

## Ultrastructure of *Wolbachia* are Found in Somatic and Reproductive Tissue of *Drosophila simulans* and *D. melanogaster*

ENDANG SRIMURNI KUSMINTARSIH

Faculty of Biology, Jenderal Soedirman University  
Jalan Dr Soeparno No 63, Grendeng, Purwokerto 53122, Indonesia

Ultrastructural observations shows *Wolbachia* in the testis of *Drosophila melanogaster*; where *Wolbachia* and cytoplasm are excluded from the mature sperm, and also found either in somatics and reproductive organ of *D. simulans*. Details of the ultrastructure of *Wolbachia pipientis* from the reproductive and somatic organs of *D. melanogaster* harbour *Wolbachia*-induce popcorn-effect and *D. simulans* harbour *Wolbachia*-induce cytoplasmic incompatibility are described as follows: the ultrastructure of *Wolbachia* in both of them were similar, distribution of *Wolbachia* in *D. Simulans* was not restricted to the reproductive organs, they were also found in somatic tissues (muscle). *Wolbachia* can be present without causing detrimental effects. They are pleomorphic (rod shaped, elongate, oval or slightly bent). The size varies between 0.04  $\mu\text{m}$  and 0.47  $\mu\text{m}$  in diameter and between 0.26  $\mu\text{m}$  and 1.2  $\mu\text{m}$  in length. In a few cases *Wolbachia* seem to have been undergoing fission, but are still joint together by a vacuolar membrane. The microorganisms appear to have two membranes, a host membrane and a bacterial membrane. However, in some cases the microorganism seems to be surrounded by only one membrane, the possibility why only one membrane was visible might be due to loss during the embedding process.

Key words: *Wolbachia*, ultrastructure, *Drosophila simulans*, *D. melanogaster*; cytoplasmic incompatibility, popcorn-effect

Pengamatan dengan elektron mikroskop menunjukkan bahwa *Wolbachia* dan sitoplasma pada testis *Drosophila melanogaster* yang terinfeksi *Wolbachia* penyebab popcorn-effect, keluar dari sperma yang dewasa; demikian juga *Wolbachia* ditemukan pada jaringan soma dan organ reproduksi dari *D. simulans* yang terinfeksi *Wolbachia* penyebab ketidakserasian sitoplasma. Pengamatan *W. pipientis* secara terperinci dengan mikroskop elektron dari organ reproduksi dan soma *D. melanogaster* terinfeksi *Wolbachia* mempunyai bentuk morfologi sama, penyebaran *Wolbachia* pada *D. simulans* tidak hanya terbatas pada organ reproduksi, melainkan juga pada jaringan soma pada otot. Keberadaan *Wolbachia* tidak menimbulkan kerusakan, bentuknya bervariasi dari bentuk batang, memanjang, oval atau agak membengkok. Ukurannya bervariasi, diameter antara 0.04  $\mu\text{m}$  dan 0.47  $\mu\text{m}$  dan panjang antara 0.26  $\mu\text{m}$  dan 1.2  $\mu\text{m}$ . Pada beberapa kasus, *Wolbachia* ditemukan sedang dalam proses pembelahan, namun masih saling menempel yang dihubungkan dengan membran vakuola. Secara umum dinding sel *Wolbachia* terdiri atas dua lapisan, yaitu satu membran dari inangnya dan satu membran dari bakteri itu sendiri, tetapi dalam pengamatan ini ditemukan *Wolbachia* yang hanya dikelilingi oleh satu membran, hal ini karena membran tersebut hilang ketika dilakukan proses embeding.

Kata kunci: *Wolbachia*, *Drosophila simulans*, *D. melanogaster*; ketidakserasian sitoplasma, efek popcorn

*Wolbachia* are bacteria endosymbiont that infect a wide variety of mainly Arthropod that optimize their own fitness by manipulating the reproduction of their host (Werren *et al.* 2008). Studies have shown that *Wolbachia* is an obligate endosymbiont most prominently found in the cytoplasmic of reproductive tissue of all described host, as well as in a number of other tissues depending on the particular *Wolbachia*/host combination Lassy and Karr 1996; McGraw *et al.* 2002; Fu *et al.* 2010). Infections with *Wolbachia* have been associated with host reproductive abnormalities such as cytoplasmic incompatibility, feminization, parthenogenesis, male killing and the popcorn-effect. Lo *et al.* (2007) and Pfarr *et al.* (2008) propose that all endobacteria of arthropods and nematodes commonly called *Wolbachia* be formally declared as *Wolbachia pipientis*. Other host that infect

by *Wolbachia* is filarial nematodes (McLaren *et al.* 1975; Kozek 1977; Kozek and Marroquin 1977).

Cytoplasmic incompatibility in arthropods was reported for the first time by Ghelelovitch 1950 and studied in detail by Laven 1967. Crossed between different strains of mosquitoes failed to produce progeny, Laven reported that cytoplasmic factors were involved. However, cytoplasmic incompatibility was not associated with the present of *Wolbachia* until the 1970's. *Wolbachia* were identified as the causative agent of cytoplasmic incompatibility in *Culex pipiens* by Yen and Barr (1973). Hoffmann *et al.* (1986) were the first to describe cytoplasmic incompatibility in *D. simulans*.

Popcorn-effect inducing *Wolbachia* was reported by Min and Benzer (1997), Zakaria *et al.* (2009). They discovered that *Wolbachia* produce an unusual effect in tissues as brain, eyes (retina) and muscle of the *D. melanogaster*. A sudden massive degeneration of the fly's cells occurs in response to bacterial multiplication.

\*Corresponding author: Phone: +62-0281-638794,  
Fax: 62-0281-631700, E-mail: [endang\\_sk@lycos.com](mailto:endang_sk@lycos.com)

As this resembles a backing process of popcorn in a microwave, hence it has been termed the “popcorn-effect”.

Due to the small dimensions of *W. pipientis*, the identification using light microscopy has usually been considered unreliable and most investigations have been based on electron microscopy. The ultrastructure of the genus *Wolbachia* have been looked at in reproductive organs of mosquitoes especially of the genera *Culex* and *Aedes* (Hertig 1936; Wright *et al.* 1978) and *Tribolium confusum* (O'Neill 1989).

Hertig (1936) described the characteristic of *Wolbachia*, was exhibiting an enlarged form, usually 0.5-1.3  $\mu\text{m}$  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. Wright *et al.* (1978) and Wright and Barr (1980) described ultrastructural the *Wolbachia* in *Culex pipiens* as a coccoid ( $\leq 1.0 \mu\text{m}$  diameter) to bacilliform (0.25 x 1.5  $\mu\text{m}$ ) with an outer cell wall, inner plasma membrane, and cytoplasm of ribosome and filamentous deoxyribo nucleic acid strands. *W. pipientis* is always surrounded by a third membrane of host origin. Yen (1975) reported that in gonads cells are filled with the organism but exhibit no obvious pathologic characteristic. 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 microorganism are removed with cytoplasm and the mature sperm contain no *Wolbachia*.

Furthermore, Wright and Barr (1980) described *Wolbachia* in *Aedess cutellaris* as follows: they were pleomorphic and vary in shape from spherical to elongate. The diameters of them varied from 0.4  $\mu\text{m}$  to 1.1  $\mu\text{m}$ . Those diameters at the small end of the range 0.4  $\mu\text{m}$ -0.7  $\mu\text{m}$  are of elongate form. The length of elongate forms measured up to 2  $\mu\text{m}$ . Furthermore, they reported that *Wolbachia* is surrounded by a single host vacuole membrane. 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. 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 of the host. The average length of

*Wolbachia* had a minimum of 0.54  $\mu\text{m}$  and a maximum of 1.03  $\mu\text{m}$ . Mahilum *et al.* (2003) was also reported that the bacteria in *Culex pipiens* exhibited 3 enveloping membranes. Oh *et al.* (2001) reported that *Wolbachia* in the egg cells of *Nephila clavata* has three enveloping membranes.

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 0.57  $\mu\text{m}$  and width of 0.28  $\mu\text{m}$ . The organisms 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 outer two and is likely to be of host origin. The internal structure consisted of cytoplasm and a network of nucleic acid filaments.

The presence of *Wolbachia* in non-reproductive tissues has been largely ignored. 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.

## MATERIALS AND METHODS

The tissues (testis, ovaries, brain, muscle, and eyes) of *D. melanogaster*<sup>w<sup>1118</sup></sup> and *D. simulans* DSR obtained from School of Biological Sciences, University of Wales, Bangor, United Kingdom were taken from flies dissected in Phosphate buffer saline (PBS) and put in Karnofsky's fixative consisting of 0.4% paraformaldehyde and 0.1% glutaraldehyde in sodium cacodylate, 10% w/v sucrose, pH 7.2 (5 mL 0.4 M buffer [sodium cacodylate] plus 2 mL 2% paraformaldehyde, 0.8 mL of 2% glutaraldehyde and 1 g sucrose with addition of distilled water to 10 mL so that the final concentration of fixative is 2%) for 1 h, then washed twice using 0.4 M sodium cacodylate buffer. The tissues were then put in 1% osmium tetroxide for 1 h. Washed twice with water and put in 2% uranyl acetate and kept in the fridge (4 °C) overnight. After fixation the material was dehydrated using ethanol, the tissues were then treated with 100% propylene oxide for 15 min and transferred to Spurr's resin and 100% propylene oxide (1:1) and slowly rotated in a rotor overnight. They were embedded in Spurr's resin 100% in an oven at 60 °C overnight (Spurr 1969). Sections were stained with uranyl acetate, after which they were examined with a Philips EM 60 kv.

## RESULTS

Using electron microscopy it was shown that *Wolbachia* were present in the muscle and testis of *D.*

*simulans* (Fig 1 and 2) and in the eye (Fig 3), brain, muscle, ovary, and testes of *D. melanogaster* (Fig 4-12). Flies used for examination is about 20 individual flies. Numbers of bacteria found in different kind of tissues of *D. melanogaster* and *D. simulans* (Fig 1-12).

The ultrastructure of *Wolbachia* in *D. 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* reveals only two basic forms. The most common form is an elongated cylinder with convex ends typical of bacterial rods. The second form of *Wolbachia* that has been encountered is a barrel shape that is oval like an egg. The bent rod shape is often discovered in muscle (Fig 1 and 6) tissues and eye (Fig 3). The size varies between 0.04  $\mu\text{m}$  and 0.47  $\mu\text{m}$  in diameter and between 0.26  $\mu\text{m}$  and 1.2  $\mu\text{m}$  in length.

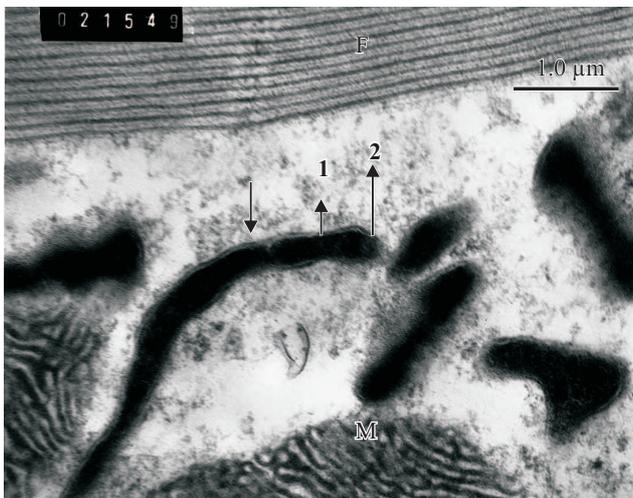


Fig 1 *Drosophila simulans* (DSR) muscle tissue infected with electron dense *Wolbachia*. Arrow, this could be four *Wolbachia* joined together by an isthmus (x 53 000). These could be four *Wolbachia* joined together or the bacteria have been undergoing division and one still connected by an isthmus of cytoplasm. The diameter of *Wolbachia* is 0.12  $\mu\text{m}$  and the length ranges from 0.3 to 0.5  $\mu\text{m}$ . The cells are oval, bent rods, straight rods or elongate in form. These bacteria are enveloped by host (1) and bacterial (2) membranes. M, mitochondria; and F, fibril of muscle.

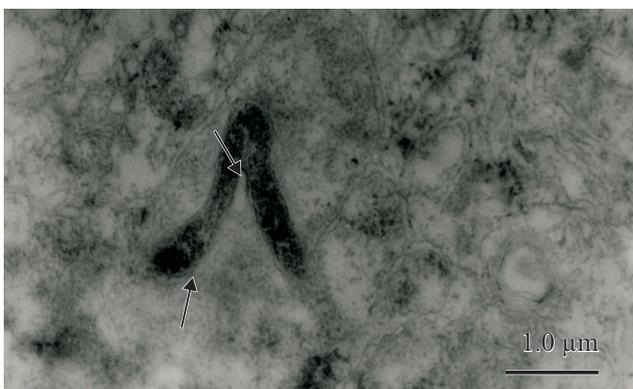


Fig 2 Two tightly-connected, electron dense *Wolbachia* in *D. simulans* testis (x 42 000). Arrow: *Wolbachia* sharing a host membrane and appear to have been undergoing fission. The diameter of *Wolbachia* ranges from 0.14 to 0.19  $\mu\text{m}$  and the length was 0.8 and 0.9  $\mu\text{m}$ .

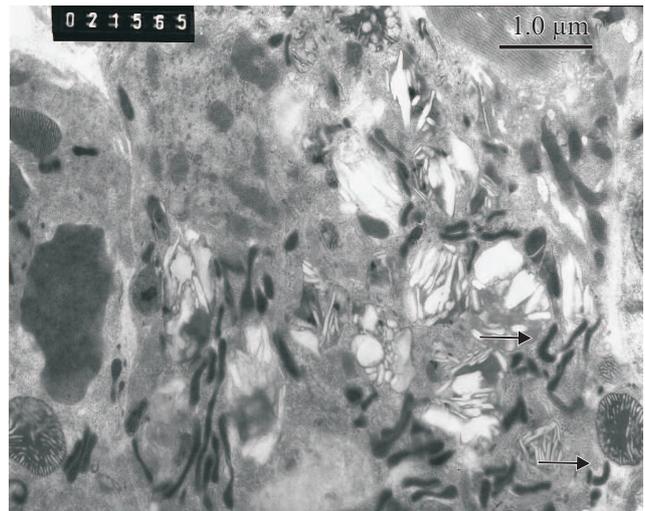


Fig 3 Electron dense *Wolbachia* in *D. melanogaster* ( $w^{1118}$ ) eye (x 21 000). Arrow: *Wolbachia*. The cells are slightly bent, straight rods or of oval shape. The diameter of *Wolbachia* ranges from 0.08 to 0.12  $\mu\text{m}$  and the length ranges from 0.3 to 0.56  $\mu\text{m}$ . These bacterial is enveloped by bacterial membrane.

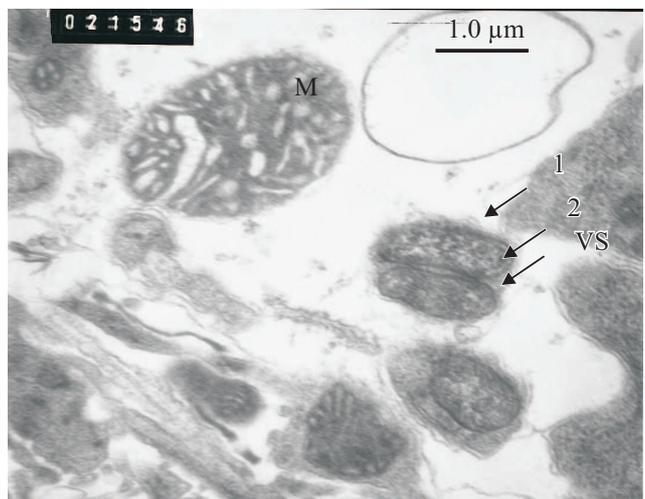


Fig 4 Two tightly-connected, electron dense *Wolbachia* in the *D. melanogaster* ( $w^{1118}$ ) brain sharing an irregular host membrane and appear to have been undergoing fission. The diameter of *Wolbachia* is 0.15  $\mu\text{m}$  and the length ranges from 0.4 to 0.43  $\mu\text{m}$ . There is a vacuolar space between the vacuole membrane (1) and the bacterial membrane (2); M, mitochondria.

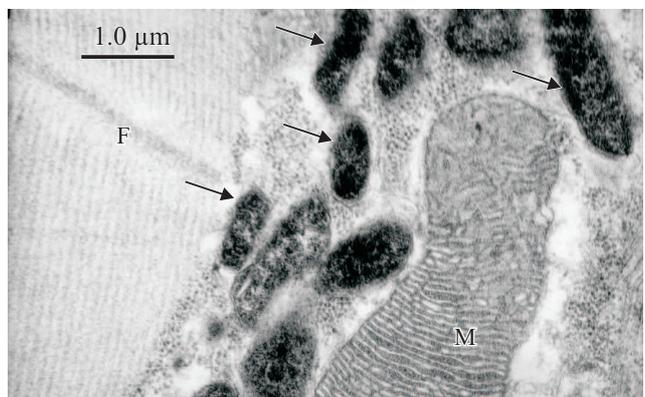


Fig 5 *D. melanogaster* ( $w^{1118}$ ) muscle tissue infected with electron dense *Wolbachia*. The cells are oval or rod shaped, surrounded by mitochondria (M). The diameter of *Wolbachia* ranges from 0.15 to 0.4  $\mu\text{m}$  and the length ranges from 0.3 to 1.2  $\mu\text{m}$ . These bacteria is enveloped by bacterial membrane. (x 36 000). M, mitochondria; F, fibril of muscle; arrow, *Wolbachia*.

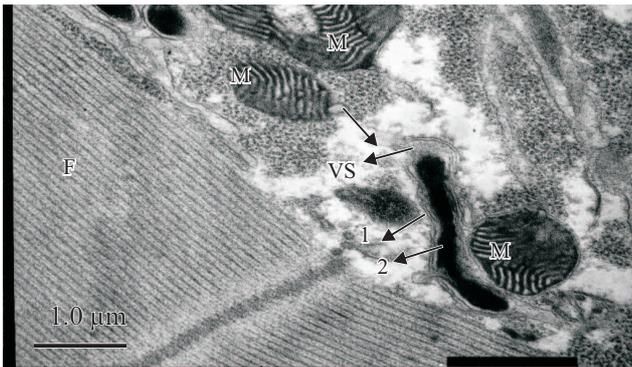


Fig 6 *Drosophila melanogaster* ( $w^{1118}$ ) muscle tissue infected with electron dense *Wolbachia* enveloped by a double membrane, which could be part of the endoplasmic reticulum or part of the vacuolar host membrane and the third membrane is the bacterial membrane. There is a vacuolar space (vs) between the vacuole membrane (1) and the bacterial membrane (2). 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 0.12  $\mu\text{m}$  and the length is 0.8  $\mu\text{m}$  (x 36 000). M, mitochondria; F, fibril of muscle; arrow: *Wolbachia*.

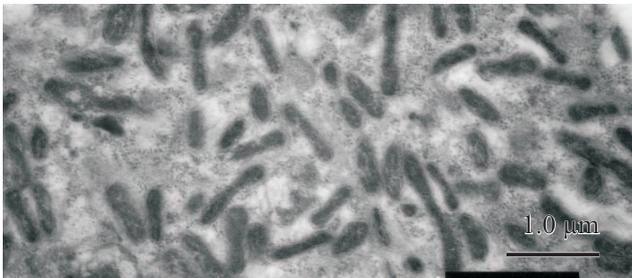


Fig 7 A high magnification image of *Wolbachia* emphasising the enormous numbers of electron dense *Wolbachia* in the waste basket of the testis of *D. melanogaster* ( $w^{1118}$ ) showing the bacterial membrane (x 40 000). The cells are oval, rod shaped or elongate. The diameter of *Wolbachia* ranges from 0.04 to 0.07  $\mu\text{m}$  and the length ranges from 0.24 to 0.33  $\mu\text{m}$ .

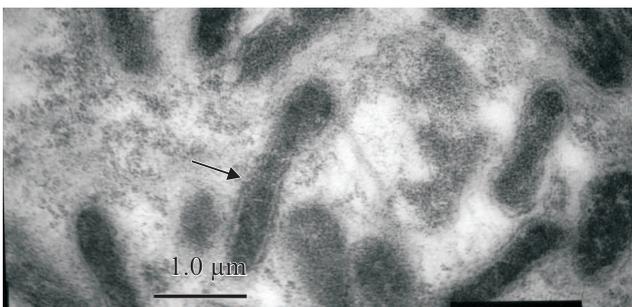


Fig 8 Using higher magnification (x 96 000) two membranes (host membrane and bacteria membranes). Stresses that even on the waste basket every single *Wolbachia* cell is still enveloped by a host membrane. The diameter of *Wolbachia* ranges from 0.07 to 0.08  $\mu\text{m}$  and the length ranges from 0.28 to 0.38  $\mu\text{m}$ .

## DISCUSSION

All measurement of length and diameter will very widely, the result show between 0.26  $\mu\text{m}$  and 1.2  $\mu\text{m}$ . Hertig (1936) described the characteristics of *Wolbachia*, was exhibiting an enlarged form, usually 0.5-1.3  $\mu\text{m}$  in diameter. The precise orientation of *Wolbachia* in the plane of the picture is uncertain. It is not possible to define whether any transection is exactly in the mid plane or at any other location. All

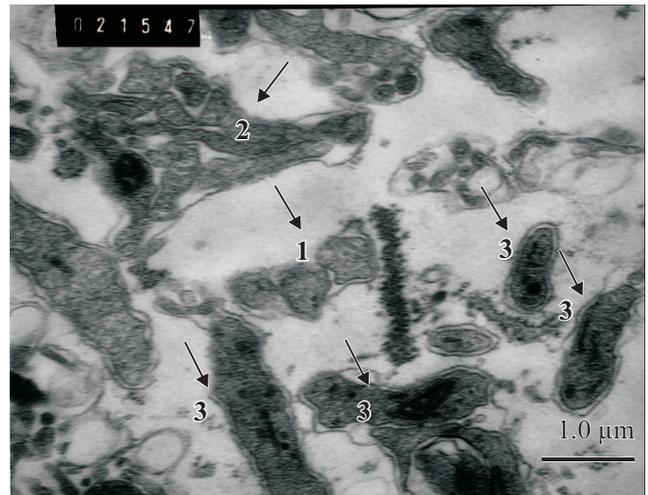


Fig 9 An unidentified membrane-bound structure surrounded by host membrane in *D. melanogaster* ( $w^{1118}$ ) ovary. Arrow 2, this could be a group (11 organisms) of rod shape, electron light *Wolbachia*. Arrow, points to round structure and presumably this is a transection of *Wolbachia*. The arrow 3 points to electron dense *Wolbachia* sharing a vacuolar membrane. The diameter of *Wolbachia* ranges from 0.1-0.17  $\mu\text{m}$  and the length ranges from 0.28 to 0.69  $\mu\text{m}$ . (x 49 000).

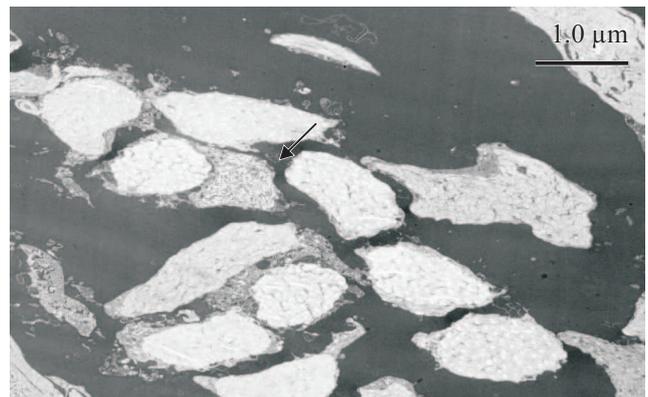


Fig 10 The removal electron dense of *Wolbachia* and cytoplasm from the developing spermatid of the testis *D. melanogaster* ( $w^{1118}$ ). The structure receiving the cytoplasm and *Wolbachia* is called a waste basket. The *Wolbachia* cells are oval, rod shaped or elongate.

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 (Fig 1, 2, 4, 6).

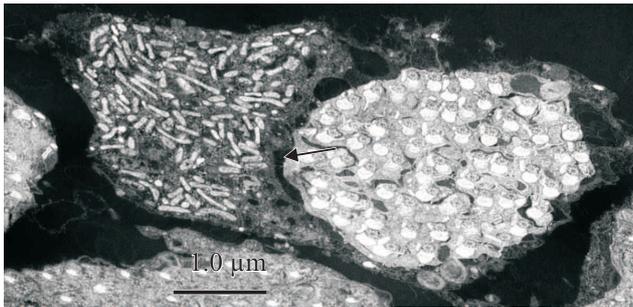


Fig 11 The removal of electron dense *Wolbachia* and cytoplasm from the developing spermatid of the testis of *Drosophila melanogaster* ( $w^{118}$ ) (x 16000). This picture shows the same object as Fig 12 but on a positive film.

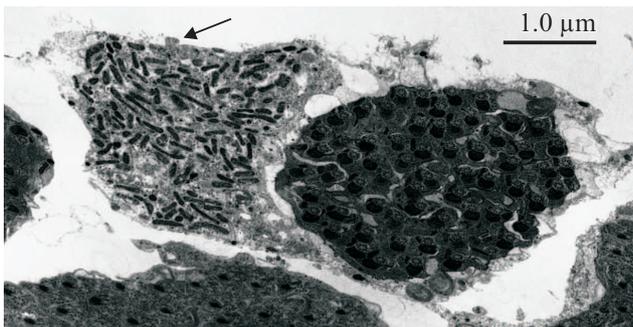


Fig 12 The removal of electron dense *Wolbachia* and cytoplasm from the developing spermatid of the testis of *D. melanogaster* ( $w^{118}$ ) on negative film. The structure receiving the cytoplasm and *Wolbachia* is called a waste basket. The *Wolbachia* cells are oval, rod shaped or elongate.

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 (Fig 4, 6). In one case Fig 6, a double membrane could be clearly seen as part of the host membrane and the third membrane being the bacterial membrane. Furthermore, in Fig 1, 2, 4, and 6 the shape of the bacteria as bent and probably constituted two bacteria (Fig 2, 4), three bacteria (Fig 6) or four bacteria (Fig 1) 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 (Fig 2, 3, 5, 7), however, when the picture was taken using higher magnification (x 96 000) (Fig 8), 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. Zhukova (2008) was also reported that essential envelope disturbances and other structural alterations have been revealed in the bacteria

present in the ovarian cell cytoplasm of the flies. It was found an unidentified membrane-bound structure in the ovary tissue of *D. melanogaster* (Fig 9). It probably is a group of 11 organisms of electron dense light *Wolbachia* (arrow 2). The arrow 1 in Fig 9 points to presumably 3 round electron light *Wolbachia* and the arrows 3 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 (1986) also reported that *Wolbachia* appeared to be absent from sperm of *D. 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 in *Dahlbominus fuscipennis* were never seen in mature spermatozoa and that these microorganisms were probably lost along with excess cytoplasm.

Here, it was shown *Wolbachia* in the testis of *D. melanogaster* (Fig 10,11, 12), where *Wolbachia* and cytoplasm are excluded from the mature sperm. The structure receiving the cytoplasm and *Wolbachia* is called a waste basket. This situation is apparently similar to that in *C. 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 (testis). They were also found in somatic tissue (muscle). The ultrastructure of *Wolbachia* were similar to the description of *W. pipientis* from *D. melanogaster*, including the occurrence of a cytoplasmic isthmus connecting two cells (Fig 2, 4) or four cells (Fig 1) at a late stage of division. Especially in Fig 1 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. These 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*.

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