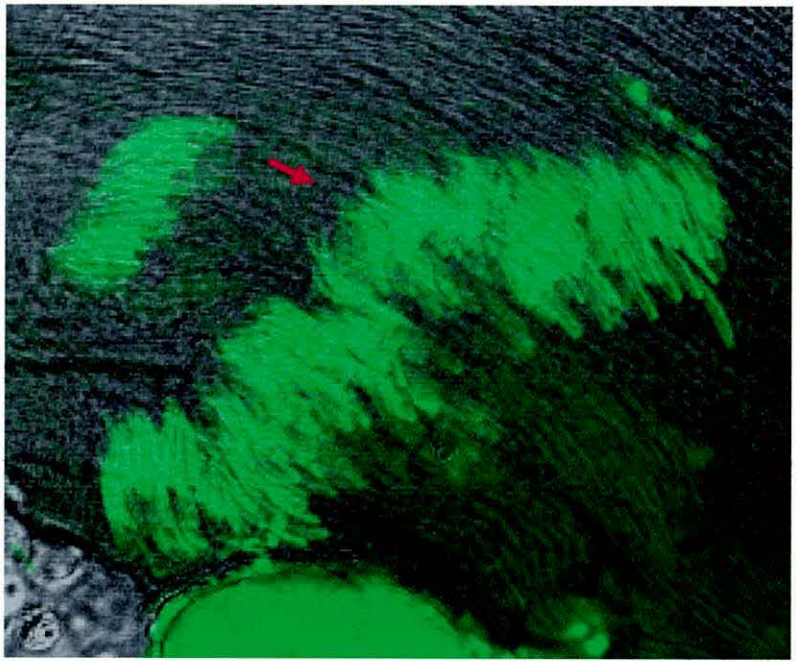


Fig. 3-57 Sperm cysts of *D. melanogaster* popcorn



Testis *Drosophila simulans* treated with tetracycline (DSRT)

Fig. 3-58, 59, 60 and 61 show there is no *Wolbachia* in the sperm cells of *D. simulans* treated with tetracycline (DSRT) during individualisation. Red arrows indicate sperm bundles.

Fig. 3-58 Sperm cysts of *D. simulans* tetracycline

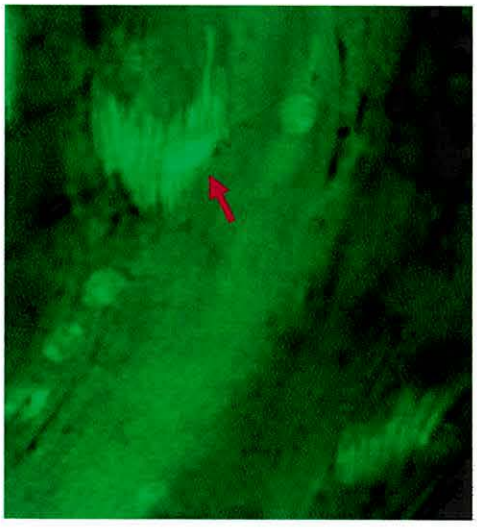


Fig. 3-59 Sperm cysts of *D. simulans* tetracycline

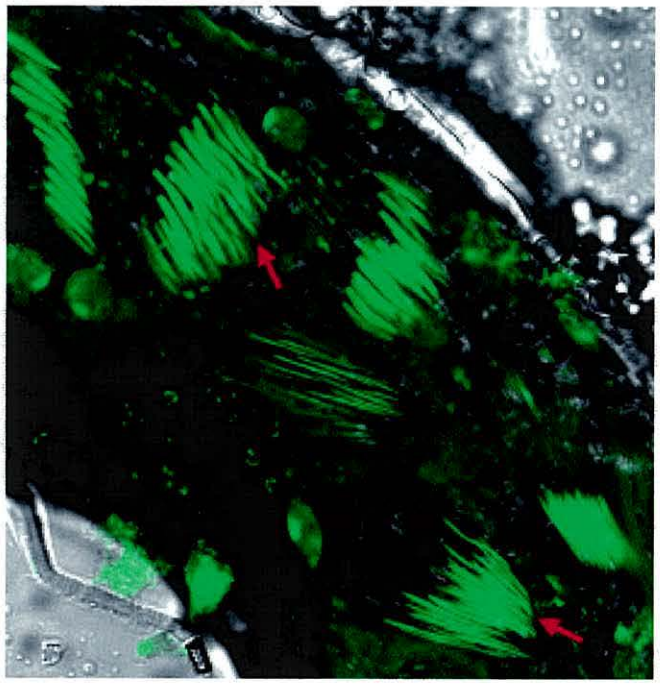


Fig. 3-60 Sperm cysts of *D. simulans* tetracycline

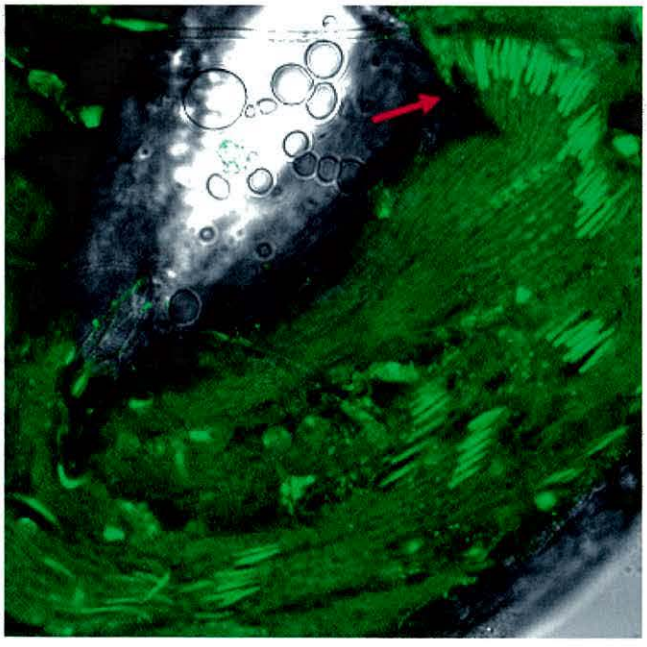


Fig. 3-61 Sperm cysts of *D. simulans* tetracycline

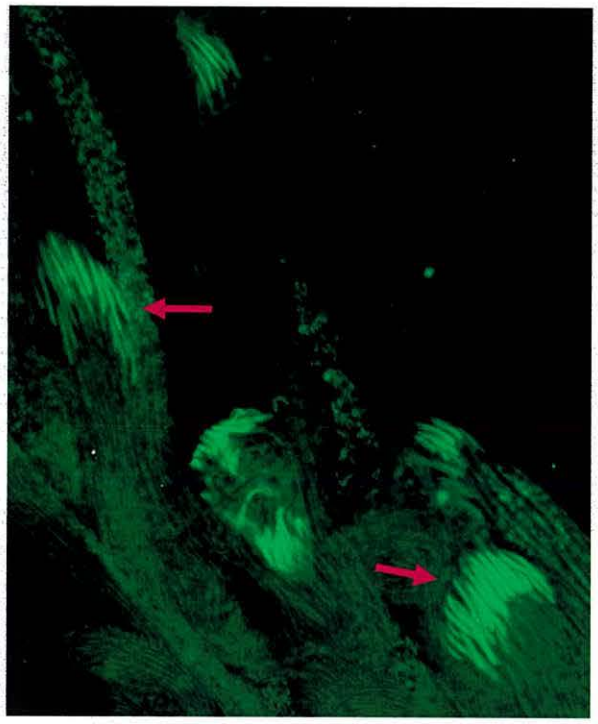


Fig. 3-62. Confocal images of cysts leaving from the tubulus of *D. simulans* treated with tetracycline (DSRT).

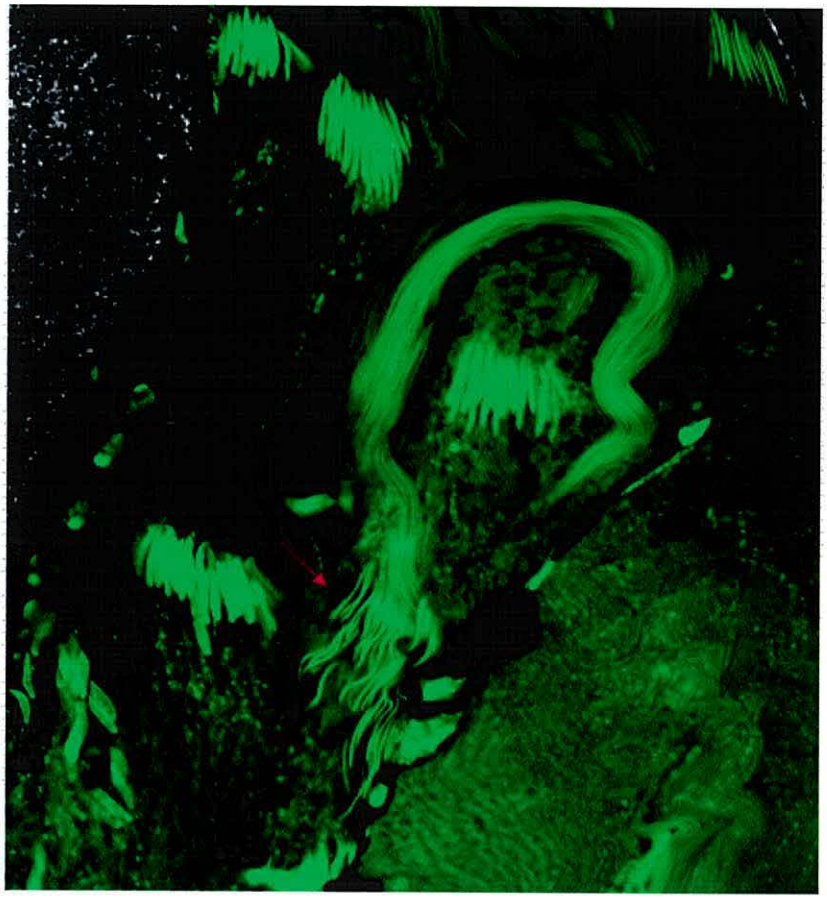


Fig. 3-62 shows sperms bundle/cysts of *D. simulans* treated with tetracycline (DSRT) leaving from the tubulus (red arrow).

Ovaries *Drosophila simulans*

Fig. 3-63, 64, 65 and 66 show ovaries of *D. simulans* portions of several follicles (yellow arrow) and nurse cells (red arrow).

Fig. 3-63 Ovaries *D. simulans* Riverside

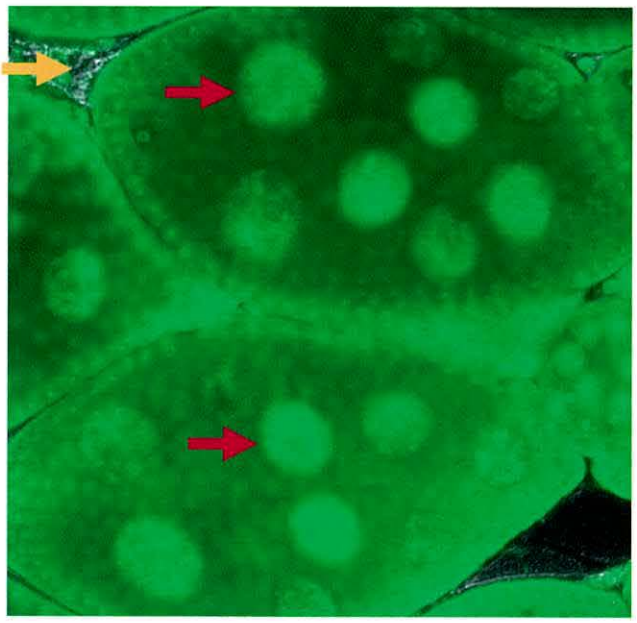


Fig. 3-64 Ovaries *D. simulans* Riverside

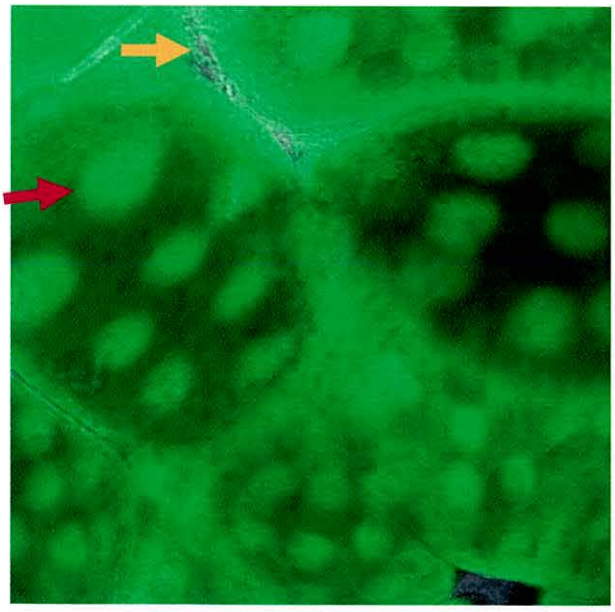


Fig. 3-65 Ovaries *D. simulans* Riverside

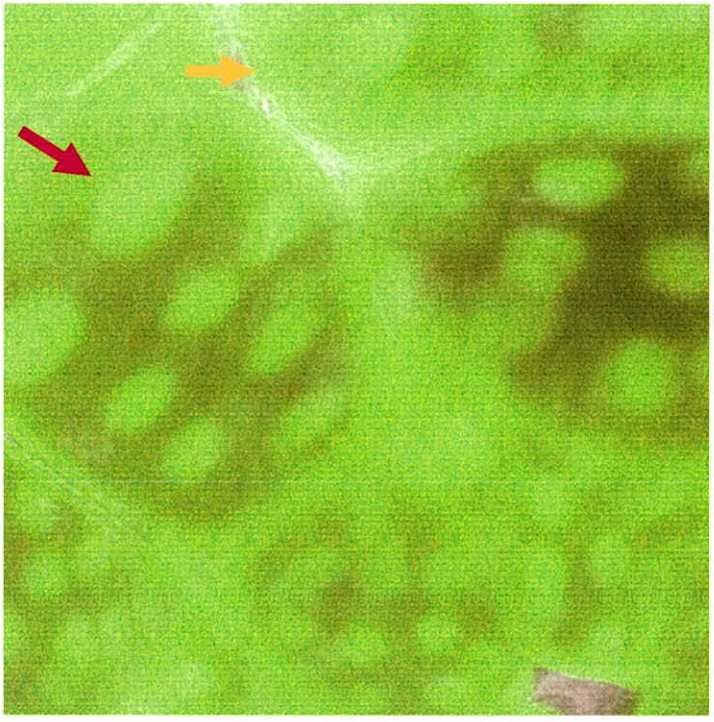
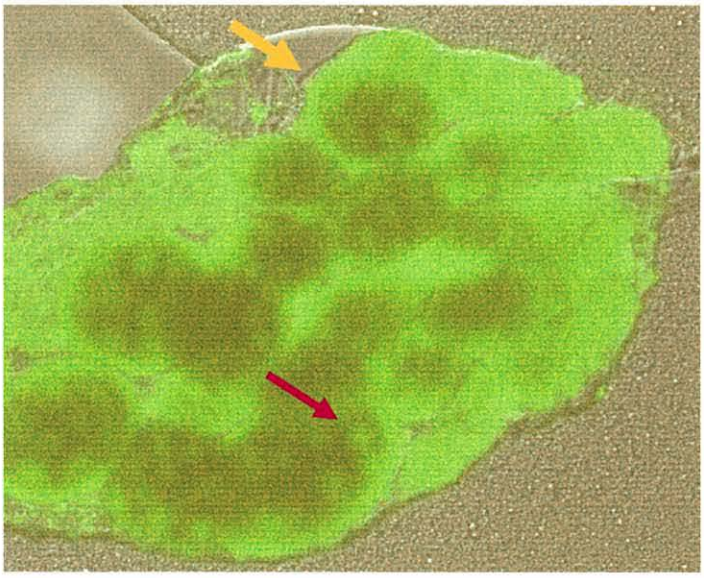


Fig. 3-66 Ovaries *D. simulans* Riverside

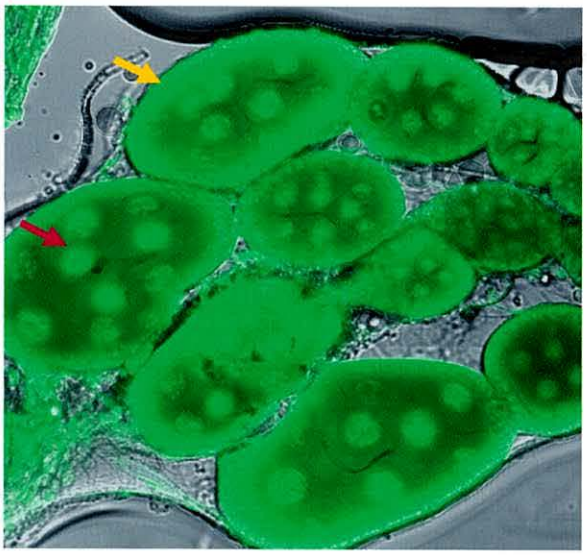


Ovaries *Drosophila simulans* treated with tetracycline

Fig. 3-67 and 68 show ovaries of *D. simulans* treated with tetracycline (DSRT) showing portions of several follicles (yellow arrow) and nurse cells (red arrow).

Fig. 3-67. Ovaries *D. simulans* tetracycline

Fig. 3-68 Ovaries *D. simulans* tetracycline



3.5 DISCUSSION

The ultrastructure of *Wolbachia* in *Drosophila melanogaster* can be described as follows, they are pleomorphic, oval, the shaped of bent rods, straight rods and of an elongate form. The analysis of the form of several hundred of *Wolbachia* (see table 3-1) reveal only two basic forms. The most common form is 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 has been encountered is a barrel shape that is oval like an egg and shows a maximum diameter along its length. The bent rod shape is often discovered in eye (Figs. 3-24 and 3-25) and muscle (Figs. 3-3, 3-17, 3-18 and 3-38) tissues. The size varies between 0.04μm and 0.47μm in diameter and between 0.26μm and 1.2μm in length. The precise orientation of *Wolbachia* in the plain of the picture is uncertain. It is not possible to define whether any transection is exactly in the mid plain or at any other location. All measurement of length and diameter will very widely. A minimum diameter or length of *Wolbachia* cannot be defined. The uncertainty of the transection plain does not allow us to estimate any minimum dimension. However, the maximum dimension can be assigned with considerable confidence. This confidence grows forward from the large number of *Wolbachia* per viewing area as well as the numerous, independent tissues examined. If we restrict ourselves to measuring the diameter of only perfect circles, we can be certain that we are measuring the shorter of the two dimensions and are not confusing the diameter with any measurement of the length. In both the rod and the barrel the maximum of the form and

therefore the longest distance is always the minimum of the longest length. In a few cases *Wolbachia* seem to have been undergoing fission, but are still joint together by a vacuolar membrane (Figs. 3-16, 3-17, 3-18, 3-28, 3-29, 3-30, and 3-38).

The microorganisms appear to have two membranes, a host membrane and a bacterial membrane. In some cases a vacuolar space was found between host membrane and bacterial membrane (Figs. 3-1, 3-7, 3-27, 3-36 and 3-38). In one case Fig. 3-38 a double membrane could be clearly seen as part of the host membrane and the third membrane being the bacterial membrane. Furthermore, in Figs. 3-16, 3-17, 3-18 and 3-38, the shape of the bacteria as bent and probably constituted two bacteria (Figs. 3-16, 17, 28, 29 and 30), three bacteria (3-38) or four bacteria (3-18) in the process of cell division, and were still connected by an isthmus of cytoplasm. In some cases the microorganisms seem to be surrounded by only one membrane (Figs. 3-31, 32, 33, 34, 35), however, when the picture was taken using higher magnification (x 96,000) (Fig. 3-36), the bacteria clearly showed two membranes. They consisted of a host membrane and a bacterial membrane and between host membrane and bacterial membrane there was a vacuolar space. Another possibility why only one membrane was visible might be due to loss during the embedding process and/or attributed to the very thinness of the cell wall (Musgrave *et al.,* 1962) or to the absence of certain substances from the membranes resulting in insufficient staining (Kreig, 1963). I found an unidentified membrane-bound structure in the eye tissue of *D. melanogaster* (Fig. 3-20). It probably is a group

of 11 organisms of electron dense light *Wolbachia* (red arrow). The green arrow in Fig 3-20 points to presumably 3 round electron light *Wolbachia* and the pink arrows indicate electron dense *Wolbachia* sharing a vacuole membrane.

Yen and Barr (1971) and Yen (1975) reported that *Wolbachia* appear to be absent from mature spermatids of *Culex pipiens.* Binnington and Hoffmann (1989) also reported that *Wolbachia* appeared to be absent from sperm of *Drosophila simulans.* During the change from syncytial to individualized stage, there is a loss of cytoplasm from *Drosophila* spermatids, and the *Wolbachia* are shed with excess cytoplasm during individualization. When the spermatocytes develop into spermatids the cytoplasm and bacteria are shed from sperm during maturation, indicating that the bacteria must in some way 'condition' the developing spermatid, this effect being carried over to the mature spermatozoon. Byers and Wilkes (1970) reported that RLO's (Rickettsia-like organisms) in *Dahlbominus. fuscipennis* were never seen in mature spermatozoa and that these microorganisms were probably lost along with excess cytoplasm.

Here, I show *Wolbachia* in the testis of *D. melanogaster* (Figs. 3-31, 32, 33, 34, 35 and 36), where *Wolbachia* and cytoplasm are excluded from the mature sperm. This situation is apparently similar to that in *Culex pipiens* and *D. simulans*, in which *Wolbachia* were absent in mature sperm. The fate of the *Wolbachia* is still unclear. They are probably reabsorbed into the cell. *Wolbachia* might exert their effect

during spermatid development and the mature spermatozoon might carry this effect in the absence of *Wolbachia.*

The distribution of *Wolbachia* in *D. simulans* was not restricted to the reproductive organs (ovary and testis). They were also found in somatic tissue (muscle). The ultrastructure of *Wolbachia* were similar to the description of *W. pipientis* from *Drosophila melanogaster,* including the occurrence of a cytoplasmic isthmus connecting two cells (Figs. 3-16 and 17) or four cells (Fig. 3-18) at a late stage of division. Especially in Fig. 3-18, the bacteria present a very long elongate shape connected by an isthmus of cytoplasm, it could be four bacteria that have been undergoing fission. This is probably due to the limited space in the muscle cells. Similarly, the *Wolbachia* found in eye tissue, which is filled with dense structures, displayed the same shape of bent bacteria. There is probably not enough space for the bacteria to expand.

These results (*Wolbachia* found in *D. simulans* muscle tissues) are supported by Dobson *et al.* (1999). They reported that *Wolbachia* infect various tissues other than the gonads in *D. simulans* (and some other species). Therefore my findings support their observation that *Wolbachia* causing CI in *D. simulans* infect somatic tissue as well as the gonads. I have also shown that the distribution of *Wolbachia* in *D. simulans* is the same as the distribution of the 'popcorn-effect' *Wolbachia* in *D. melanogaster.*

The presence of *Wolbachia* in *Stomoxys calcitrans* is 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* were found in somatic tissues of *S. calcitrans* brain and muscle, were morphologically similar to the *Wolbachia* present in *D. melanogaster*

To provide evidence that *Stomoxys calcitrans* did not contain bacteria before injection with *Wolbachia,* the various tissues, muscle, brain and eyes, were examined by electron microscope and PCR. The Figs. 3-8, 9, 10, 11, 12, 13, 14 and 15) show tissues of *Stomoxys calcitrans,* where no *Wolbachia* or any other bacterial structures were detected. This also supported by PCR analysis (Fig. 2-5) where no bands are present in the gel electrophoresis. This indicates that *S. calcitrans* were negative for *Wolbachia* .

Figs. 3-40, 41 and 42 show scanning electron micrograph of the reproductive tissues of *D. melanogaster*, the sperm cells. The ovary shows an unidentified structure.

The *Wolbachia* that were found in *S. calcitrans*, *D. simulans* and *D. melanogaster* have an ultrastructure that exhibit the basic morphological characteristics of *Wolbachia* previously described by

Hertig (1936), Byers and Wilkes (1970), Yen and Barr (1971) and Yen (1975), Larsson (1983), O'Neill (1989), Kambhampati *et al.* (1992) and Popov *et al.* (1998). The cells were pleomorphic (rod shaped, elongate, oval or slightly bent). *Wolbachia* can be present without causing detrimental effects. *Wolbachia* having only arthropods as natural hosts are therefore presumably non-pathogenic and not restricted to mycetocytes. Some mitochondria are found in infected cells and these appear to be normal, autophagic vacuoles were never observed. The 'popcorn-effect' bacteria that cause rupturing of the cells and sudden death of their host are not taken up by autophagy of the cell. The bacteria presumably multiply rapidly in the cell, resulting in the cell to be filled with bacteria to the point of bursting. The bacteria were localised in the cytoplasm of the examined cells, and always found intracellularly and therefore the bacteria will not be recognised by the immune system of their host.

Table 3-1. Diameter and length of *Wolbachia.*

Figures	Size	
	Diameter (μM)	Length (μM)
Fig. 3-1	0.16	0.66
Fig. 3-2	0.15	1.1
Fig. 3-3	0.11 0.07 0.14 0.11	0.33 0.26 0.7 0.4
Fig. 3-4	0.1	0.6

	0.1	0.5
	0.1	0.75
Fig. 3-5	0.13	0.7
	0.2	0.5
Fig. 3-6	0.16	0.5
	0.16	0.4
	0.11	0.8
Fig. 3-7	0.06	0.4
Fig. 3-16	0.14	0.8
	0.19	0.9
Fig. 3-17	0.07	0.9
	0.1	0.5
Fig..3-18	0.12	0.3
	0.12	0.5
Fig. 3-19	0.06	0.28
Fig. 3-20	0.1	0.14
	0.12	0.16
	0.14	0.17
	0.17	0.69
	0.12	0.57
	0.14	0.28
	0.16	0.43
Fig. 3-21	0.13	0.64
	0.08	0.45
	0.2	0.75
	0.1	0.33
	0.1	0.87
	0.1	0.4
	0.2	0.4
Fig. 3-22	0.19	0.75

	0.1	0.6
Fig. 3-23	0.1	0.4
	0.1	0.4
Fig. 3-24	0.12	0.56
	0.08	0.4
	0.08	0.3
Fig. 3-25	0.08	0.5
	0.1	0.4
	0.08	0.4
	0.08	0.3
	0.1	0.28
Fig. 3-26	0.15	0.6
	0.1	0.2
	0.1	0.3
	0.06	0.2
	0.1	0.2
	0.1	0.3
Fig. 3-27	0.08	0.33
Fig. 3-28	0.1	0.5
	0.15	0.5
Fig. 3-29	0.15	0.4
	0.15	0.43
Fig. 3-30	0.3	0.7
	0.24	0.56
	0.16	0.6
	0.3	0.5
Fig. 3-35	0.06	0.3
	0.07	0.33
	0.06	0.4

	0.04	0.25
	0.06	0.24
	0.04	0.25
Fig. 3-36	0.07	0.38
	0.08	0.28
Fig. 3-37	0.4	1.2
	0.2	0.4
	0.4	0.66
	0.26	0.62
	0.47	0.47
Fig. 3-38	0.12	0.8

Figs. 3-44 - 3-51 show that mature cysts of *Drosophila simulans* contain abundant *Wolbachia* moved from the cysts during individualisation. This confirm previous work by Clark and Karr (2002) that *Wolbachia* was observed within a large percentage of developing spermatocytes and spermatids of young males of *D. simulans* Riverside (DSR). This might be correlated with the expression of that the cytoplasmic incompatibility in its host. The hypothesis that *Wolbachia* modify maturing sperm and then *Wolbachia* and cytoplasm are excluded from the mature spermatozoon, is under pinned by the electron microscopy results describe earlier (Figs. 3-31, 32, 33, 34, 35 and 36). However, in a single case shown in Fig A-4.9 *Wolbachia* could not be recognised in a sperm cyst of *D. simulans* Riverside. This shows that not all sperm cells are infected with *Wolbachia* and the consequence of this is that cytoplasmic incompatibility cannot 100%. However, the sperm cysts in testes of *D. melanogaster* infected with 'popcorn-effect'

Wolbachia did not show *Wolbachia.* This might be correlated with the fact that the popcorn strain of *Wolbachia* does not induce cytoplasmic incompatibility in its host.

Spermatogenesis in adult *Drosophila* has been detailed by Clark *et al.* (2003). They reported that spermatogenesis begins at the germinal proliferation centre at the apical bud of the testis where the gonial stem cells and cyst progenitor cells are located. A primary gonial cell buds off from the germ-line stem cell and is surrounded by two somatically derived cyst cells, which bud off the nearby cyst progenitor cells. Subsequently, the primary spermatogonial cell undergoes four rounds of mitotic division before entering meiosis, it moves down the tube of the testis away from the apical hub. Early cysts are generally found toward the inner curve of the coiled testis and the individualisation complex is seen as a bugle proceeding along the cyst, pushing the stripped away material into the waste bag. Fig. 3-62 shows a bundle of cysts leaving from the tubulus.

CHAPTER 4

EFFECTS OF TETRACYCLINE AND TEMPERATURE ON THE ADULT LIFE SPAN OF *DROSOPHILA MELANOGASTER* INFECTED WITH *WOLBACHIA* INDUCING THE 'POPCORN- EFFECT'

4.1 INTRODUCTION

There is evidence that environmental factors such as temperature, food quality, larval density, antibiotics and bacterial density effect the level of cytoplasmic incompatibility caused by *Wolbachia.* Clancy and Hoffmann (1998) reported that *Wolbachia* cause cytoplasmic incompatibility in *Drosophila simulans* and that transmission rate may be associated with environmental factors such as temperature, nutrition, larval density and antibiotic treatment. They reported that low levels of nutrition (yeast and raw sugar in 10%, 30%, 60% and 100%) of the normal amount (sucrose (4.8%), dead yeast (3.2%), agar (1.8%) and both propionic acid and methyl hydroxybenzoates as preservatives and rearing males at high temperature led to an increase in compatibility. However, exposing infected females to a high temperature did not influence their compatibility with infected males. Exposure to 25°C reduced the density of *Wolbachia* in embryos compared with a 19°C treatment. It was also reported that larval crowding (twenty eggs per vial with 10mL medium and 120 eggs in vials containing 10mL of 100% normal medium) had no effect on compatibility.

Opposite to Clancy and Hoffmann (1998), Sinkins *et al.* (1995b) claimed that incompatibility levels and transmission fidelity were found to be higher when a double-infected line of *D. simulans* was raised at low larval density (100 eggs added to 25ml medium) compared to a high density (400 eggs added to 5ml medium).

Kambhampati *et al.* (1993) showed that tetracycline treatment of *Aedes albopictus* resulted a partial, but significant, restoration of compatibility. The inability of tetracycline treatment to restore compatibility was also reported by O'Neill (1989) where antibiotic treatment in one strain of *Tribolium confusum* was able to eliminate *Wolbachia,* whereas an identical treatment of another strain was unsuccessful removing the symbiont. It was suggested that the ineffectiveness of tetracycline in *Aedes albopictus* might due to of resistance by the symbionts to one or more antibiotics or to the detoxification of the antibiotic by the host before it reaches the symbionts.

Breeuwer and Werren (1993) reported that tetracycline treatment (1mg/ml in 10% sucrose) of female *Nasonia vitripennis* had a dramatic effect on bacterial densities in their eggs. On the first day after treatment tetracycline had effectively eliminated cytoplasmic bacteria and caused reduced or absent the microorganism in the eggs. Werren and Jaenike (1995) reported that in *Drosophila recens Wolbachia* were 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.*

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 *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* bacteria and fungi *Penicillium* can cure *Tribolium* flour beetles of infection with *Wolbachia.*

Stouthamer *et al.* (1990) investigated *Trichogramma* harbouring parthenogenesis-inducing *Wolbachia.* When cured by antibiotics tetracycline hydrochloride, sulfamethoxazole or rifampicin (100mg/ml honey) the result was the appearance of male offspring because the microorganisms responsible for thelytoky were killed. However, treated with gentamycin, penicillin G and erythromycin, male offspring did not occur. When reared at an elevated temperature >30°C, the females produced male offspring as well. Stouthamer (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. Fujii, *et al.* (2001) reported feminisation-inducing *Wolbachia* in *Ephestia kuehniella.* Those cured by tetracycline produced male offspring. Min and Benzer (1997) reported that treatment with 0.25mg/ml of tetracycline eliminated the bacteria, restoring normal life span of *Drosophila melanogaster* infected with 'popcorn-effect' *Wolbachia.*

Previous experiments showed that the expression of cytoplasmic incompatibility, parthenogenesis and feminisation in many hosts of *Wolbachia* is influenced by environmental factors such as temperature. Therefore, it might also affect *Wolbachia* inducing 'popcorn effect'. To determine the possible role of the environment on the 'popcorn-effect' inducing *Wolbachia*, *Drosophila melanogaster* infected with *Wolbachia* 'popcorn-effect' will be cured with 0.25mg/ml tetracycline (as in the previous experiment by Min and Benzer, 1997) and reared at 20°C 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.

4.2 AIM

To determine the effects of tetracycline treatment and temperature on the life span of *D. melanogaster* harbouring the 'popcorn-effect' inducing strain of *Wolbachia.*

4.3 MATERIALS AND METHODS

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 0.25mg/ml of tetracycline in the media. The strain was treated for three generation and tested for infection status with PCR (See chapter 2) utilizing 81F and 691F 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 (2.3cm diameter and 9.5cm in length) with 10

replicates. To examine the effect of temperature, flies were reared in different temperature such as at 20°C and 29°C.

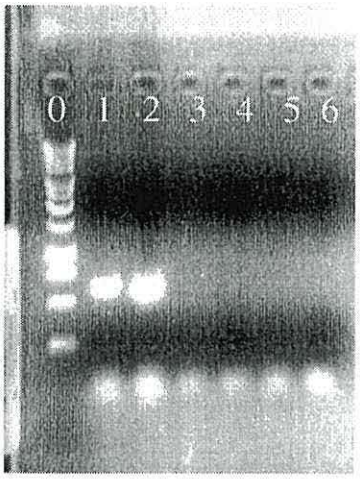
Statistical analysis

An effect of tetracycline and temperature in *Drosophila 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.

4.4 RESULTS

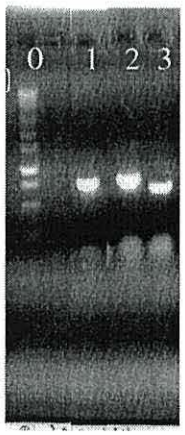
The effect of tetracycline on the *Wolbachia* inducing 'popcorn-effect' was analysis by PCR and the results are shown in Fig. 4-1. It shows the gel electrophoresis of untreated and treated *Drosophila melanogaster* infected with *Wolbachia* inducing 'popcorn-effect'. Lane 1 and 2 are samples from untreated. The band indicates that these were positive for *Wolbachia.* However, in lanes 3, 4, 5 and 6 are samples from treated, where no band is visible. This indicates that there were no *Wolbachia* any longer.

Fig. 4-1 Gel electrophoresis of *Drosophila melanogaster* (W^{1118}) untreated and treated with tetracycline .



0: 1 Kilobase ladder
1 and 2: *Drosophila melanogaster* (W^{1118}) untreated
3, 4, 5, 6: *Drosophila melanogaster* (W^{1118}) treated with tetracycline *wsp* fragment of *Wolbachia.*

Fig. 4-2 Gel electrophoresis of *Drosophila melanogaster* (W^{1118}) untreated with tetracycline at 29°C and 20°C.



0: 1 Kilobase ladder
1: *Drosophila melanogaster* infected with 'popcorn'effect maintained at 29°C
2: *Drosophila melanogaster* infected with 'popcorn'effect maintained at 20°C
line 1 and 2: *wsp* fragment of *Wolbachia*
3: *Drosophila melanogaster* infected with 'popcorn'effect at 20°C ribosomal of nucleus

Fig. 4-3 Percentage of adult survivors of *Drosophila melanogaster* (w^{1118}) infected with the 'popcorn-effect' inducing *Wolbachia* when untreated and treated with tetracycline and maintained at 29°C.

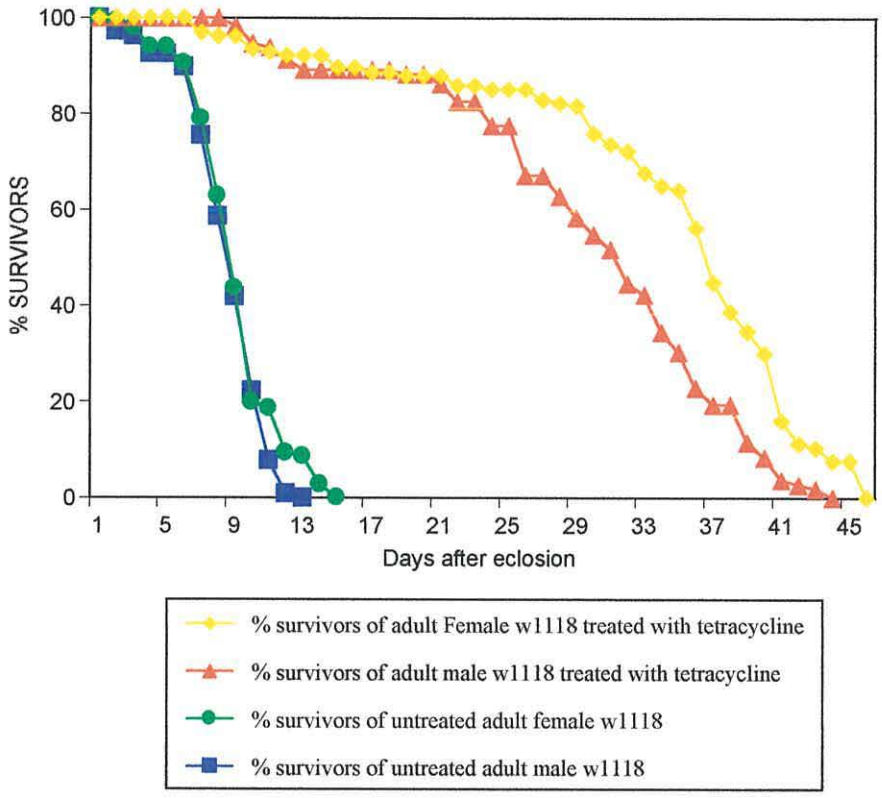


Figure 4-3 shows the life span of *Drosophila melanogaster* infected with *Wolbachia* 'popcorn-effect' untreated and treated with tetracycline at 29°C. The life span of treated *Drosophila melanogaster* is longer than that of untreated.

Fig. 4-4 Percentage of adult survivors of *Drosophila melanogaster* (w^{1118}) infected with the 'popcorn-effect' inducing *Wolbachia* when untreated and treated with tetracycline and maintained at 20°C

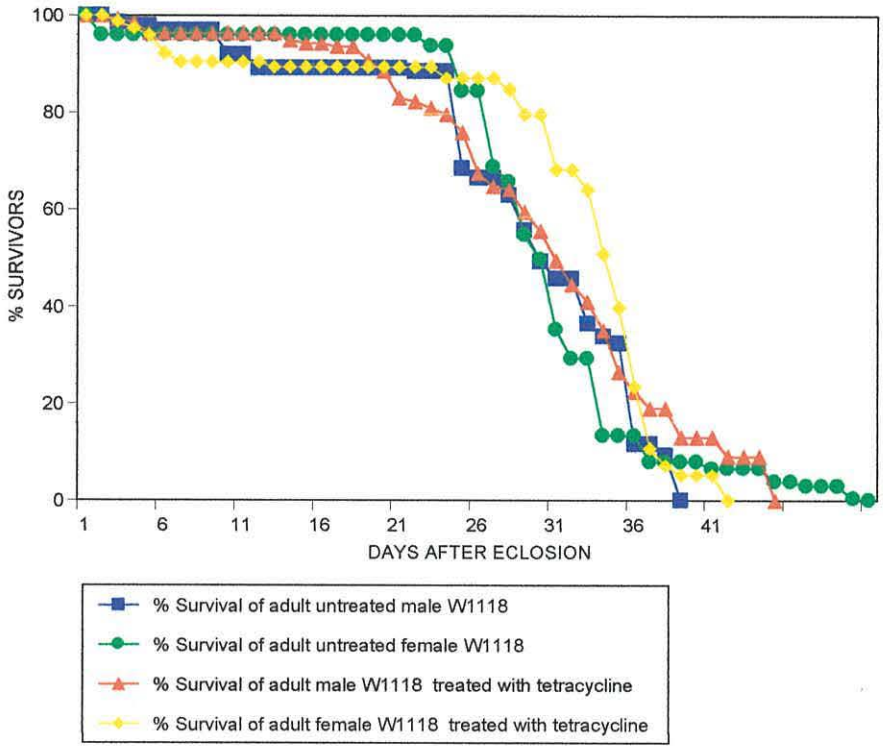


Figure 4-4 shows the life span of *Drosophila melanogaster* infected with *Wolbachia* 'popcorn-effect' untreated and treated tetracycline at 20°C. This figure apparently shows that there is no difference in the life span of *Drosophila melanogaster* between untreated and treated. Unlike the life span of *Drosophila melanogaster* at 29°C where infected flies have a shorter life span than treated flies. Therefore, there is no 'popcorn-effect' in the *Drosophila melanogaster* at low temperature (20°C).

Table 4-1 Data of life span (days) of *Drosophila melanogaster* w^{1118} (untreated and treated with tetracycline) at 29°C and 20°C belonging to Fig. 4-3 and 4-4.

No	29°C				20°C			
Replication	*Male* W^{1118} untreated	Female W^{1118} untreated	*Male* W^{1118} treated	Female W^{1118} treated	*Male* W^{1118} untreated	Female W^{1118} untreated	*Male* W^{1118} treated	Female W^{1118} treated
1	11	11	31	44	38	51	34	37
2	11	10	44	38	33	37	42	31
3	10	13	45	40	38	32	36	36
4	10	8	45	47	37	30	21	31
5	12	8	41	46	30	27	35	38
6	10	8	44	49	39	34	45	36
7	9	14	45	42	31	34	35	42
8	12	11	43	48	26	32	39	37
9	10	9	32	45	31	27	29	36
10	7	9	43	46	25	25	26	34

Table 4-2 Univariate Analysis of Variance of data in Table 4-1

Descriptive Statistics

Dependent Variable: Logarithm

Treatment	Temperature	Sex	Mean	Std. deviation	N
Untreated	20°C	Male	1.5110	0.06922	10
		Female	1.5087	0.08780	10
		Total	1.5098	0.07696	20
	29°C	Male	1.0040	0.06822	10
		Female	0.9961	0.08817	10
		Total	1.0001	0.07684	20
	Total	Male	1.2575	0.26853	20
		Female	1.2524	0.27656	20
		Total	1.2550	0.26907	40
Treated	20°C	Male	1.5243	0.09964	10
		Female	1.5523	0.03973	10
		Total	1.5383	0.07520	20
	29°C	Male	1.6123	0.06143	10
		Female	1.6471	0.03546	10
		Total	1.6297	0.05198	20
	Total	Male	1.5683	0.09234	20
		Female	1.5997	0.06091	20
		Total	1.5840	0.07883	40
Total	20°C	Male	1.5177	0.08378	20
		Female	1.5305	0.06999	20
		Total	1.5241	0.07647	40
	29°C	Male	1.3082	0.31835	20
		Female	1.3216	0.34031	20
		Total	1.3149	0.32533	40
	Total	Male	1.4129	0.25307	40
		Female	1.4260	0.26457	40
		Total	1.4195	0.25733	80

F - Test of Between-Subject Effects

Dependent Variable: Logarithm

Source	Type III Sum of Square	df	Mean Square	F	Sig.
Corrected	4.858[a]	7	0.694	133.890	< 0.001
Intercept	161.190	1	161.190	31098.134	< 0.001
Treatment	2.165	1	2.165	417.727	< 0.001
Temperature	0.875	1	0.875	168.861	< 0.001
Sex	3.437E-03	1	3.437E-03	0.663	0.418
Treatment*Temperature	1.807	1	1.807	348.651	< 0.001
Treatment*Sex	6.674E-03	1	6.674E-03	1.288	0.260
Temperature*Sex	1.780E-06	1	1.780E-06	0.000	0.985
Treatment*Temperature*Sex	1.957E-04	1	1.957E-04	0.038	0.846
Error	0.373	72	5.183E-03		
Total	166.422	80			
Corrected total	5.231	79			

The analysis above shows highly significant difference (< 0.001) between treatment (treated and untreated) vs temperature (29°C and 20°C). However, there is no significant difference between treatment and sex (male and female). The detailed calculation of individual significance is as below:

Calculation of the threshold of significant

Treatment	Temperature	
	20°C	29°C
Untreated	1.51	1.00
Treated	1.54	1.63

N = 20
Error MS = 0.005183
Mean of logs
Log x = 1
X = 10

31.6	10
34.7	42.7

$$\mathbf{Q}\ (4, 72) \times \sqrt{\frac{\text{error MS}}{N}}$$

$$3.73 \times \sqrt{\frac{0.005183}{20}} = 0.06$$

2 means are significant different if they differ by more than 0.06. Therefore, at 20°C untreated and treated are not significant different, at 29°C untreated and treated are significant different, at 29°C untreated are significant different with untreated and treated at 20°C.

4.5. DISCUSSION

When *Drosophila 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. 4-3). The difference in life-span is highly significant (Table 4-2). However, there is no significant difference in sex (male and female). Tetracycline eliminates *Wolbachia* and restores the life-span of male and female flies at 29°C. This is the same temperature for which the 'popcorn-effect' of *Wolbachia* was originally described (Min and Benzer, 1997). Whereas when reared at 20°C (Fig. 4-4) the life span of the flies is no longer significantly different between untreated and treated (Table 4-2), although *Wolbachia* can still be detected when the flies were reared at 20°C (Fig. 4-2). Therefore,

apparently there is no 'popcorn-effect' at 20°C. It is 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 controls the bacteria under optimal physiological condition and *Wolbachia* does 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. In McGraw *et al.* (2002) they 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, naïve 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 is already deadly for uninfected *D. simulans* flies. The authors failed to make any observations at physiological temperatures.

The potential of the 'popcorn-effect' for the manipulation of vector insects has been repeatedly stressed, e.g., Bourtzis and Braig (1999). The ideal would have been 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' is in

effect a laboratory artefact and is not expressed under physiological conditions puts an early end to these hopes.

CHAPTER 5

DENSITY OF *WOLBACHIA* IN *DROSOPHILA MELANOGASTER*

5.1 INTRODUCTION

Drosophila melanogaster is known to harbour *Wolbachia* that cause the early death of its adult host. Min and Benzer (1997) explain that death occurs early as a result of rupturing of the cell caused by the proliferation of *Wolbachia* within it. They note that *Wolbachia* are present in low numbers through development in the embryonic, larval and pupal stages but that, as soon as the adults emerge, the bacteria start to multiply rapidly. However, the number of *Wolbachia* causing the 'popcorn-effect' in *D. melanogaster* is unknown. Until recently there has been no *in vitro* system for culturing *Wolbachia* within insect cells (O'Neill *et al.,* 1997). The fastidious nature of *Wolbachia,* together with the difficulty in counting bacteria numbers in tissue 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 measurement. Using real-time PCR, I studied *Wolbachia* in order to determine whether the number of *Wolbachia* is related to the early death of its host. The crucial question in this case is whether *Wolbachia* increases linear or geometrically. If *Wolbachia* is able to freely reproduce, I would expect a geometrical increase in *Wolbachia* number. If there is a linear increase in *Wolbachia* number, then there are either strong constrains by the host or there is a *Wolbachia* population in which statistical run-aways ultimately dictate the physiological outcome

(not knowing exactly when and how many will change behaviour). The link between control of the intracellular *Wolbachia* population and the physiological or pathological consequences for the host might be so tight that the - in principal random - lack of response by a single *Wolbachia* cell to host control can eventually lead to a catastrophic sequence of events. This means that the outcome of such an interaction is statistical in nature. The increase in the density of *Wolbachia* from the egg to the adult fly may also be attributed to the increase in their total volume per cell. There are two larval moults in *D. melanogaster*. During each moult both the internal organs and the body wall grow. Internal organs (such as malpighian tubules, muscle, fat body and intestine) grow by an increase in cell size, the number of cells in the organ remaining constant. In contrast, imaginal discs grow by cell multiplication. Therefore, cell division occurs mainly between immature stages and not in the adult fly where the fly is practically post mitotic.

Wolbachia density has been examined using microscopy combined with fluorescent staining (Bressac and Rousset, 1993), dot blots of nuclei acid and Western blots of protein (Bourtzis and O'Neill, 1998; Rousset *et al.,* 1999). However, there is a weaknesses to these methods, as microscopy it is not able to measure the number of *Wolbachia* in the whole body of an insect. Therefore, a more accurate and easier detection method detection method for *Wolbachia,* nuclei and mitochondria would provide a more accurate and faster analysis of the relationship between bacterial density and the 'popcorn-effect', between the number of bacteria and the number of nuclei and between cells of the host

and the number of mitochondria. A more accurate method is using the real-time PCR. Real-time PCR has the ability to measure the concentrations of nucleic acid over a vast dynamic range, is highly sensitive and has the capacity to process many samples simultaneously. Unlike traditional 'endpoint' measurement of PCR products, real-time PCR provides immediate information about the kinetics of the PCR. It also provides accuracy and produces reliable as well rapid quantification of results (Pfaffl, 2001). In real-time PCR, a fluorescent reporter is used to monitor the PCR reaction as it occurs. The simplest detection technique for newly synthesised PCR product in real-time PCR uses SYBR Green I fluorescence dye that binds specifically to the minor groove of double-stranded DNA (Morrison *et al.,* 1998). The incorporation of the fluorescent dye during each round of amplification allows for the rapid detection and quantification of DNA without the need for post-PCR processing, such as gel electrophoresis or radioactive hybridization (Heid *et al.,*1996).

In real-time PCR threshold cycles (Cr), in which the fluorescence begins to increase from the back ground level, are measured. The number of molecules in the samples is determined from the threshold cycle (Cr) on the basis of a standard curve. There is a linear relationship between the log of the starting amount of template and the corresponding threshold cycle during real-time PCR (Sambrook and Russell, 2001). 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 number. This standard curve can then be used to

determine the starting amount for each unknown template, based on its threshold cycle number.

The quantification of *Wolbachia* in insecticide-resistant mosquitoes, *Culex pipiens,* using real-time quantitative PCR has been reported by Berticat *et al.* (2002) and the results show that all resistant strains exhibit a significantly higher *Wolbachia* density than the susceptible strains. However, *Wolbachia* density did not differ between the resistant strains. These results are the same for mosquitoes reared in the laboratory and mosquitoes collected from the field.

Noda *et al.* (2001a) reported the detection of *Wolbachia* using real-time quantitative PCR (RTQ-PCR) in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera* for three time points adult females (0, 4 and 9 days) and for four time points of adult males (0, 4, 9 and 14 days). The number of *Wolbachia* in the females is higher than in the males of both species. However, in the males of *S. furcifera wolbachia* decreased from $6.2x10^5$ at 0 days old to $3.3x10^5$ at 14 days old and was less than one tenth of that in the males of *L. striatellus.*

There are several steps to working with real-time PCR. Firstly, primers need to be designed to provide a product of less than 200bp. Sequence is needed to design these primers. I cloned and sequenced a fragment of the *wsp* gene of *Wolbachia,* the 16S gene of mitochondria and the Na^+ pump α subunit of nuclei. I used these genes because they are present as single copies in the

genome (Braig, 1998, Farrel cited in Lebovitz, *et* al., 1989 and Bensaadi-Merchermek *et al.*, 1995).

5.2 AIM

To determine the copy number of *Wolbachia,* nuclei and mitochondria per single organism.

5.3 MATERIALS AND METHODS

DNA extraction from *D. melanogaster*

25 whole flies or 50 whole larvae or 100 eggs were homogenized by hand in a 1.5mL microcentrifuge tube with a sterile polypropylene pestle in 400µl of homogenizing buffer (100ml of 1M tris-HCL, pH 9.0; 200ml of 0.5M EDTA, pH 8.0; 100ml of 10% SDS and 600ml water to make a total volume of 1 litre). After homogenization they were put in a heat block for 30 minutes at 65°C. After heating, 68µl potassium acetate (8M) was added and the liquid mixed. It was placed on ice for 30 minutes and then centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred into a new 1.5ml microcentrifuge.

200µl isopropanol was added and mixed by inversion of the tube. It was allowed to stand at room temperature for 5 minutes and was then centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded. The pellet containing DNA was vacuum/air-dried and the DNA dissolved in 200µl of TE (Tris-EDTA) pH 7.4. It was extracted with 1 volume of Phenol:chloroform:isoamyl alcohol (25:24:1). 1/3 volume of 7.5M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol were added to the aqueous phase and mixed by inversion

of the tube. It was placed on dry ice for at least 10 minutes and centrifuged at 12,000 rpm for 10 minutes. The supernatant was decanted and the pellet washed by addition of 400µl of 70% ethanol. It was then recentrifuged at 12,000 rpm for 5 minutes and the supernatant discarded. The DNA pellet was vacuum-dried and the DNA dissolved in 100µl TE (pH 7.4).

Materials and methods for cloning, restriction enzyme digestion and sequencing see Appendix.

Design of primer

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, is the length of amplicon (< 200 bp). The reason for using the short product is that a straight-line relationship of starting amount versus threshold cycle should be obtained. 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 percentage GC value within primer sets. *Wsp* was generally used as the primer for determining *Wolbachia* bacterial number, 16S for determining mitochondria number and the Na^+ pump α subunit for determining nuclei number.

DNA standards used for determining the density of bacteria, mitochondria and nuclei using real-time PCR

The standards used were the *wsp* plasmid for *Wolbachia* numbers, the 16S plasmid for mitochondria numbers and the sodium pump α subunit plasmid for nuclei numbers. A stock of plasmids was measured by Pico Green. 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 25pg/ml of dsDNA (50pg dsDNA in a 2 ml assay volume) with a standard spectrofluorometer.

Measurement of dsDNA molecules of the plasmid using Pico Green:

The number of plasmid standard in real time PCR was calculated using Pico Green dsDNA quantification reagent (Molecular Probe). The following procedures was followed:
The standard range used was: 100, 50, 25, 12.5 and 3.125ng. 5µl standard was diluted in 1 x TE to a final volume of 500µl and 100µl were used in every well of the plate. Samples were diluted 1 in 400 and 100µl was used in each well. The Dye was diluted 200x in TE, a 100µl was used. The plate was incubated for 2-5 minutes in darkness. The intensity was read in the fluorescent spectrometer (Vector).

The standard dilution started at starts 10^7 copies/µl for *wsp*, 16S and sodium pump α subunit plasmids. Standard graphs were prepared from data at the same time as the test samples.

A 10-fold dilutions series were prepared from 10^7 to 10^5 copies/µl for *wsp* and 10^7 to 10^4 copies/µl for 16S and sodium pump α

subunit plasmid. Then 5µl of each different dilution plasmid was added to one of the skirted tubes containing master mix.

*i*Cycler real-time PCR

The initial concentration of target sequences can be expressed as the fractional cycler number (C_r) required to achieve a present threshold of amplification. A plot of (C_r) against the log $_{10}$ of the initial copy number of a set of standard DNAs yields a straight line. The target sequences in an unknown sample may be easily quantified by interpolation into this standard curve. Unlike other forms of quantitative PCR, internal standards are not required in real-time PCR. The ability to quantify the amplified DNA during the exponential phase of the PCR, when none of the components of the reaction are limiting, results in an improved precision in the quantitation of target sequences.

Real-time PCR amplification efficiencies

Real-time PCR efficiencies were calculated from the given slopes in the *i*Cycler. The corresponding real-time PCR efficiencies (E) of one cycle in the exponential phase was calculated according to the equation $E = 10^{(-1/slope)} - 1$.

For the *i*Cycler reaction, a master mix of the following reaction components was prepared: The new *Wolbachia* and nucleus primers are: 5'- CCG GTT GAA TTT TTA GGA T – 3' for forward *Wolbachia* primer/*wspe* and the reverse primer is 5' – CTT TGG AAC CCG CTG TGA ATG A – 3'. Whilst primer for nucleus/ *Ne* is: 5' – GAG CGG CCG CCA GTG TGA –and

reverse primer is 5' – GGG TGA GGG TGC CGG TCT TAT C – 3'.

12.5µl PCR Master Mix, 2X (Promega)
0.5µl forward primer (20µM)
0.5µl reverse primer (20µM)
0.5µl SYBR Green 1/dye (1:10,000 dilution)
5µl of template is added to the standard or unknown (for negative control no template is added)
6µl nuclease free water

Finally, 25µl aliquots of each mix were pipetted into the rows of a 96-well thin-well PCR plate. The plate was covered with a piece of optically clear sealing tape and the plate was placed in the *i*Cycler IQ detection system. PCR was carried out at 95°C for 2.3 minutes followed by 40 cycles of 15 secs at 95 °C and 20 secs at 55 °C and 30 secs 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 α subunit and unknowns were investigated [egg, larvae, adult (day 1), adult (day 5), adult (day 8) and adult (day 12)]. Before conducting the experiment, I set up and ran the external well factor plate.

Statistical analysis

The density of *Wolbachia,* and nuclei in *Drosophila melanogaster* infected with *Wolbachia* 'popcorn-effect' in egg, larvae and adult will be analysed by using one way ANOVA. All data are transformed to logarithm before performing one way ANOVA.

5.4 RESULTS

Result of restriction enzyme and sequencing see Appendix.

Design of primers

The regions for the PCR product within the *wsp*, 16S and Na^+ pump α subunit genes were chosen manually.

Design of *wsp* gene primers

Fig. 5-1. Amplification range of the new *wsp* primers

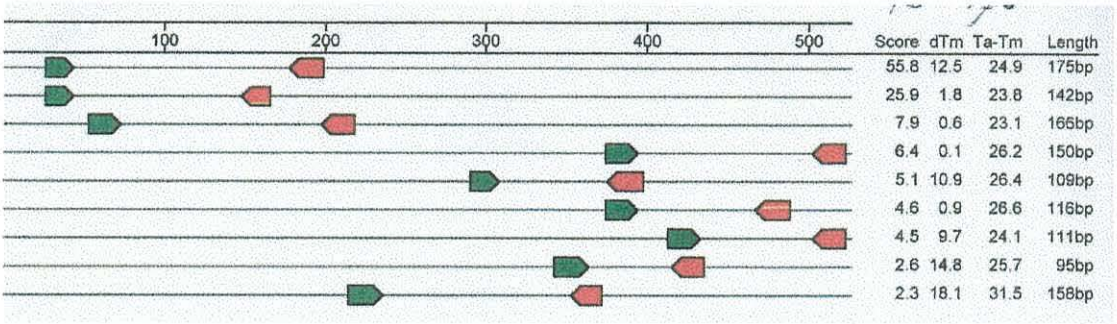


Figure 5-1 shows prospective primer combination and localisation within the gene. The primer pairs are ranked according to their quality. The quality is determined by a unique annealing site, the absence of significant primer dimer pairs within and with the primers themselves and between the primers, the length of the product and a high and matching annealing temperature of the primers. This quality is expressed in the score value which is ideally as high as possible. This figure also lists the score (55.8), the melting temperature difference between the primers (12.5), the difference between annealing and melting temperatures (24.9) and the length of the amplified product (175bp).

Fig.5-2. Summary of the new *wsp* primers

Upper Primer: 19-mer 5' CCGGTTGAATTTTTAGGAT 3' Lower Primer: 22-mer 5' CTTTGGAACCCGCTGTGAATGA 3'		
DNA 250 pM, Salt 50 mM	Upper Primer	Lower Primer
Primer Tm Primer Overall Stability Primer Location	46.1 °C -36.2 kc/m 26..44	58.6 °C -43.4 kc/m 200..179
Product Tm - Primer Tm Primers Tm Difference Optimal Annealing Temperature	24.9 °C 12.5 °C 48.7 °C	
Product Length Product Tm (%GC Method) Product GC Content Product Tm at 6xSSC	175 bp 71.0 °C 36.6% 92.6 °C	

Figure 5-2 shows that the forward primer of *wsp* is 5'- CCG GTT GAA TTT TTA GGA T – 3' (T_m 46.1°C) and that the reverse primer is 5' – CTT TGG AAC CCG CTG TGA ATG A – 3' (T_m 58,6°C).

Fig. 5-3. Dimers pairs of *wsp.*primers

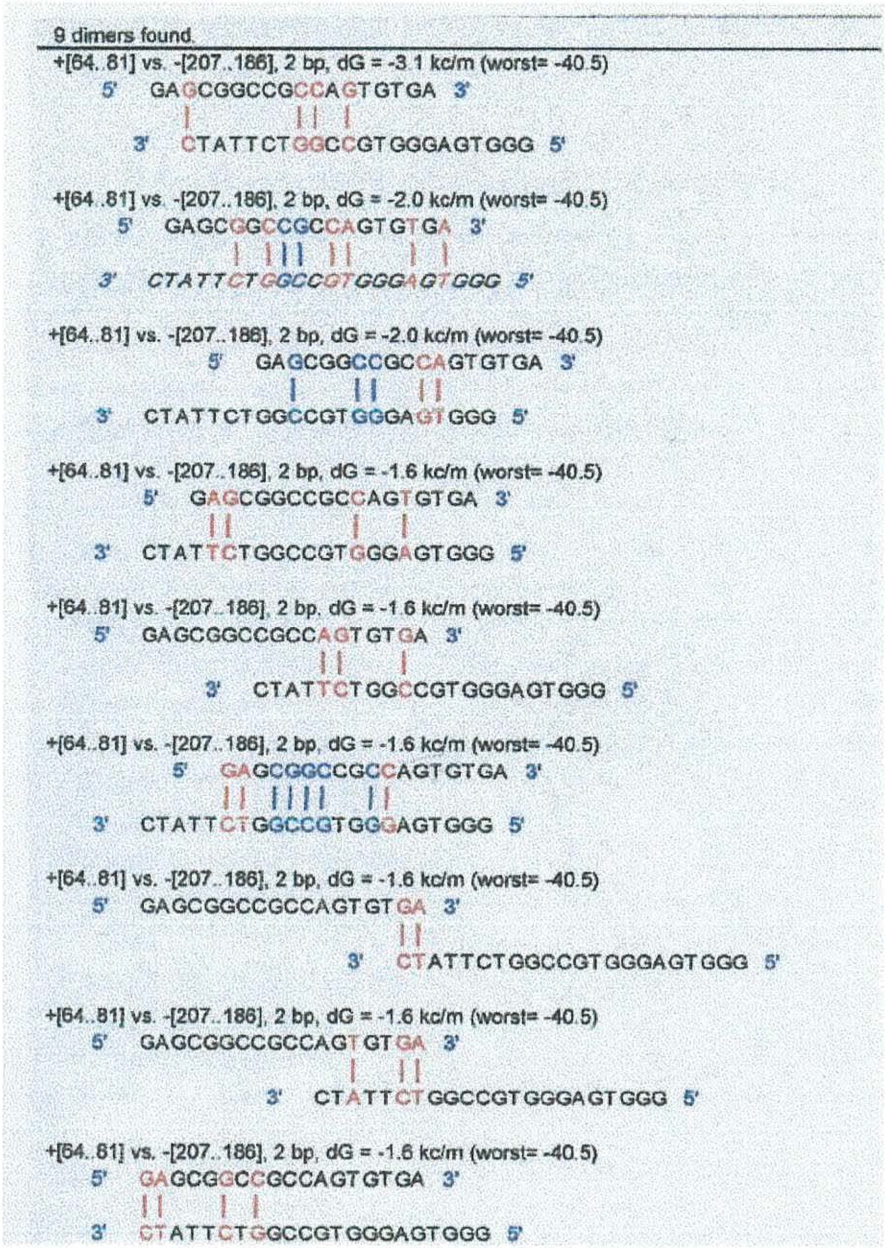
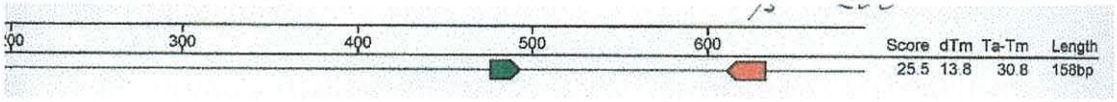


Figure 5-3 shows 9 different hybridization positions that these particular primers can form. Energy of hybridization = (Δ Gibbs free energy). A dimer is ranked according to the hybridization energy with the highest listed first/top, it also calculates the value for the worst case. The program will indicate if the values would interfere with the PCR amplification.

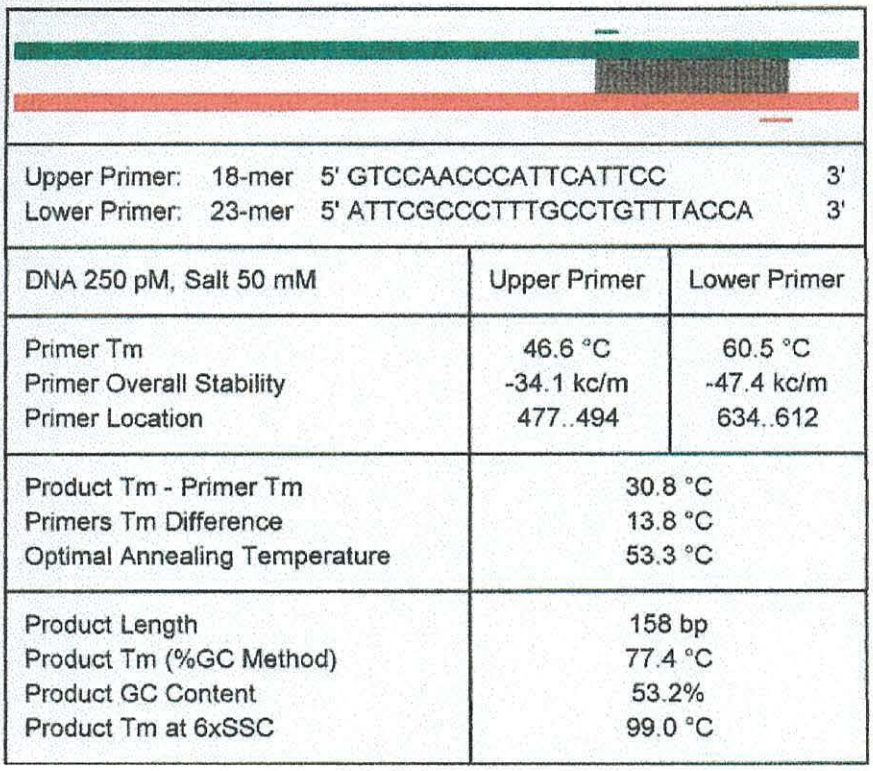
Design of 16S gene primers

Fig. 5-4. Amplification range of the new 16S primers.



Similar to Fig. 5-2, figure 5-4 shows the primers to have a score of 25.5, the melting temperature difference between the primers (13.8), the difference between annealing and melting temperatures (30.8) and the length of the amplified product (158 bp).

Fig. 5-5. Summary of the new 16S primers



Upper Primer: 18-mer 5' GTCCAACCCATTCATTCC 3'
Lower Primer: 23-mer 5' ATTCGCCCTTTGCCTGTTTACCA 3'

DNA 250 pM, Salt 50 mM	Upper Primer	Lower Primer
Primer Tm	46.6 °C	60.5 °C
Primer Overall Stability	-34.1 kc/m	-47.4 kc/m
Primer Location	477..494	634..612
Product Tm - Primer Tm	30.8 °C	
Primers Tm Difference	13.8 °C	
Optimal Annealing Temperature	53.3 °C	
Product Length	158 bp	
Product Tm (%GC Method)	77.4 °C	
Product GC Content	53.2%	
Product Tm at 6xSSC	99.0 °C	

Figure 5-5 shows that the forward primer of 16S is 5' – GTC CAA CCC ATT CAT TCC – 3' (T_m 46.6°C) and that the reverse primer is 5' – ATT CGC CCT TTG CCT GTT TAC CA – 3' (T_m 60.5°C)

Fig. 5-6. Primer dimers of the new 16S primers

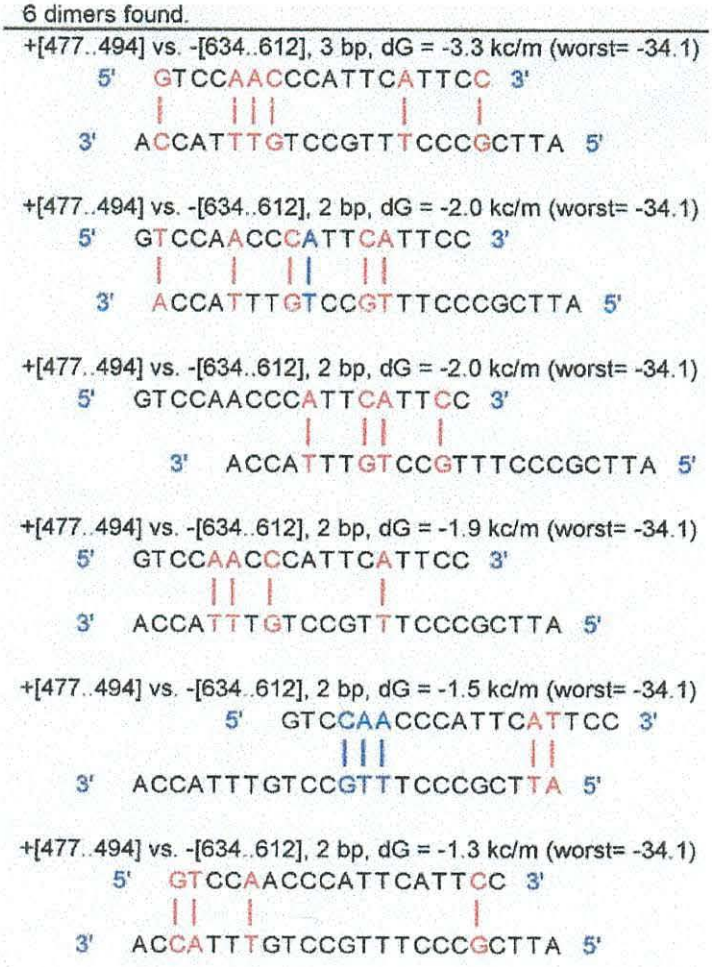
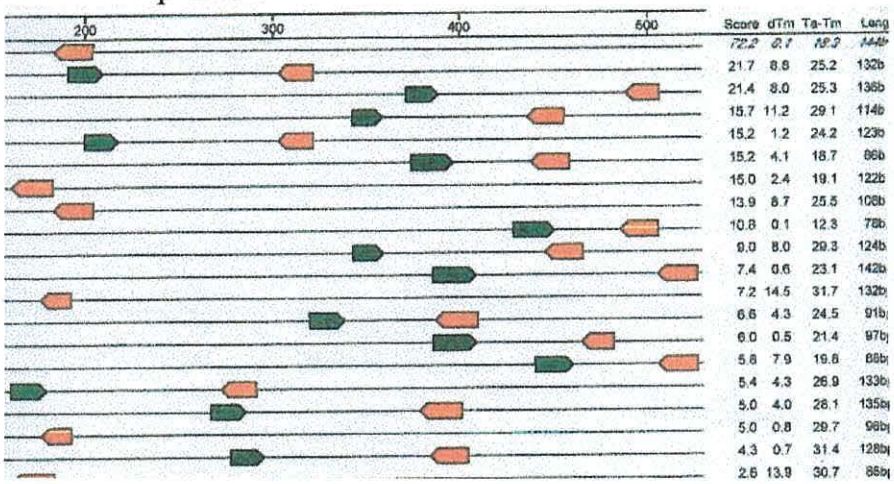


Figure 5-6 shows 6 different hybridization positions that these particular primers can form.

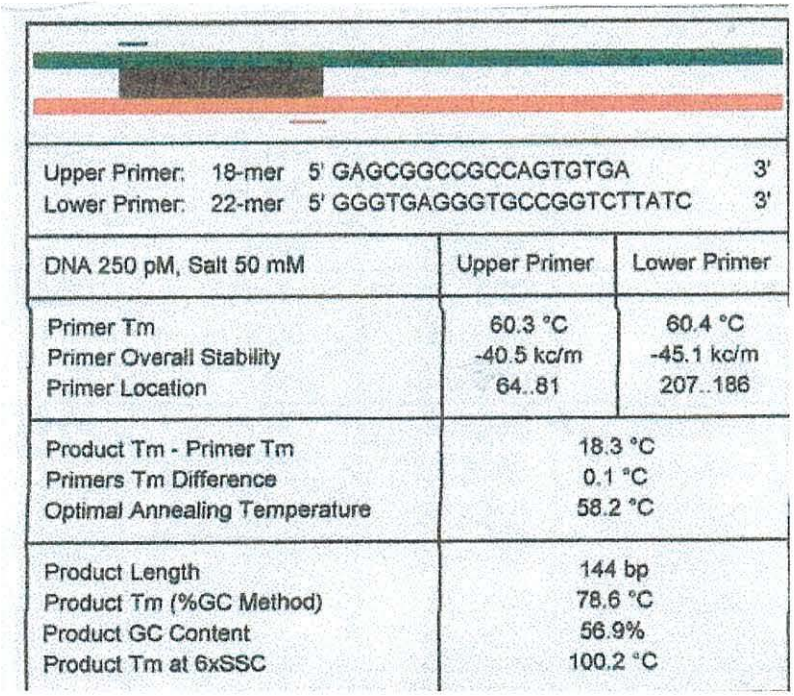
Design of Na^+ pump α sub unit gene primers

Fig. 5-7. Amplification range of the new Na^+ pump α sub unit primers



Similar to fig.5-2, figure 5-7 shows the primers to have a score of 72.2, the melting temperature difference between the primers (0.1), the difference between annealing and melting temperatures (18.3) and length of the primer product (144 bp).

Fig. 5-8. Summary of the new Na^+ pump α sub unit primers.



Upper Primer: 18-mer 5' GAGCGGCCGCCAGTGTGA 3'
Lower Primer: 22-mer 5' GGGTGAGGGTGCCGGTCTTATC 3'

DNA 250 pM, Salt 50 mM	Upper Primer	Lower Primer
Primer Tm	60.3 °C	60.4 °C
Primer Overall Stability	-40.5 kc/m	-45.1 kc/m
Primer Location	64..81	207..186
Product Tm - Primer Tm	18.3 °C	
Primers Tm Difference	0.1 °C	
Optimal Annealing Temperature	58.2 °C	
Product Length	144 bp	
Product Tm (%GC Method)	78.6 °C	
Product GC Content	56.9%	
Product Tm at 6xSSC	100.2 °C	

Figure 5-8 shows that the forward primer of the Na^+ pump α sub unit is 5' – GAG CGG CCG CCA GTG TGA – 3' (T_m 60.3°C) and reverse primer is 5' – GGG TGA GGG TGC CGG TCT TAT C – 3' (T_m 60.4°C)

Fig. 5-9. Primer dimers of the new Na^+ pump α sub unit primers.

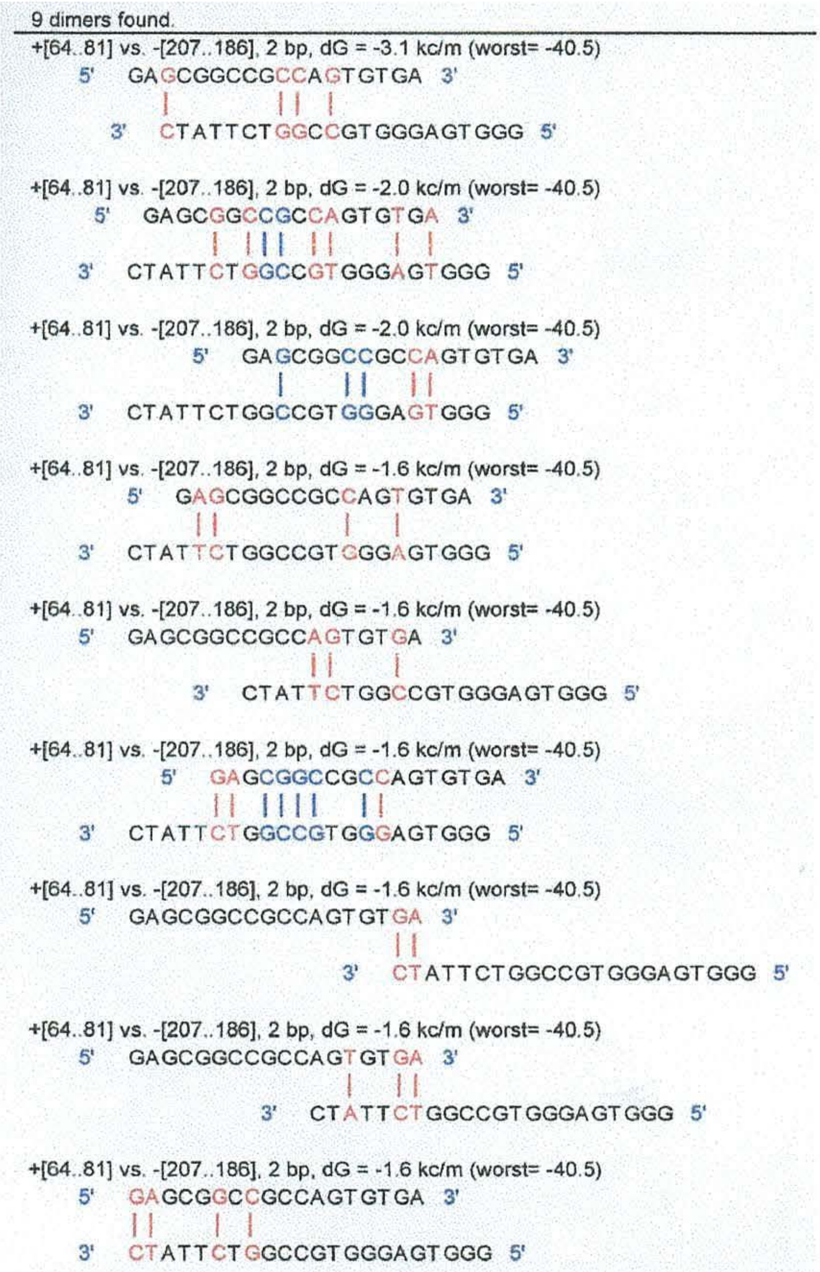


Figure 5-9 shows 9 different hybridization positions that this particular primer can form.

The new primers were named *Wspe, Ne* and *Me*. *Wsp, N* and *M* stand for *Wolbachia*, nuclei and mitochondria respectively, whilst e indicates that the primers are designed by Endang.

Fig. 5-10. Standard curve of dsDNA quantification

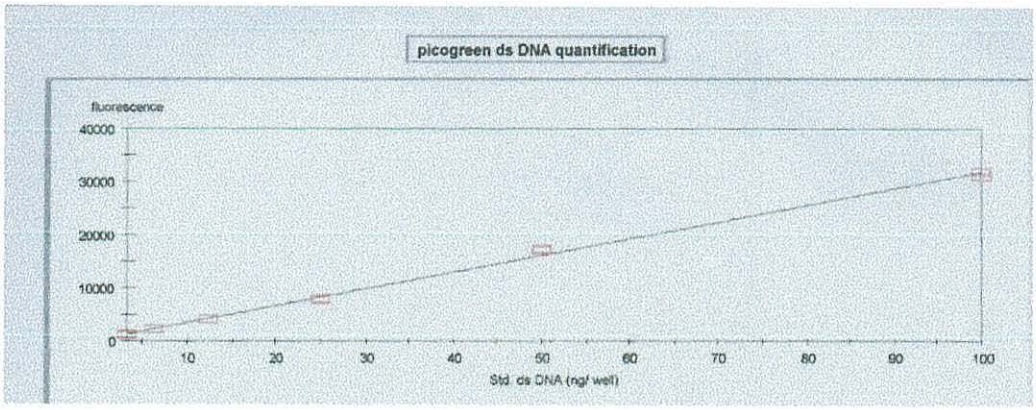
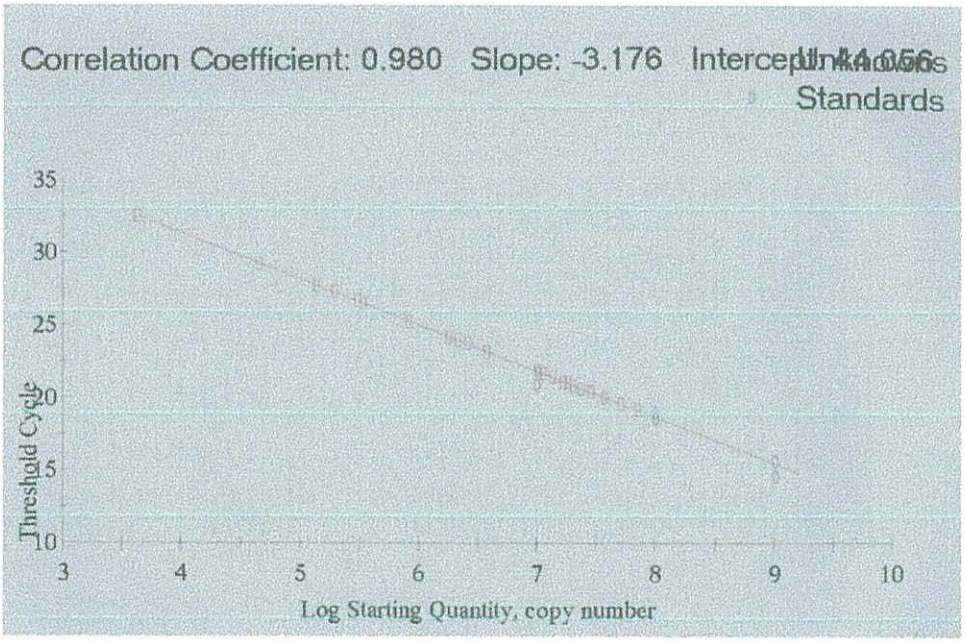


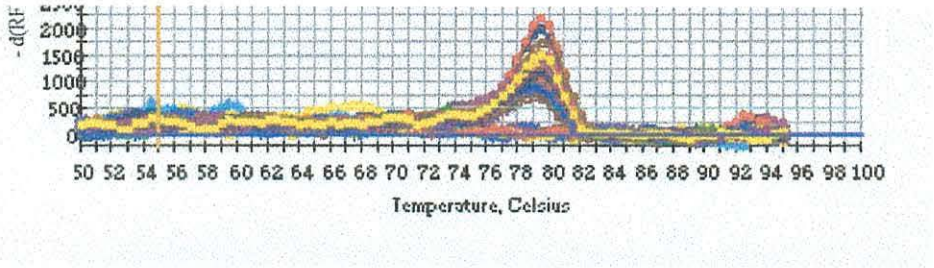
Figure 5-10 is the standard curve of dsDNA quantification using Pico Green.

Fig. 5-11. The standard curve of *Wspe* in real-time PCR.



The standard curve gives a correlation coefficient of 0.980 and slope of – 3.176. The efficiency was calculated using the equation mentioned above and resulted in a high real-time PCR efficiency rate (100%). The correlation coefficient indicates 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.

Fig. 5-12. **Melting curve of *Wspe* product in real-time PCR.**



The curve shows that the melting temperature resulted in one peak of the product at 79°C. This indicates that the melting temperature of all DNA templates both standard and unknown, are the same. It also indicates that the amplified products are homogeneous and reassures that the correct product has been specifically amplified. This is a good melting curve result. The colours differentiate between each template.

Fig. 5-13. Number of *Wolbachia* in *D. melanogaster* infected with 'popcorn-effect' inducing *Wolbachia.* Standard deviation of data are shown

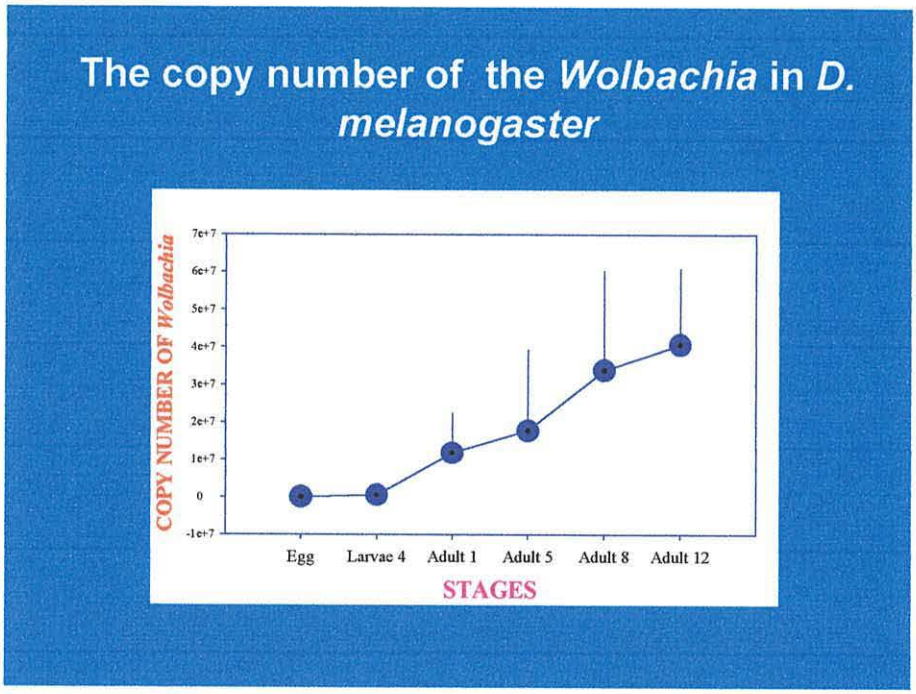


Figure 5-13 is the graph of the copy number of *Wolbachia* in *D. melanogaster* infected with 'popcorn-effect' inducing *Wolbachia.* It shows an increase in the number of *Wolbachia* from eggs, larvae to adults day 1, day 5, day 8 and day 12.

Table. 5-1. *Wolbachia* copy number in *D. melanogaster*

Stages	Copy number R I	Copy number R II	Copy number R III
Eggs	32×10^3	6×10^3	21×10^3
Larvae 4 days	836×10^3	222×10^3	512×10^3
Adults 1 day	24×10^6	5×10^6	6×10^6
Adults 5 days	0.6×10^6	42×10^6	11×10^6
Adults 8 days	64×10^6	13×10^6	25×10^6
Adults 12 days	33×10^6	25×10^6	64×10^6

R = replication

Table 5-2. Descriptive statistics of data of Table 5-1

Stages	N	Mean	Standard Deviation	Standard Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
		log	log	log	log	log	log	log
1	3	4.20	0.36	0.21	3.31	5.10	3.80	4.50
2	3	5.66	0.29	0.17	4.94	6.38	5.35	5.92
3	3	6.96	0.36	0.21	6.06	7.86	6.71	7.38
4	3	6.94	0.64	0.37	5.36	8.51	6.20	7.30
5	3	7.44	0.35	0.20	6.56	8.31	7.10	7.80
6	3	7.58	0.21	0.12	7.07	8.09	7.40	7.80
Total	18	6.46	1.26	0.29	5.84	7.09	3.80	7.80

Table 5-3.Test of homogeneity of variances

Levene Statistic	Df1	Df2	Sig
1.654	5	12	0.220

The variances are homogenous and do not show any significant difference. Therefore one way ANOVA is justified.

Table 5-4. One way Anova of the copy number of *Wolbachia*

	Sum of Square	**df**	**Mean Square (MS)**	**F**	**sig**
Between Group	25.216	5	5.043	33.038	p<0.001
Within Group	1.832	12	0.153		
Total	27.047	17			

Q (6, 12) = 4.25 Q = Q table

Error MS

Within Group Ms = 0.153

n = 3

$$Q \times \sqrt{\frac{\text{error MS}}{n}} = 4.25 \times \sqrt{\frac{0.153}{3}} = 1.073$$

4.20 5.66 6.96 6.94 7.44 7.58

2 means are significant different if their means differ by more than 1.073. Therefore 1 differs with 2, 3, 4, 5 and 6;

2 differs with 3, 4, 5 and 6.

1. The copy number of *Wolbachia* in eggs is significant different with that of larvae day 4.
2. The copy number of *Wolbachia* in larvae day 4 is significant different (< 0.001) with that of adults day 1, day 5, day 8 and day 12.

The analysis shows that the increase in number of *Wolbachia* from egg to adult (day 12) is significant. However, there is no

significant difference in the copy number of *Wolbachia* found in adults (day 1, 5, 8 and 12).

Fig. 5-14. Gel electrophoresis of *Wspe* amplification

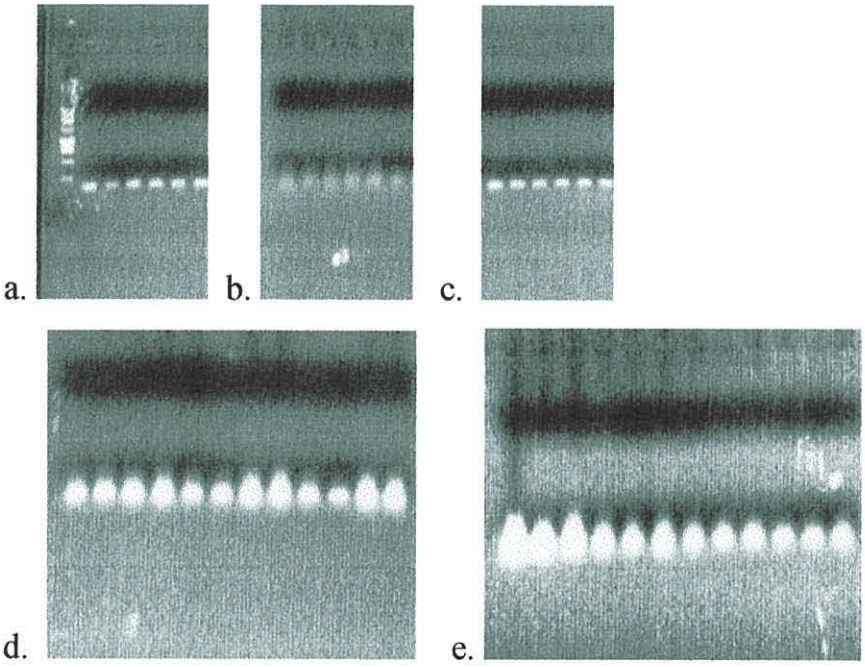
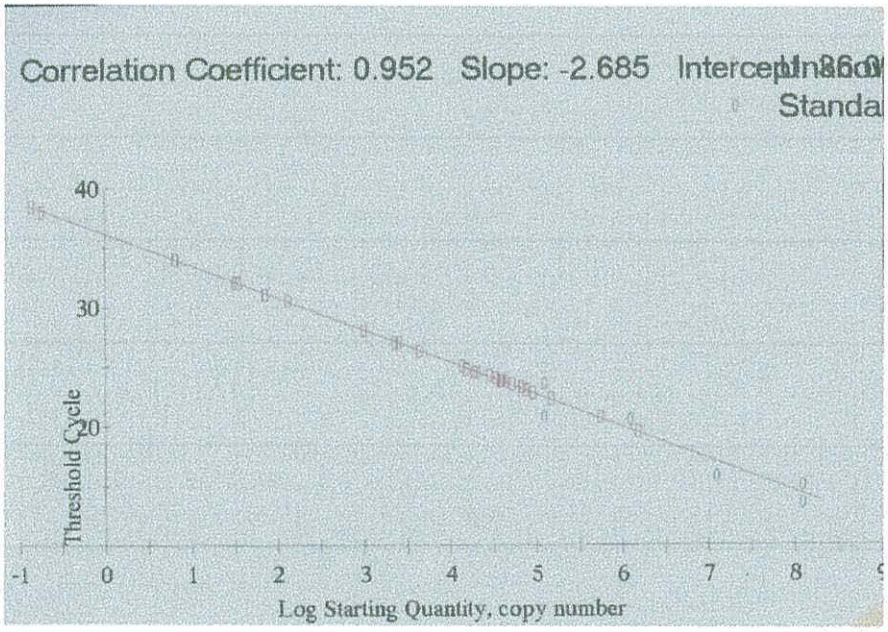


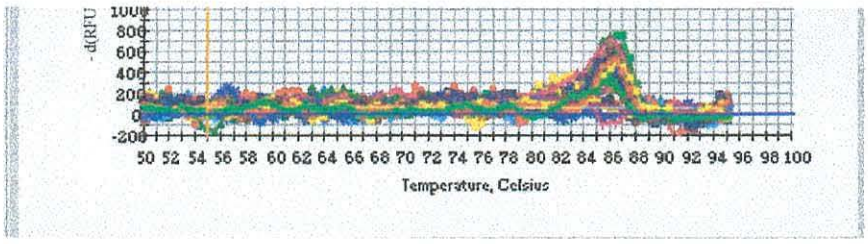
Fig. 5-14. Gel electrophoresis of one kilobase ladder and a, standards; b, egg samples; c, larval samples; d, adults day 1 and days 5 samples and e, adults day 8 and days 12 samples.

Fig. 5-15. The standard curve of the *Ne* primers in real-time PCR



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

Fig. 5-16. Melting Curve of *Ne* product in real-time PCR



The curve shows that the melting temperature was obtained in one peak of the product at 86°C and this indicates that the melting temperature of all DNA templates both standard and unknown, are the same. It indicates that the amplified products are homogeneous and the T_m provides reassurance that the correct product has been specifically amplified.

Fig.5-17. Copy number of nuclei in *Drosophila melanogaster*. Standard deviation of data are shown

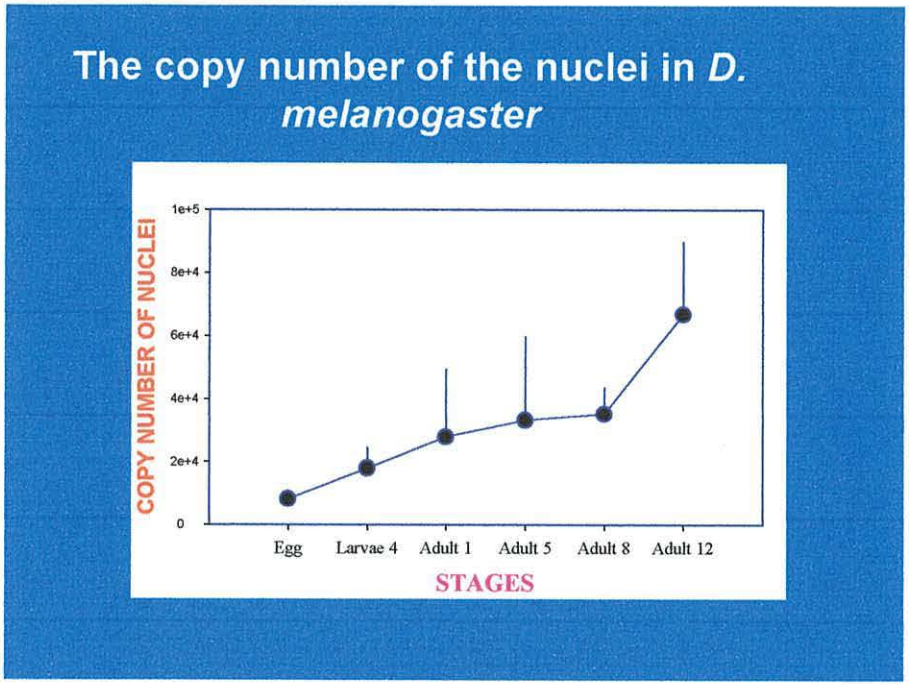


Figure 5-17 is the graph of the number of nuclei in *D. melanogaster* infected with 'popcorn-effect' inducing *Wolbachia.* It shows an increase in the number of nuclei from eggs, larvae to adults day 1, day 5, day 8 and day 12.

Table 5-5. Nuclei copy number in *D. melanogaster*

Stages	Nuclei Copy number R I	Nuclei Copy number R II	Nuclei Copy number R III
Eggs	6×10^3	8×10^3	10×10^3
Larvae 4 days	13×10^3	25×10^3	16×10^3
Adults 1 day	25×10^3	50×10^3	8×10^3
Adults 5 days	16×10^3	20×10^3	64×10^3
Adults 8 days	25×10^3	40×10^3	40×10^3
Adults 12 days	40×10^3	80×10^3	80×10^3

R = replication

Table 5-6. Descriptive statistics of data of Table 5-5

Stages log	N	Mean log	Standard Deviation log	Standard Error log	95% Confidence Interval for Mean		Minimum Log	Maximum log
					Lower Bound log	Upper Bound log		
1	3	3.90	0.10	0.58	3.65	4.15	3.80	4.00
2	3	4.24	0.15	0.09	3.86	4.61	4.10	4.40
3	3	4.34	0.40	0.23	3.33	5.34	3.90	4.70
4	3	4.44	0.32	0.19	3.64	5.24	4.20	4.80
5	3	4.54	0.11	0.07	4.25	4.82	4.40	4.60
6	3	4.80	0.17	0.10	4.37	5.23	4.60	4.90
Total	18	4.37	0.35	0.08	4.20	4.54	3.80	4.90

Table 5-7. Test of homogeneity of variances

Levene Statistic	Df1	Df2	Sig
2.252	5	12	0.116

The variances are homogenous and do not show any significant difference. Therefore one way ANOVA is justified.

Table 5-8. One way ANOVA of the copy number of nuclei

	Sum of Square	df	Mean Square (MS)	F	sig
Between Group	1.376	5	0.275	4.810	0.012
Within Group	0.686	12	0.057		
Total	2.062	17			

Q (6, 12) = 4.25 Q = Table Q.

Error MS
Within Group Ms = 0.057

N = 3

$$Q \times \sqrt{\frac{\text{error MS}}{n}} = 4.25 \times \sqrt{\frac{0.057}{3}} = 0.58$$

3.9 4.0 4.3 4.4 4.5 4.8

2 means are significant different if their means differ by more than 0.58, therefore, 1 differs with 5 and 6.
The copy number of nuclei in eggs is significant different (0.012) with the copy number of nuclei in adults day 8 and 12 and the number of nuclei in the larvae day 4 is significant different with the number of nuclei in adults days 12.

Fig. 5-18. Gel electrophoresis of *Ne* product

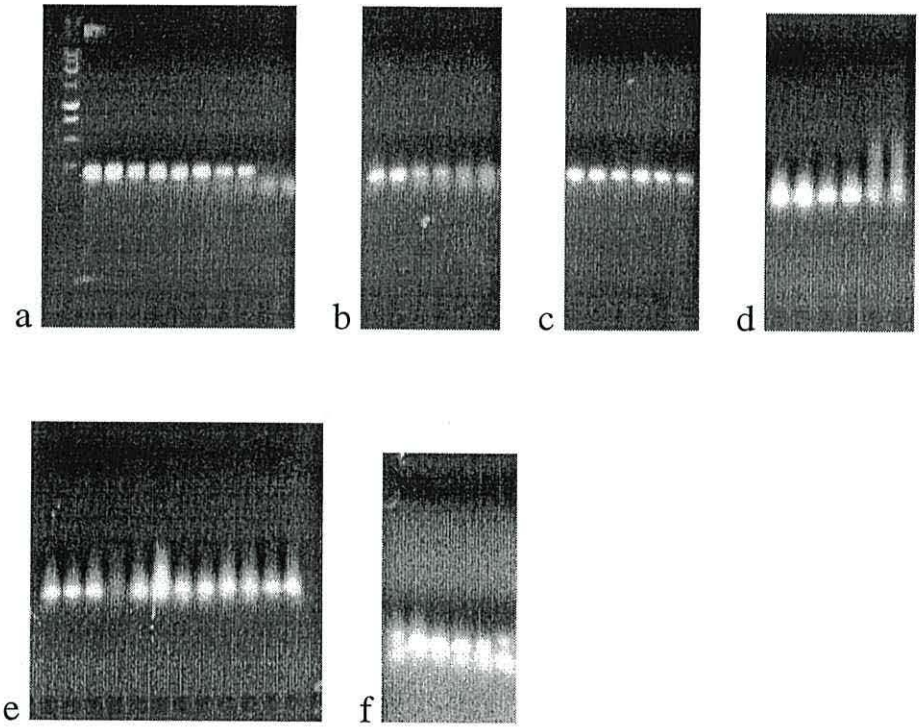


Fig. 5-18 Gel electrophoresis of one kilobase ladder and
a, standards; b, egg; c, of larval day 4; d, adults day 1;
e, adults days 5 and day 8; and f, adults days 12

Due to a lack samples the number of mitochondria could not be determined.

5.5 DISCUSSION

Min and Benzer (1997) discovered that *Wolbachia* infection in *Drosophila melanogaster* was responsible for the early death of the flies. They reported that the bacteria are present in low number during development through the embryonic, larval and pupal stages. When the adult flies emerge the bacteria start to multiply rapidly. However, the number of *Wolbachia* involved is not known. In this study I examined the quantity of *Wolbachia* causing the 'popcorn-effect' in *D. melanogaster* and the number of nuclei present in *D. melanogaster.*

The quantity of *Wolbachia* and nuclei were examined using the real-time PCR. Mean copy number of *Wolbachia* present in eggs was 20×10^3 and 524×10^3 were present in larvae day 4. After the adults emerged, the bacteria reached 12×10^6, 18×10^6, 34×10^6 and 41×10^6 in adults aged 1, 5, 8 and 12 days, respectively. The copy numbers of *Wolbachia* in the egg by confocal microscopy have been reported by Boyle *et al.* (1993). They showed that there were 20×10^3 *Wolbachia* in eggs. My results are similar. The number of *Wolbachia* increases significantly from eggs to larvae to adults. This is similar to the report by Min and Benzer (1997). According to Min and Benzer, as soon as the adult flies emerge the bacteria start to multiply rapidly, causing the sudden death of their host. However, my statistical analysis shows that this is not the case.

The copy number of nuclei was 8×10^3; 18×10^3; 28×10^3; 33×10^3; 35×10^3 and 67×10^3 in eggs, larvae (day 4), adults (day 1),

adults (day 5), adults (day 8), and adults (day 12), respectively. Statistical analysis shows that there is a significant difference between eggs and the adults 8 and 12 and between larvae day 4 to adults 12. I assume that some of cells in the adult fly at day 12 are apoptotic, and therefore slightly higher. Cell division mainly occurs between immature stages and practically will not occur in adults other than the reproductive tissue.

The increase in the copy number of *Wolbachia* is attributed to the increase in the age of the fly and most likely, the increase in volume of the fly's cells, to make it possible for the bacterial cells to multiply. The number of nuclei in eggs is 8×10^3 whilst the number of *Wolbachia* in the egg is 20×10^3 and therefore I can assume that a cell contains at least 2.4 *Wolbachia*. In larvae day 4, the number of nuclei is 12×10^3, whilst the number of *Wolbachia* is 52×10^3 and therefore I can assume that in a cell contains at least 45 *Wolbachia*. Further, the number of *Wolbachia* in a cell of adults day 1, 5, 8 and 12 are 422, 535, 964 and 610, respectively. The differences seen in adult flies are statistically not significant. 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 number of *Wolbachia*.

The quantity/density of *Wolbachia* causing cytoplasmic incompatibility (CI) in *Drosophila simulans* was detected using competitive/quantitative PCR by Sinkins *et al.* (1995a). Comparisons were initially made between three strain of *Drosophila simulans*: DSR (*D. simulans* Riverside), DSCH (*D.*

simulans Coffs harbour) and DSH (*D. simulans* Hawaii) and between three strain of *Aedes albopictus* (Houston, Koh Samui and Mauritius). Results showed that *Wolbachia* density in DSR was highest, this strain showing the strongest expression of CI. *Wolbachia* density in DSCH was found to be intermediate between that of DSR and DSH (the relative densities being 100, 60 and 20 respectively. In this method, the number of *Wolbachia* cannot be measured absolutely, and therefore we do not know the exact number of *Wolbachia*.

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* is low and increased very little during the life span 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). In my results 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.,* 2001a). This is a reasonable as *Wolbachia* causing CI do not rupture the cell. However, *Wolbachia* in *D. melanogaster* cause the 'popcorn-effect' in which cells rupture as a result of massive multiplication of bacteria within them.

Conclusions

Transfecting the naïve host, *Stomoxys calcitrans*, with 'popcorn-effect' *Wolbachia* from *Drosophila melanogaster* has lead to new insights regarding the possible dynamics of horizontal transmission of *Wolbachia.* Somatic replication of *Wolbachia* in its new host was unrestricted. However, access to the germ-line cells of the ovaries and replication of *Wolbachia* in these cells were not adequate to secure stable vertical transmission and infection of the population or species. While these limitations to the replication of *Wolbachia* in ovarial cells might initially be interpreted as 'the' bottleneck to new host-parasite associations, it also raises the opposite question whether *Stomoxys calcitrans* rather might represent the outcome of a successful elimination of *Wolbachia* and the first example of host 'immunity' against *Wolbachia.* The data showing somatic tissues fully supporting *Wolbachia* replication and maintenance advocate this view of a past co-evolution of *Wolbachia* and *Stomoxys.* It indeed suggests a new approach and technique to investigate the evolutionary past of *Wolbachia.*

On the level of cellular biology, this selection pressure must have resulted in various molecular differentiations in both *Wolbachia* and germ cells. Structural features of the germ cells, especially mechanisms responsible for intracellular movement and localisation of vacuoles might have been shaped to a much greater extend by intracellular parasites such as *Wolbachia* than previously suspected. Molecular comparison of different *Wolbachia* strains might identify proteins responsible for these host interactions which then might be used as a bait to identify their host counter parts.

The work on the cellular localisation of 'popcorn-effect' *Wolbachia* in *Drosophila melanogaster* corroborates this line of approach. 'Popcorn-effect' *Wolbachia* still can rescue cytoplasmic incompatibility but can no longer induce it. *Wolbachia* are absent from developing sperm cysts. This suggests that the first response of the host is to overcome the phenotype induced by its endosymbiont, then its transovarial transmission. This, again, seems to be accomplished by excluding it from germ cells in the testes. This exclusion or rather displacement, most likely, happens later during sexual maturation of the male flies. On an evolutionary scale, this might be followed by displacement and finally exclusion from the germ cells in both sexes at increasingly earlier stages of development until *Wolbachia* no longer might be able to follow the pole nuclei during pole cell formation. This hypothesis also proposes the most important host factor for establishing stable infections. Whatever cellular mechanism leads pole nuclei to their destination during early embryogenesis is likely to be hijacked by *Wolbachia* as well.

The 'popcorn-effect' has been identified as a temperature sensitive trait of the 'popcorn-effect' *Wolbachia* strain. This establishes this strain also as the first temperature sensitive mutant of *Wolbachia* and as such as the first genetic tool for investigating the intracellular interaction of this reproductive parasite and its host.

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APPENDIX

Cloning and sequencing methods

For cloning, the TOPO Cloning Kit was used. It is a faster method compared to others. Until now, filamentation temperature sensitive mutant Z, *ftsZ* and *wsp* genes have not been sequenced for the 'popcorn-effect' inducing strain of *Wolbachia* in *Drosophila melanogaster.*

For *wsp*, the forward and reverse primers are: *wsp* 81F (5' TGG TCC AAT AAG TGA TGA AGA AAC 3') and *wsp* 691R (5' AAA AAT TAA ACG CTA CTC CA 3'). For 16S, the forward and reverse primers are: 16Sb (5' CCG GTC TGA ACT CAG ATC ACG T 3') and 16 Sal (5' CGC CTG TTT ACC AAA AAC ATT T 3') and for Na^+ pump α subunit, the forward and reverse primers are: S 1124 (5' AG CGT ATG GC (C/A) TC (A/G) AAG AAC TG 3') and A 1669 (5' AG(C/T) TCC ATG TAG GCA TTG TTG A 3'). Primers amplify 610 bp, 511 bp, and 545 bp, respectively.

For *ftsZ*, the forward and reverse primers are ZWF 70 (5' GTT GTC GCA AAT ACG GAT GC 3') and ZWR 1184 (5' CTT AAG TAA GCT GGT ATA TC 3'), the forward and reverse primers are ZHF (5' CCG TAT GCC GAT TGC AGA GCT TG 3') and ZHR (5' GCC ATG AGT ATT CAC TTG GCT 3'). Primers amplify 1114 bp and ± 816 bp, respectively.

PCR was performed according to chapter 2. Analysis of 10µl of each sample by agarose gel electrophoresis was carried out. If a bright band was detected, then cloning followed. PCR products from *D. melanogaster* with bright bands were directly cloned into

a TA cloning vector Kit (Invitrogen) 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), according to outlined specifications

Cloning

10µl of each PCR product was analysed by agarose gel electrophoresis. 6µl of TOPO Cloning Reagent was prepared containing sterile water (1µl) and TOPO Vector (1µl). Fresh PCR product (4µl) was added. This was then mixed gently and incubated at room temperature for 30 minutes. The tube was then placed on ice. One-shot chemical transformation (TOPO TA Cloning from Invitrogen) followed. "One-Shot" *E. coli* was thawed on ice. To this was added 2µl of the TOPO Cloning Reagent and it was mixed by clicking. Incubation for 30 minutes at 42°C (without shaking) followed.

Plating

250µl of SOC medium or LB (Luria-Bertani) medium was added to the cells (at room temperature). The tubes were capped and shaken at 37°C for one hour. 50µl from each transformation tube was spread onto an LB plate containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 100µg/ml Ampicillin. 50µl IPTG (isopropyl- β-D-thiogalactopyranoside) was added. It was incubated overnight at 37°C. One white colony was selected, put in LB medium and shaken overnight at 37°C. A bacterial pellet

was derived from spinning the medium [the LB medium (6ml) containing bacteria] at 12,000 rpm.

Purification of the plasmid.

For purification of the plasmid DNA, I used QIAprep Miniprep from QIAGEN.

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consisted of three basic steps: (1) Preparation and clearing of a bacterial lysate (2) Adsorption of DNA onto the QIAprep membrane, and (3) Washing and elution of plasmid DNA. Endonucleases were efficiently removed by a brief wash step with Buffer PB to ensure that plasmid DNA was not degraded. Salts were efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA was then eluted from the QIAprep column with 50-100µl of Buffer EB or water. The complete procedures were as follows. Bacterial pellet was resuspended in 250µl of buffer P1 (wash buffer) in a microcentrifuge tube. Subsequently, 250µl of buffer P2 (lysis buffer in NaOH/SDS) was added and the tube was 4 – 6 times gently inverted to mix it. 350µl of buffer N3 was added and the tube gently inverted 4-6 times, then centrifuged for 10 minutes. During centrifugation, a QIAprep spin column was placed in a 2ml collection tube. The supernatants was applied the QIAprep column by decanting, and centrifuged for 60 seconds, then flow-through was discarded. The QIAprep spin column was washed by addition of 0.5ml of buffer PB and centrifuged for 60 seconds; again the flow-through was discarded. The QIAprep

spin column was washed by addition of 0.75ml of buffer PE and centrifuged for 60 seconds and then the flow-through was discarded. Any residual washing buffer was removed by centrifugation for an additional 1 minute. The QIAprep spin column was placed in a clean 1.5ml microcentrifuge tube. To elute the DNA, 50µl water was added to the centre of each QIAprep spin column, allowed to stand for 1 minute and then centrifuged for 1 minute.

Restriction digest

In order to verify the presence of an insert, the plasmids were digested using *EcoR* 1 and the products were run on 0.7% agarose gel. Using ultra-violet light, a photograph of the gel was taken. In order for the restriction enzyme to work it was mixed with 15µl of water, 2µl of buffer H and 2µl of template (1µl *EcoR* 1 being added last in the *Appligene oncor block*). It was incubated at 37°C for 1-2 hours.

Sequencing

For DNA sequencing, I used Dye Terminator Cycle Sequencing on the CEQ 2000 with Quick Start Kit. The sequencing reagent was prepared in a 0.2ml thin-walled tube. All constituents were kept on ice whilst preparing this reagent. They were added in the order listed below:

d H_20 (to adjust total volume to 20µl)	0-9.5µl
DNA template (see template preparation in Table A-1).	0.5-10µl
47 Sequencing primer (1.6 pmol)	2.0µl
DTCS Quick Start Master Mix	8.0µl
Total	20.0µl

DTCS Premix reaction consists of:

Component	Volume
10X Sequencing Reaction Buffer	200µl
dNTP Mix	100µl
ddUTP Dye terminator	200µl
ddGTP Dye terminator	200µl
ddCTP Dye terminator	200µl
ddATP Dye terminator	200µl
Polymerase Enzyme	100µl
Total	1200µl

These reactions were mixed and aliquot 200µl. The aliquot was stored at -20°C non frost free freezer.

Template preparation was probably the most critical factor in obtaining good sequence data from the CEQ 2000. Firstly, for the plasmid DNA templates, the following pre-heated treatment had improved both signal strength and current stability. The plasmid DNA was heated at 96°C for 1 minutes. Secondly, the amount of template DNA for sequencing reactions was calculated. The amount of template DNA used in sequencing reaction was dependent on the form of the DNA. It was important to quantitate the amount (moles) of DNA when performing the DNA sequencing reaction. This was important because the molar amount of template needed to be known so that the minimum molar ratio of primer to template was achieved (primer to template ratio $\geq$ 40:1). In addition, knowing the amount of template was important so that too much template was not used in the sequencing reaction.

There are several methods available estimate the amount of DNA in a sample. However, most of them are very inaccurate. The most commonly used, and probably the least accurate, is measuring the absorbance at 260nm. Even good quality DNA samples will contain material other than DNA that absorbs at 260nm. This will lead to an erroneously high reading and too little DNA will be added to the reactions. Some relatively new fluorescent methods using dyes such as PICO Green (Molecular Probe) are better, but care must be taken in setting standard curves. Furthermore, agarose gels are one of the other way of assessing the quality and quantity of the DNA in a sample. The technique is common, easy to do and is also useful for assessing quality of preparations. Quantitation of the amount of DNA in an unknown sample can be made by comparison to a known amount of standard DNA such as HindIII digested lambda DNA (Lobidel *et al.*, cited in Sambrook and Russell, 2001). In my work, I used the agarose gel method to calculate the amount of DNA for sequencing with modification. The procedure was as follows: DNA was diluted in water in 1:4; 1:16; 1:64 etc. Then 1µl of dilution was dropped onto 0.7% agarose gel, incubated for one hour at room temperature and using ultra-violet light a photograph of the gel was taken. To calculate the amount of DNA with this method, it was assumed that the dot, which come up in the dilution 1:4 is equal with 10µg of DNA.

The following table can be used to estimate DNA concentration for template sequencing.

TABLE A-1. For estimating the dsDNA concentration

Size (Kilobase Pairs)	Ng for 25fmol	Ng for 50fmol	G for 100fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500	1500

Ng = nano gram, f mol = femto mole, g = gram

After calculation the amount of DNA, the sequencing reagent for *ftsZ* (*ZWFR, ZHFR*), *wsp,* 16S and Na^+ pump α subunit genes was made up as follows:

DTCS Quick Start Master Mix	8µl
Primer	2µl
Template	1.1µl (heated at 96°C for 1 minute)
Water	8.9µl
Total	20.0µl

Thermal cycling:

96°C	20 seconds
50°C	20 second
60°C	4 seconds

(for 30 cycles followed by holding at 4°C)

Ethanol precipitation

For each sample, a sterile 1.5ml plastic tube was labelled. To each of these was added 4µl fresh Stop Solution (1.5M NaOAc + 50mM EDTA) made by mixing equal volumes of 3M NaOAc and 100mM EDTA and adding of 1µl of 20mg/ml glycogen. The sequencing reagent was transferred to the appropriately labelled 1.5ml microcentrifuge tube and mixed thoroughly. 60ml cold 95% v/v ethanol/dH_2O was added from the freezer (-20°C) and mixed thoroughly. Immediately it was centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed carefully with a micropipette (the pellet should be visible). The pellet was rinsed 2 times with 200µl 70% v/v ethanol/dH_2O from the freezer (-20°C) and centrifuged immediately at 14,000 rpm for 2 minutes after each rinse. After the final centrifugation, all of the supernatant was removed carefully with a micropipette and vacuum dried for 40 minutes. The sample was resuspended in 40µl of the Sample Loading Solution.

Sample preparation for loading into the CEQ

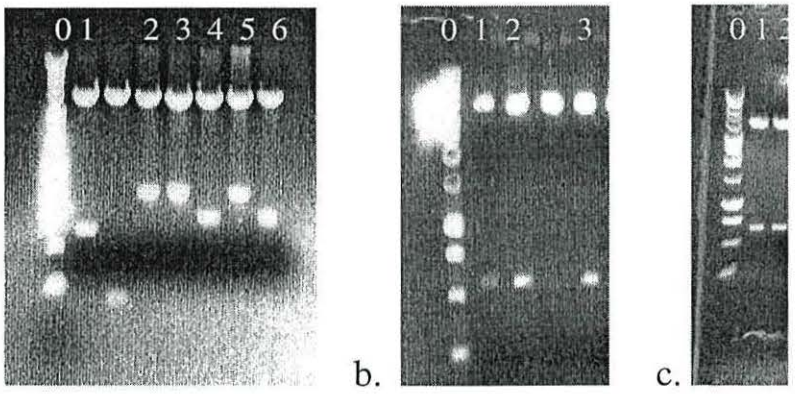
The resuspend samples were transferred to the appropriate wells of the CEQ sample plate (P/N 609801). Each of the resuspended samples were overlaid with one drop of light mineral oil. The

sample plate was loaded into the CQE and the procedure commenced. The result was analysed using DNA STAR

RESULTS

Plasmids from 5 different amplifications were found to contain the expected inserts of about 1114 bp and 816 bp for *ftsZ*, 610 bp for *wsp*, 511 bp for 16S and 545 bp for the Na^+ pump α subunit gene fragments. The gel electrophoresis of the restriction digest is shown in Fig. A-1:

Fig. A-1. Restriction digest of plasmids containing *wsp, ftsZ,* 16S and Na^+ pump α subunit fragments.



a,. lane 1: restriction enzyme treatment of *wsp* fragment
lane. 2, 3 and 5: Restriction enzyme treatment of *ftsZ* fragment (*ZW* primers)
lane. 4 and 6: Restriction enzyme treatment of *ftsZ* fragment (*ZH* primers)
b, lane 1, 2 and 3: Restriction enzyme treatment of 16S fragment
c, lane 1and 2: Restriction enzyme treatment of Na^+pump α subunit fragment
a,b,c, 0: One kilobase ladder

Table A-2. *ftsZ* fragment of both (*ZW* and *ZH*) amplification

```
  1 TTGTTGTCGC AAATACGGAT GCTCAAGCAC
 31 TAGAAAAGTC GTTATGTGAT AAAAAAATGC
 61 AGCTTGGCAT TAATTTAACT AAGGGGCTTG
 91 GTGCTGGTGC TTTGCCTGAT GTTGGCAAAG
121 GTGCAGCAGA AGAGTCAATT GATGAGATTA
151 TGGAGCATAT AAAAGATAGT CATATGCTTT
181 TCATCACAGC AGGAATGGGC GGTGGTACTG
211 GAACCGGTGC AGCACCGGTA ATTGCAAAAG
241 CAGCCAGAGA AGCAAGAGCC GCAGTTAAGG
271 ATAGAGCGCC AAAAGAAAAA AAGATATTGA
301 CTGTTGGAGT TGTAACTAAA CCGTTCGGTT
331 TTGAAGGTGT GCGCCGTATG CCGATTGCAG
361 AGCTTGGACT TGAAGAACTG CAAAAATACG
391 TGGATACACT TATTGTCATT CCAAATCAGA
421 ATTTATTTAG AATTGCAAAT GAAAAAACTA
451 CATTTTCTGA TGCATTTAAA CTTGCTGATA
481 ATGTTCTGCA CATTGGCATC AGAGGAGTAA
511 CTGACTTGAT GGTCATGCCA GGGCTTATTA
541 ATCTTGACTT CGCTGATATA GAAACAGTAA
571 TGAGCGAGAT GGGCAAAGCG ATGATCGGCA
601 CCGGAGAGGC AGAAGGAGAA GATAGAGCAA
631 TTAGTGCTGC AGAGGCTGCA ATATCTAATC
661 CATTGCTTGA TAATGTATCA ATGAAAGGT 689
```

Table A-3. *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 A-4. 16S fragment

```
  1 TTTGAACGGC TACACCCAcA AATTATATCT
 31 TAATCCAACA TtCGAGGTCG CAATCTTTTT
 61 TATCGATATG AACTCTCCAA AAAAATTACG
 91 CTGTTATCCC TAAAGTAACT TAATTTTTTA
121 ATCATTATTA ATGGATCAAA TATTCATAAA
151 TTTATGTTTT TAAAAATTAA AAGTTTTTTA
181 AATTTTAATA TCACCCCAAT AAAATATTTT
211 TATTTATTAA AATTTAATTA ATCTATATAA
241 TTAAAATAAA AAAAAATATA AAGATTTATA
271 GGGTCTTCTC GTCTTTTAAA TAAATTTTAG
301 CTTTTTGACT AAAAAATAAA ATTCTATAAA
331 AATTTTAAAT GAAACAGTTA ATATTTCGTC
361 CAACCATTCA TTCCAGCCTT CAATTAAAAG
391 ACTAATGATT ATGCTACCTT TGCACAGTCA
421 AAATACTGCG GCCATTTAAA ATTTTCAGTG
451 GG
```

Table A-5. 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
```

The sequences reported in this report show similarity with GenBank database (http:www.ncbi.nlm.nih.gov) accession nos. 311274, 2078546, 13173392, 8573429 and 18487812 for *ftsZ, wsp,* 16S and Na^+ pump α subunit sequences, respectively. The sequences had identities of 95%, 100%, 98%, 100% and 97%, respectively.

Calculation of copy number of DNA standard

The DNA standard was calculated based on the following equations:

The length of the plasmid is 3,908bp. Average molecular weight of dNMP is assumed to be 330. Therefore, the molecular weight of the double standard DNA of 3,908 bp is: 3,908x330x2 = 2.58 x 10^6.

The DNA standard was calculated based on the concentration number that is read on the Fluorometer machine times (x) 4, because the samples were diluted 1 in 400 and I used 100µl for measuring. For instance, when a *Wolbachia* plasmid sample read 43 on the fluorometer, the concentration of *Wolbachia* in 1µl was 43 x 4 = 172ng/µl. The volume was 50µl, therefore the total amount of *Wolbachia* plasmid as a standard in a tube was:

172 ng/µl x 50 µl = 8600 x 10^{-9} g = 8.6 x 10^{-6} g.

Avogadro number is = 6 x 10^{23}

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

$$= \frac{8.6 \times 10^{-6} \times}{2.58 \times 10^{6}} \quad 6 \times 10^{23} = 20 \times 10^{11} \text{ copies/50µl}$$

$$= 0.4 \times 10^{11} = 4 \times 10^{10}$$

The samples used in iCycler were diluted 1 in 100, therefore:

$$\frac{4 \times 10^{10}}{100} = 4 \times 10^{8}$$

When 5µl of PCR template was used, the standard was = $5 \times 4 \times 10^{8} = 20 \times 10^{8}$

Calculation copy number of *Wolbachia*

Wolbachia amount (the number of *Wolbachia*) in unknown samples was calculated based on a single egg, larva or adult as follow:

The number of eggs, larvae and adults used for DNA extraction were, 100, 50 and 25 respectively in 100µl solution. I used 5µl for templates. Therefore, in each individual contained of 0.2, 0.4 and 0.8×10^{b}. *Wolbachia.*

^{b} = log starting quantity of each sample.

Sample and instrument preparation (making a knife, cutting the specimen using piramitome and ultratome LKB and making pioloform solution) for Electron Microscopy

The specimens were removed from the embedding mould and the correct size of holder was chosen for each specimen. There are four types of holder: G holder with LKB easy mould block, G holder with gelatine capsule-embedded block, D holder with gelatine capsule-embedded block and K holder with flat-embedded block. When clamped, the space between jaws of the K holder was paralleled. The space between the jaws of holders G, D and E were paralleled or decreased towards the tip. The 2 small Allen screws were adjusted until the distance between the jaws decreases slightly towards the tip. The specimen was placed

in the orientation head and locked into specimen holder with the large screw.

The specimen was trimmed using a glass knife made using a knife maker (LKB 7800B). The glass strip was held against the white plate and the strip pushed against the stud (did not remove hand from the strip). The clamping head was lowered until it touched the glass and clamped securely. The fork was placed under the end of the strip. It was scored by turning the breaking knob clockwise. The knob was reset immediately. The clamping head shaft was raised and the glass square removed by means of the fork

The square was placed between the two glass holders and fastened by lowering the clamping head. The fork was placed under the glass and the glass was scored. The DPA-lever was moved until the damping pad touched the glass (did not exceed the 45 mark on the lever). The square was broken and the breaking knob was reset immediately. The DPA-lever was reset and the scoring shaft supported with the right hand whilst the clamping head was raised. The scoring shaft was pushed in and the rear glass holder was pushed backwards using the fork.

A trimming knife was inserted to the correct height and the clearance angle was adjusted to 4°. The specimen arm was locked and the orientation head and specimen were inserted (with the arch in the vertical plane). The knife height was adjusted. It was ensured that all clamps were locked.

The orientation head was locked in the arm. The microscope was switched to position "A" for diffuse light. The knife edge was brought into focus and the specimen arm freed. The knife stage rotation was set to zero (0°) and the knife was advanced towards the specimen. The specimen arm was held with the left hand and moved down past the knife edge and the specimen face was trimmed. The small wheel (1 click = 1μm) was rotated and advanced to approximately 4μm per cut. The microscope was pulled to the forward position where the specimen face was viewed. The specimen was trimmed until the required area of specimen face was exposed. The exposure of the required area was shown in the specimen face view. The orientation head was loosened and rotated clockwise through 90° so that the arch was seen to be horizontal. The knife was rotated through 30° to the right (adjusted to the lateral position of the knife). A suitable depth was trimmed to form the shorter of the two parallel sides of the specimen face. The knife was withdrawn, and rotated 30° to the left. The specimen was trimmed to the relevant depth and the knife withdrawn. The orientation head was loosened and turned back to the first position (arch vertical) then rotated approximately 5° to the left of vertical. The left-sloping side of the block face of the specimen was trimmed up to the relevant area of the specimen. The knife was rotated 30° to the right side and the orientation head was tilted 5° to the right side of vertical for the right-sloping side and the remainder of the specimen was trimmed. The orientation head was rotated and the sample was then sectioned with a fresh glass knife. Ultrathin sections were cut from the prepared mesa. Many mesas were prepared at different levels within the specimen in order to trace interesting

structures throughout the depth of the specimen. Semithin or survey sections were cut from the specimen. An area of interest was selected by comparing it to the block face and a mark was made on the block face with a needle using the eyepiece graticule for accurate measurement of the area. The block face was viewed by tilting the microscope in forward position. A glass knife with a good left hand corner was inserted. The specimen was locked in the arm. The orientation head was inserted so that the specimen was at the height of the knife. Side-ways knife adjustment was used to bring the left corner of the knife a measured distance from the reference point on the specimen face. The knife stage was rotated 1° anti-clockwise. This gave the material surrounding the mesa a slight slope. I started trimming into the block by 1μM advance steps. The side was trimmed away to a depth of 20μM. The knife was retracted with the macro feed. The orientation head was rotated anti-clockwise through 90° and the knife adjusted laterally to align with the left corner to trim away the reference mark by 20μM. The knife was retracted and the head was rotated 90° to the final position. The knife was aligned to the specimen. The specimen was trimmed to a depth of 20μM and ready for ultrathin sectioning of the selected area (I did not adjust the specimen height again before sectioning).

The knives that had already been made were placed on the hot deck with the cutting edge protruding over the edge. The knives were heated for about half a minute. The truf was placed on the wax dispensing deck so that the lift edge of the truf fitted into groove. It was then mounted directly on to the warm knife. The upper edge was flushed with the knife and allowed to cool. The

truf was sealed out the edges with dental wax from the well. The truf was thrown away with the knife when sectioning was completed. In this way, contamination of the through liquid and the sections was avoided.

The pioloform solution was filtered into a Coplin jar (when humidity was above 30%, the desiccator was used. The desiccator lid was replaced quickly and the humidity allowed to drop below 10% before proceeding). The slide was dipped into the solution evenly. It was removed and the film drained by sliding it against the inner surface of the Coplin jar. Filter paper was placed beneath the jar to aid drainage. The slide was dried for one minute. When humidity was high, this procedure was done in the desiccator. The water level in the staining jar was adjusted so that it was level with the dish lip. The water surface was cleaned by sweeping it with lens paper and, double parallel lines were scored along the four sides of the slide using a fresh razor blade. The slide was submerged slowly in a water-filled staining dish at a 45^{o} angle. The film was parted from the slide (the film was seen as it began floating free if the lamp was properly adjusted) by the water surface tension. Forcep tips were used, gently positioning the freed film to one side, removing the slide and attaching the film to the lip of the dish. Grids were placed, flat surface down, in a row until the film was lined with grids. Placing grids on folds or on any discoloured area in the film was avoided. The parafilm strip was aligned over the film containing grids. The parafilm was overlapped at both ends to the full extent of the grid preparation. The film was attached to one end to the parafilm and evenly lowered until the film was

completely covered, then released. After a few seconds any trapped air pockets were disappeared. One corner of the parafilm was gripped using forceps and removed from the preparation. Water from the grid area was not removed. Excess parafilm framing the preparation was trimmed away using scissors. The trimmed preparation was placed in the filter paper-lined petridish. Each was anchored by placing indenting parafilm with a blunt object at four or five sites along the edge of the strip and covered with the dish top.

An evaluated glass knife was inserted with a truf to the correct height. The clearance angle was set to 4^{o} and the specimen arm locked. The orientation head was inserted with the trimmed specimen to the same height as the knife and the diffuse light switched on. The microscope was pulled to a forward stop position and was looked at the block face to ensure that the specimen face was at the same height as the knife. The upper and lower parallel edges of the face were aligned to the knife-edge (unlocked and rotated to achieve this). It was locked when the adjustment was completed. The diffuse light was rotated to "A" position for alignment when the specimen approached the knife. The microscope was tilted slightly towards the silver indicator band. The specimen face was ensured to be at the same height as the knife. The light was adjusted until the flat specimen face was shinning brightly. The specimen face was like a mirror, reflecting the image of the knife as a dark shadow. The knife was advanced to the specimen further until this reflection was seen. The silver space between the knife and its reflection was the actual distance. This reflection was used to align the knife to specimen in the

horizontal plane. When the knife was closer to the left side of the specimen, the knife was rotated anti-clockwise until parallel to its reflection in the specimen face. The specimen arm was freed and the "Manual" control brought the specimen to the height of the knife. The same reflection was used as before for vertical alignment. The width of the silver space between the knife and the specimen was examined at the base of the specimen face. The specimen and high quality knife were aligned with one another. The knife was tilted to 4^{o} (and checked that all the clamps were locked). The diffuse light was used at "A" position to see the reflection of the knife in the block face. The knife was advanced until it was close to the specimen without touching it. The "Manual" control was used to bring the specimen below the knife-edge and the light was adjusted to the "S" position. The disposable syringe was used to fill the over fill slightly and the level was adjusted with the screw until the surface was horizontal and shinning brightly. The specimen was brought to the "UP" position with the "Manual" control and the cutting speed was set to 2nm per second. The "feed switch" was turned off and "Advanced" to the back dot (about 80nm). It was switched to "auto". The knife was advanced to the specimen using 0.1μM click of the micro feed. When the first section was cut, it was switched to "feed". Feed was adjusted until the correct colour of the section was obtained. When sufficient sections were cut, it was switched from "Auto" to "Manual" and the sections collected (the feed stopped automatically). If more sections from the specimen were required, it was switched back to "Auto" (the feed started automatically). When a long pause was made for changing the knife or the specimen it was always important to switch to

"reset" for 10 minutes. In this way, the advance was reset to ensure that the total advance was available for the next series of sections. It was switched to "Reset" if the red thermal feed lamp began to flash. There was a wide range of cutting parameters on Ultromote V. The cutting speed, knife clearance angle or knife angle (scored angle) were changed if the sections were unsatisfactory. To protect the knife-edge, the correct cutting speed was established as early in the cutting sequence as possible. Then the section was picked up by the grid. An LKB grid was elected that gave the section adequate support without obscuring too great an area of the specimen. The band was freed from the knife-edge. The grid was picked up in the forceps and the forceps closed with a rubber ring. The grid bars if possible were aligned carefully in the forceps so that the sections were collected along the bar and not across them. The grid was lowered vertically until it was under the water surface and brought it out a small distance holding it steadily. The sections were guided with an eyelash (or small hair) towards the grid and the first sections allowed to touch the grid. The grid was removed slowly keeping it vertical. The section was pulled out unfolded with the grid. The grid was allowed to dry and stored in the LKB Grid Box.

Statistical analysis of the effect of temperature on the life cycle of *D. melanogaster.*

Table A-7. Univariate Analysis of Variance of the effects of tetracycline and temperature on the life span of *D. melanogaster.*

Between-Subjects Factors

		N
TREATMEN	.00	40
	1.00	40
TEMPERAT	20.00	40
	29.00	40
SEX	1.00	40
	2.00	40

Descriptive Statistics

Dependent Variable: Logarithm

Treatment	Temperature	Sex	Mean	Std. deviation	N
Untreated	20°C	Male	1.5110	0.06922	10
		Female	1.5087	0.08780	10
		Total	1.5098	0.07696	20
	29°C	Male	1.0040	0.06822	10
		Female	0.9961	0.08817	10
		Total	1.0001	0.07684	20
	Total	Male	1.2575	0.26853	20
		Female	1.2524	0.27656	20
		Total	1.2550	0.26907	40
Treated	20°C	Male	1.5243	0.09964	10
		Female	1.5523	0.03973	10
		Total	1.5383	0.07520	20
	29°C	Male	1.6123	0.06143	10
		Female	1.6471	0.03546	10
		Total	1.6297	0.05198	20
	Total	Male	1.5683	0.09234	20
		Female	1.5997	0.06091	20
		Total	1.5840	0.07883	40
Total	20°C	Male	1.5177	0.08378	20
		Female	1.5305	0.06999	20
		Total	1.5241	0.07647	40
	29°C	Male	1.3082	0.31835	20
		Female	1.3216	0.34031	20
		Total	1.3149	0.32533	40
	Total	Male	1.4129	0.25307	40
		Female	1.4260	0.26457	40
		Total	1.4195	0.25733	80

Levene's Test of Equality of Error Variances[a]

Dependent Variable: LOGARITM

F	df1	df2	Sig.
1.664	7	72	.132

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design:
Intercept+TREATMEN+TEMPERAT+SEX+TREATMEN * TEMPERAT+TREATMEN * SEX+TEMPERAT * SEX+TREATMEN * TEMPERAT * SEX

F tests of Between-Subject Effects

Dependent Variable: Logarithm

Source	Type III Sum of Square	df	Mean Square	F	Sig.
Corrected	4.858[a]	7	0.694	133.890	< 0.001
Intercept	161.190	1	161.190	31098.134	< 0.001
Treatment	2.165	1	2.165	417.727	< 0.001
Temperature	0.875	1	0.875	168.861	< 0.001
Sex	3.437E-03	1	3.437E-03	0.663	0.418
Treatment*Temperature	1.807	1	1.807	348.651	< 0.001
Treatment*Sex	6.674E-03	1	6.674E-03	1.288	0.260
Temperature*Sex	1.780E-06	1	1.780E-06	0.000	0.985
Treatment*Temperature*Sex	1.957E-04	1	1.957E-04	0.038	0.846
Error	0.373	72	5.183E-03		
Total	166.422	80			
Corrected total	5.231	79			

a. R Squared = 0.929 (Adjusted R Square = 0.922)

Calculation of the threshold of significant

Treatment	Temperature	
	20°C	29°C
Untreated	1.51	1.00
Treated	1.54	1.63

N = 20

Error MS = 0.005183

Mean of logs

Mean of logs

Log x = 1

X = 10

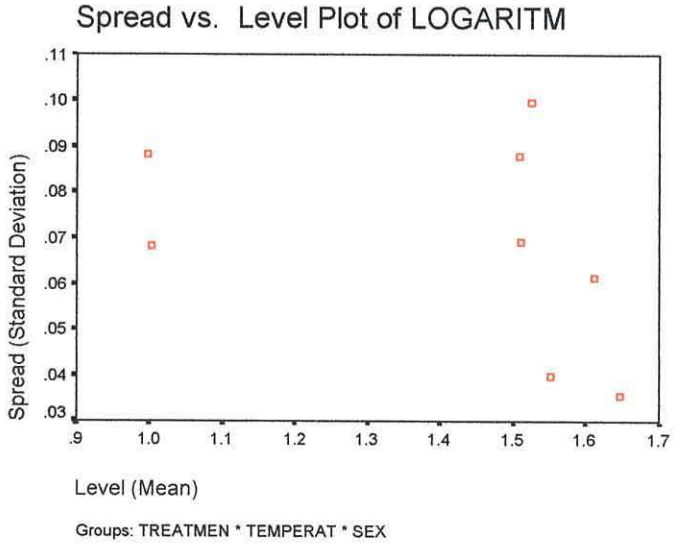
31.6	10
34.7	42.7

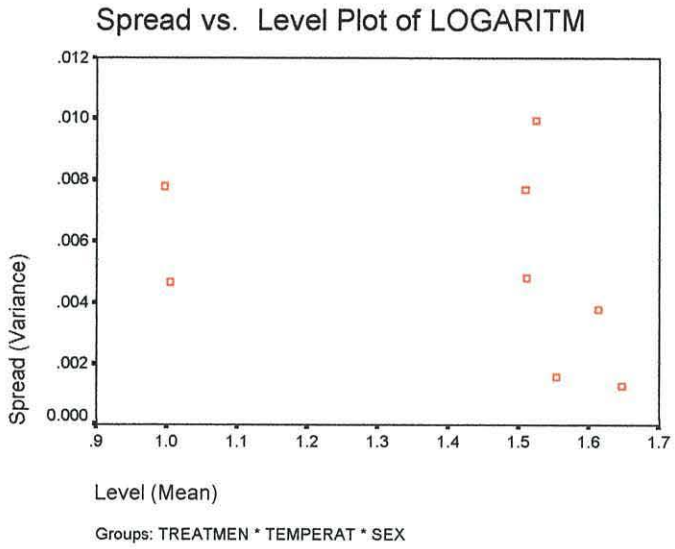
$$\mathbf{Q}\ (4, 72) \times \sqrt{\frac{\text{error MS}}{N}}$$

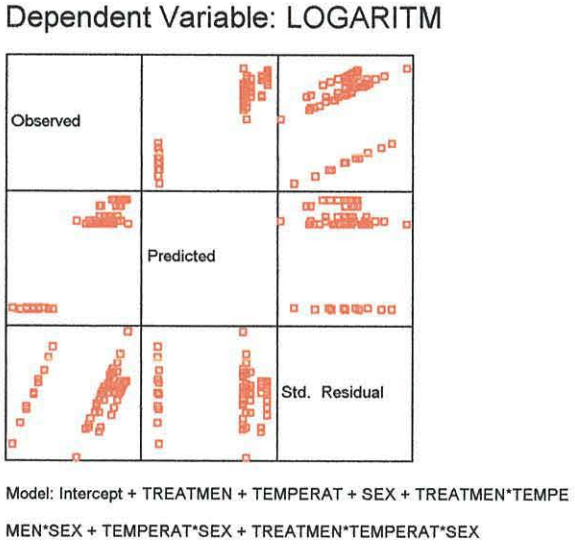
$$3.73 \times \sqrt{\frac{0.005183}{20}} = 0.06$$

2 means are significantly different if they differ by more than 0.06. Therefore, at 20°C untreated and treated are not significantly different, at 29°C untreated and treated are significantly different and at 29°C untreated are significantly different with untreated and treated at 20°C.

Spread-versus-Level Plots







The morphology of *Drosophila melanogaster*

Eggs

The egg is bilaterally symmetrical. The ventral side is convex whereas the dorsal side is flatter. The mature egg consists of two envelopes. The inner one is the vitelline membrane, which may be secreted by the egg itself. The outer membrane is the opaque chorion. The chorion is a thin but strong envelope (Strickberger, 1967; Demerec, 1994). The length of the egg is about 0.5mm. Two filaments project from the anterior surface as "water-wings"

to prevent the egg from sinking and drowning in semi-liquid medium. There is a minute pore (micropyle) on the anterior end of the egg to receive the sperm. Although large numbers of sperm enter the micropyle, only one serves to fertilize the egg. The developing embryonic tissue absorbs the others. Fertilization of the egg occurs in the uterus. Demerec (1994) reported that there is no space between the outer envelope of the egg and the uterine wall. This might prevent the escape of the gametes from the cephalic pole, where penetration of the egg takes place. Immediately after the sperm enters the egg, the first meiotic division occurs. When the division is complete, the pronucleus (the nucleus of the egg) is formed. The sperm nucleus and egg nucleus then fuse resulting in the zygote. Development starts immediately after fertilization. It is divided into two parts. The first is the embryonic period and it occurs within the egg. The second is the postembryonic period, which occurs after hatching. Development does not occur if eggs do not receive sperm.

Larvae

Larvae hatch from the egg. The larval body consists of three main parts: head, thorax and abdomen. The thorax is subdivided into three segments and the abdomen into eight segments. This means that, with the head, there are a total of twelve segments. The mouth is located in the ventral part of the head segment. Several chitineous hooks are situated around the mouth. The wall of the body is soft and flexible. It consists of an outer non-cellular cuticle. This consists of two layers; the thick lamella is the procuticle and the thin lamella is the epicuticle. The larva is

transparent and is approximately 4.5mm in length. The larvae are active feeders, crawling or burrowing in the culture medium.

Inside the body of the larva are found: salivary glands, stomach, coiled intestine, malpighian tubuli, fat bodies, ganglia, tracheae and gonads. The gonads are embedded in one of the fat bodies. The testes are much larger than the ovaries making it easy to distinguish between the sexes. Larvae grow by the process of "moulting". There are two larval moults. During each moult both the internal organs and the body wall grow. Demerec (1994) reported that internal organs (such as malpighian tubuli, muscle, fat body and intestine) grow by an increase in cell size, the number of cells in the organ remaining constant. In contrast, imaginal organs grow by cell multiplication.

At the final stage (third instar), the larvae crawl out of the food medium, usually going to the side of the culture bottle in order to look for a dry place in which to pupate.

Pupae

Pupa has an epidermis that is separated from the puparium. The pupa (known at this stage as the prepupa) is headless and has no external wings or legs. Approximately two hours after the puparium is formed the anterior spiracles appear.

Pupation occurs 12 hours after puparium formation. This stage begins with muscular contractions that result in the prepupa drawing back from the anterior end of the puparium and with the eversion of its head structures. It also ejects its larval mouth,

which was attached to the anterior end of the prepupa. Then wings, halteres and legs are formed. A typical pupa with head, thorax and abdomen is formed after the prepupal cuticle, together with tracheal linings and spiracles, is completely shed. A new pupal cuticle is formed. Once the changes are complete, the adult emerges by forcing its way through the anterior end of the pupal case.

Adult flies

There are several ways to distinguish between the male and female adult flies. In the female, the tip of the abdomen is elongated, whilst in the male it is rounded. The female abdomen has five segments, eventually becoming distended with maturing eggs. The body of the imago consists of three parts: the head, thorax and abdomen (having 20 original segments). The head bears compound eyes, ocelli, antennae and mouthparts. It consists of 6 segments: labrar, ocular, antennal, intercalary, mandibular, maxillary and labial (Schimdt *et al.*, 1994, cited in Chapman, 1998). Similarly, Gulland and Cranston (1999) reported that there are six fused head segments. They named them as the labrar, antennal, postantennal, mandibular, maxillary and labial segments.

The thorax consists of three segments: the prethorax, mesothorax and metathorax. The abdomen consists of 11 segments. The dorsal plates of the abdomen are called tergites and the anterior plates are called sternites. Seven pairs of spiracles are found on the abdomen, one pair on each of the first seven segments. The

genital opening of the female is between the eight and ninth abdominal segments (typical in Diptera), see Fig. A-2.

Fig. A-2 The morphology of adult male and female *D. melanogaster* flies (Demerec and Kaufmann, 1965)

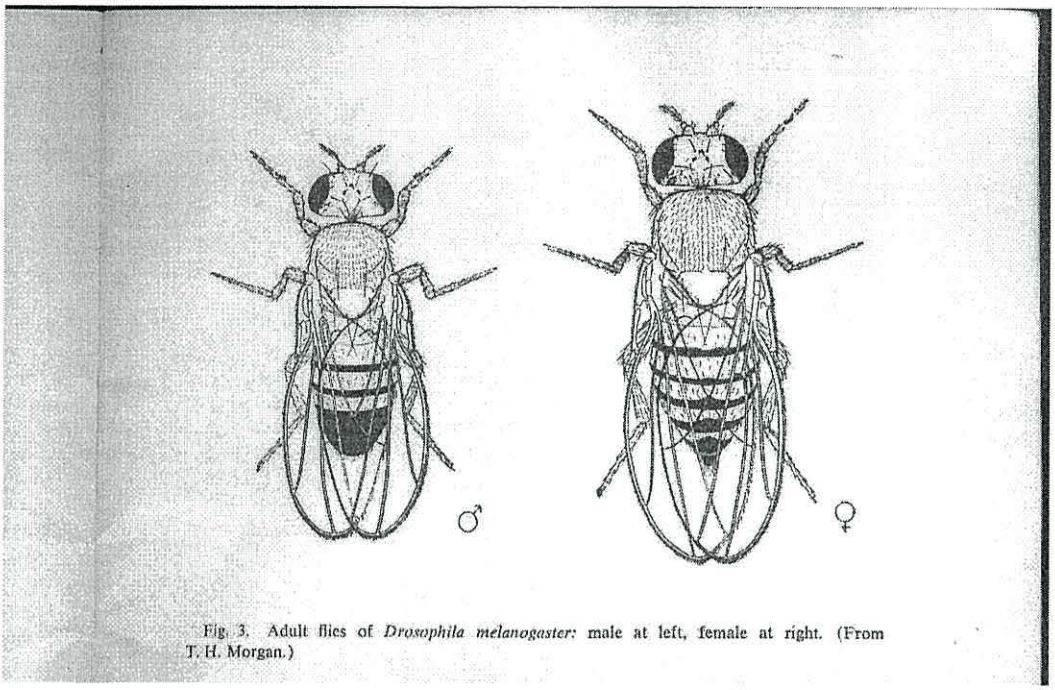


Fig. 3. Adult flies of *Drosophila melanogaster:* male at left, female at right. (From T. H. Morgan.)

The life cycle of *Drosophila melanogaster* (from egg hatching to the emergence of the imago) is about 192 hours at 25°C. The larvae pass through three instars and two larval moults. The first moult occurs at about 25 hours, the second moult at about 48 hours, and puparium formation at about 96 hours after hatching.

Table A-8. *D. melanogaster* development at 25°C [Demerec (1950) cited in Strickberger (1967)].

Hours	**Days** (Approx.)	Stage
0	0	Egg laid
0 – 22	0 – 1	Embryo
22	1	Hatching from egg (first instar)
47	2	First moult (second instar)
70	3	Second moult (third instar)
118	5	Formation of puparium
122	5	"Prepupal" moult (fourth instar)
130	5.5	Pupa: eversion of head, wings and legs
167	7	Pigmentation of pupal eyes
214	9	Adult emerges from puparium with creased and folded wings
215	9	Wings unfold to adult size

Pests of *Drosophila*

Environmental conditions such as temperature, humidity and aeration are important for the survival and proper development of *Drosophila* (Demerec, 1994). Several problems can be encountered when rearing *Drosophila.* The first problem is the growth of mould. Mould often forms a mat covering the culture medium before the adult flies have a chance to lay eggs.

The second problem that may be encountered is bacterial contamination. This may occur as a slimy exudate on the surface of the *Drosophila* culture medium. Flies may sink in this exudate

and do not thrive in these conditions. Such bacterial contamination is likely to spread through stocks quickly. The most effective control seems to be frequent transfer of adult flies to fresh culture medium.

The third problem that may be encountered is the manifestation of mites. This a more serious problem than that caused by either moulds or bacteria. The mite attaches to the flies in the adult stage. It may puncture the integument and suck the tissue fluids (Demerec, 1994). Two of the mite species that constitute a serious problem in *Drosophila* culture are the longhaired *Tyroglyphus langior* and *Histiosoma sp.* These mites spread from culture to culture by crawling and the young can crawl out of the bottle.

Fig. A-3. The morphology of adult mites found in *Drosophila* culture

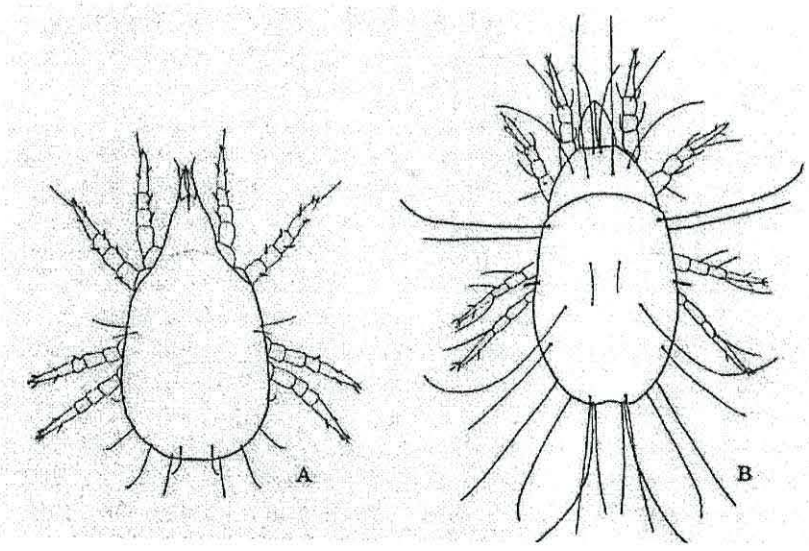


Figure A-3 shows, adult females of two species of mites. A: the dangerous laboratory mite, *Histiosoma sp*, the hypopus stage of which attaches itself to flies. B: relatively harmless long-haired

mite often found in *Drosophila* cultures and lacking a hypopus stage.