The mitochondrial heat shock protein 60 (HSP60) is up-regulated in *Onchocerca volvulus* after the depletion of *Wolbachia*

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SUMMARY

Wolbachia, a genus of endosymbiotic bacteria of filarial worms, represent novel targets for anti-filarial therapy. The efficacy of compounds against *Wolbachia* has been evaluated using antiserum raised against the 60 kDa heat shock protein (HSP60) which binds specifically to this protein in both *Wolbachia* and mitochondria. It has been shown that *Wolbachia* stains (using such specific probes) stronger than the mitochondria in untreated *Onchocerca volvulus*, whereas after the depletion of *Wolbachia* (with drugs) staining of the mitochondria is increased. Herein, immunogold electron microscopy showed that specific anti-HSP60 serum specifically labelled *Wolbachia* and filarial mitochondria, and that both have distinct localization patterns, thus allowing them to be differentiated. Immunohistochemistry of *O. volvulus* showed that HSP60 staining is increased in the mitochondria after *Wolbachia* depletion in the hypodermis, epithelia, muscles, oocytes, embryos, and developing spermatozoa. This could have been the result of the antiserum preferentially binding to the *Wolbachia* when they are present or due to increased expression of the protein in the absence of the bacteria. To address this, mRNA levels of filarial *hsp60* in *O. volvulus* were measured. After the depletion of *Wolbachia*, the transcription of *hsp60* was significantly greater (7·7 fold) compared with untreated worms. We hypothesize that the increased expression of HSP60 in the absence of *Wolbachia* is due to a disruption of the homeostasis of the endosymbiosis.

Key words: heat-shock protein 60, *Wolbachia*, filariae, *Onchocerca volvulus*, mitochondria.

INTRODUCTION

Onchocerciasis is a major public health problem in many developing countries in the tropics. In endemic areas, it is estimated that 40 million people are infected with *Onchocerca volvulus* (see Basañez *et al*. 2006; WHO, 2007). Current control regimes aim to interrupt transmission by reducing the levels of microfilariae in infected individuals. Since adult worms can survive up to 15 years, chemotherapy to control microfilariae requires multiple years of treatment with anti-microfilarial drugs. A goal of the World Health Organization is the development of new therapeutics which (i) kill microfilaria in a shorter time, (ii) sterilize or kill adult worms, (iii) can replace ivermectin for treating patients with onchoceriasis/loaiasis and/or (iv) be used if ivermectin resistance is present in worms (WHO, 2007).

Endosymbionts of the genus *Wolbachia* within a filarioid nematode were first described in the 1970s (Kozek and Figueroa Marroquin, 1977). These endosymbionts have been found in a range of species, including the major causative agents of human filariasis (Bandi *et al*. 1998; Taylor and Hoerauf, 1999). In *O. volvulus*, the bacteria are located in the hypodermis of the adult worms (both sexes), in the oocytes, developing embryos and all larval stages. The muscles, the epithelia of the digestive and genital tracts, and the sperm are devoid of *Wolbachia* (Kozek and Figueroa Marroquin, 1977; Franz, 1988; Büttner *et al*. 2003). The presence of *Wolbachia* within filariae led to the innovation of using tetracycline therapy to deplete the endosymbionts from worms (Molyneux *et al*. 2003; Taylor *et al*. 2005*a*; Hoerauf, 2006). This depletion of the endobacteria causes the degeneration of embryos, the sterilization of female worms and eventually leads to the death of adult worms (Hoerauf *et al*. 1999; Taylor *et al*. 2005*b*).

Although the hypodermis of filarial worms is syncytial, *Wolbachia* endobacteria are found in distinct zones (Franz and Büttner, 1983; Franz, 1988). An examination of an electron microscopic section of the hypodermis (with the cuticle oriented upward) reveals that there is an outer zone under the cuticle that is usually free from *Wolbachia*

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but filled with mitochondria that are densely packed together (Supplemental Fig. 1, Online version only), followed by a zone containing a low-density mix of *Wolbachia* and mitochondria. Finally, there is an inner zone with many *Wolbachia*. The ultrastructure of *Wolbachia* is morphologically distinct from that of mitochondria, being both larger (0·3–0·6 μm), surrounded by a membrane, and not as heavily stained as mitochondria. The internal structures of the endobacteria are also different from those of mitochondria, i.e. lacking the characteristic cristae of mitochondria.

Although *Wolbachia* have been detected using specific antiserum against *Wolbachia* surface protein (WSP) (Büttner *et al*. 2003; Kramer *et al*. 2003; Brattig *et al*. 2004), much of the published immunohistochemistry (both light and electron microscopic) has used various antisera against the heat shock-protein 60 (HSP60) (Hoerauf *et al*. 1999; Taylor and Hoerauf, 1999; Hoerauf, 2000; Büttner *et al*. 2003). In all cases, *Wolbachia* are seen as distinct spots in the middle and inner zones of the hypodermis and in the oocytes and embryos, but not in other tissues.

Examination by immunohistochemistry has proven invaluable for the monitoring of the efficacy of tetracyclines in depleting *Wolbachia* (Hoerauf *et al*. 1999; Hoerauf, 2000). In some instances, diffuse staining in the outer zone of the hypodermis or in the embryos in the uterus was sometimes seen following the depletion of the *Wolbachia*. This has been attributed to mitochondrial HSP60, but has not been examined. Therefore, in the present study, we examined whether mitochondrial HSP60 was specifically labelled by anti-*Yersinia* HSP60 antiserum and whether this related to the up-regulation of mitochondrial HSP60 expression in *O. volvulus*.

MATERIALS AND METHODS

Worms from human patients

Onchocercomas (=subcutaneous nodules that develop around *O. volvulus*) were collected from patients treated 6 weeks with 100 mg/day doxycycline alone or 100 mg/day doxycycline plus once with 0·15 mg/kg/day ivermectin, once with 0·15 mg/kg/day ivermectin alone or untreated, as published previously (Hoerauf *et al*. 2003). The study design was approved by both the ethics committees of the Medical Board of Hamburg, Germany as well as the School of Medical Sciences of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The procedures used were in accordance with the Declaration of Helsinki (1975 and its revisions in 1983 and 2000). Onchocercomas were extirpated 5, 12 or 18 months following the commencement of doxycycline treatment.

Worms from animals

To support the hypothesis that tetracycline has no direct effect on the embryogenesis of filariae that do not contain *Wolbachia*, *Acanthocheilonema viteae* in *Mastomys coucha* were treated as described previously (Hoerauf *et al*. 1999). Also, onchocercomas containing *Onchocerca flexuosa* (another filarial nematode without endobacteria) were collected from red deer (*Cervus elaphus*) in northern Germany (Plenge-Bönig *et al*. 1995).

Immunohistochemistry

Acanthocheilonema viteae worms and onchocercomas extirpated from patients or red deer were fixed in 4% buffered formaldehyde and embedded in paraffin. Blocks were cut in 5 μm sections and blocked with 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS). *Wolbachia* were probed with antiserum raised in rabbits against HSP60 from *Yersinia enterocolitica* (anti-Y-HSP60; diluted 1 : 1000 in TBS, 0·1% BSA) or the *Wolbachia* surface protein (WSP, diluted 1 : 4000) from *Dirofilaria immitis* as described in previous studies (Büttner *et al*. 2003; Hoerauf *et al*. 2003; Kramer *et al*. 2003; Brattig *et al*. 2004). After washing with TBS containing 0·1% Tween-20, anti-rabbit polyclonal antibody (DakoCytomation, Hamburg, Germany; D0651) was used as secondary antibody according to manufacturer's recommendations for the Alkaline Phosphatase Anti-Alkaline Phosphatase kit (APAAP) (DakoCytomation). Fast red TR salt (Sigma, Deisenhofen, Germany) was used as the chromogen and haematoxylin (Merck, Darmstadt, Germany) served as the counter-stain. The specificity of the primary antibodies was verified using a different specific antiserum or buffer instead of antiserum on consecutive sections of the respective paraffin block.

Immunogold electron microscopy

For immunogold staining, onchocercomas were fixed in 2% paraformaldehyde/0·025% glutaraldehyde and embedded in LR-White (Merck, Darmstadt, Germany). Ultra-thin sections were cut with diamond knives using a Reichert-Jung Ultra Cut E (Leica, Bensheim, Germany). Sections were then blocked for 30 min in 10% BSA in phosphate-buffered saline (PBS). The sections were incubated with rabbit anti-Y-HSP60 serum diluted 1 : 500 in PBS with 1% BSA, followed by incubation with protein A-gold (10 nm in size) diluted 1 : 50 in PBS, 1% BSA. Sections were washed after each step with PBS, 1% BSA. Staining of subcellular structures was performed in 1% uranyl acetate and lead-citrate and then examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands). The specificity of the staining was verified using

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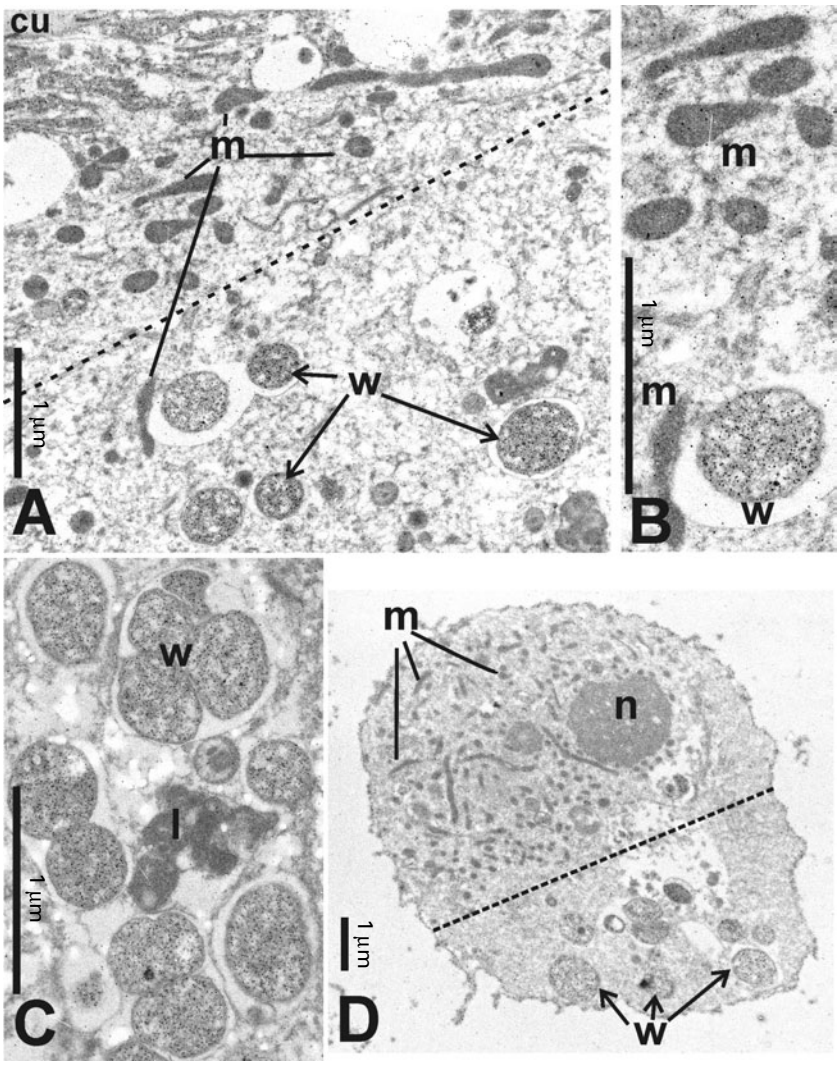

Fig. 1. Immunogold, electron microscopy of female *Onchocerca volvulus* worms in nodules from untreated patients were incubated with anti-Y-HSP60 serum to specifically localize HSP60. (A) Mitochondria (lines) are located in the outer zone of the hypodermis and *Wolbachia* (arrows) are located in the middle zone. There is also weak labelling of the outer folds of the hypodermal membrane (near to the cuticle in the upper left corner). (B) Detail of (A) showing the weaker labelling of the mitochondria and the stronger labelling of the *Wolbachia* indicative of more HSP 60 in the endobacteria. (C) In another section of the hypodermis the *Wolbachia* are strongly labelled but the lysosomes in the same zone, and often of a similar size, are not labelled. (D) *Wolbachia* are also localized to a single area of an oocyte. Dashed lines represent the division between regions where mitochondria and the endobacteria are found. cu, Cuticle; l, lysosome; m, mitochondria; n, nucleus; u, uterus; w, *Wolbachia*.

a specific antiserum (raised in rabbits) against glutathione S-transferase of *O. volvulus* (Ov-GST) (unpublished data) and diluted 1 : 500 in PBS with 1% BSA as a negative control.

Quantitative PCR

Total RNA was isolated from onchocercomas (16 untreated controls and 23 doxycycline treated) extirpated prior to ivermectin treatment, 5 months after the commencement of doxycycline treatment using the RNeasy Maxi Kit (Qiagen, Hilden, Germany). The collagenase protocol for heart and muscle tissue was followed. DNase treatment of the RNA was performed on the column. One microgram of RNA was reverse transcribed with oligo dT primers (Invitrogen, Karlsruhe, Germany) using the OmniScript kit (Qiagen). PCR primers specific to the mitochondrial gene *hsp60* of *O. volvulus* (GenBank Accession number AF121264) were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Briefly, the PCR was performed in 20 μl, containing a standard buffer (Qiagen), 4·5 mM of $MgCl_2$, 50 μM of each dNTP, 900 nM of the forward primer (CCG GTA TGG GAG GAA TGT ACT), 300 nM of the reverse primer (TAA GAC TCC CTG TTC GCT CAA), 0·2 μl of SYBR Green (1 : 1000 diluted in DMSO, Roche, Mannheim, Germany), 0·8 μg bovine serum albumin (BSA) (Roche), 2·5 U of HotStar *Taq* polymerase (Qiagen) and 2 μl of cDNA or RNA sample in the cDNA buffer without reverse

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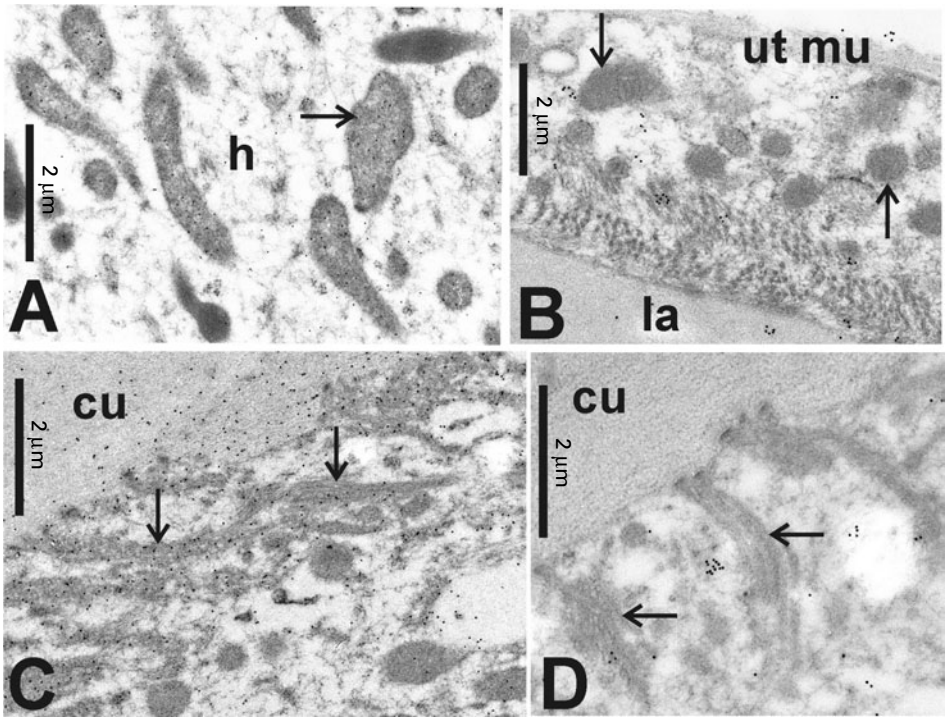

Fig. 2. Rabbit anti-HSP60 serum and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) reagents are specific. Labelling of mitochondria in the hypodermis (A, arrow), the outer folds of the hypodermal membrane (C, arrows) and the cuticle is seen in some worms and can be misjudged as non-specific staining. However, the mitochondria of a muscle cell of the uterus (B, arrows) and the lamina incubated with another rabbit antiserum against an Ov-GST are not stained. (D) Labelling of the cuticle and hypodermal membrane is also not seen with the Ov-GST antiserum (arrows). Patient was treated with ivermectin 3 days before the excision of the nodule. cu, Cuticle; hy, hypodermis; la, lamina of the uterus epithelium, ut mu, uterus muscle cell.

transcriptase as a control for genomic DNA contamination. Cycling was performed in a Light Cycler 1.0 (Roche) using the following protocol: 95 °C for 15 min, followed by 40 cycles of 94 °C for 20 sec, 58 °C for 20 sec, 72 °C for 20 sec, and 75 °C for 10 sec, the fluorescence being acquired on the FAM channel. The additional temperature step at 75 °C ensured that the signal recorded related to *hsp60* rather than primer dimers, which are sometimes detected when cDNA amounts are limiting.

The level of transcription (copies per μl) from the *hsp60* gene was estimated by comparing crossing point data for the test samples to a standard curve determined using a plasmid containing the mitochondrial *hsp60* fragment produced by PCR using the *hsp60* primers, as described previously for other genes (Heider *et al*. 2006). Transcription levels for *hsp60* were normalized against actin, as described recently (Gilbert *et al*. 2005).

Statistics

Significant differences in *hsp60* transcription were tested using the Mann-Whitney non-parametric test employing GraphPad Prism v.4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. Significance was set at $P \leqslant 0{\cdot}05$.

RESULTS

Localization of Wolbachia *using immunogold electron microscopy*

Previously, the depletion of *Wolbachia* has been monitored by immunohistochemistry using specific antisera against bacterial HSP60 and WSP (Hoerauf *et al*. 1999). To confirm the specificity of anti-Y-HSP60 serum for the endobacteria and mitochondria, the antiserum was used for immunogold staining of ultra-thin sections of worms in onchocercomas from patients not treated with doxycycline. It should be noted that 1 patient had been treated with ivermectin 3 days before the excision of the nodule. Using the anti-Y-HSP60 serum, the typical localization pattern of *Wolbachia* and mitochondria was detected, in accordance with previous results (Kozek and Figueroa Marroquin, 1977; Franz and Büttner, 1983; Franz, 1988; Büttner *et al*. 2003) (Fig. 1A). In the hypodermis, mitochondria are concentrated under the cuticle and become sparser in the middle zone where *Wolbachia* are also found (Fig. 1A). The anti-Y-HSP60 serum appeared to detect *Wolbachia* more intensely than the mitochondria (Fig. 1B).

From the immunochemical staining, it was apparent that the antiserum did not react with other organelles, such as lysosomes, granular structures

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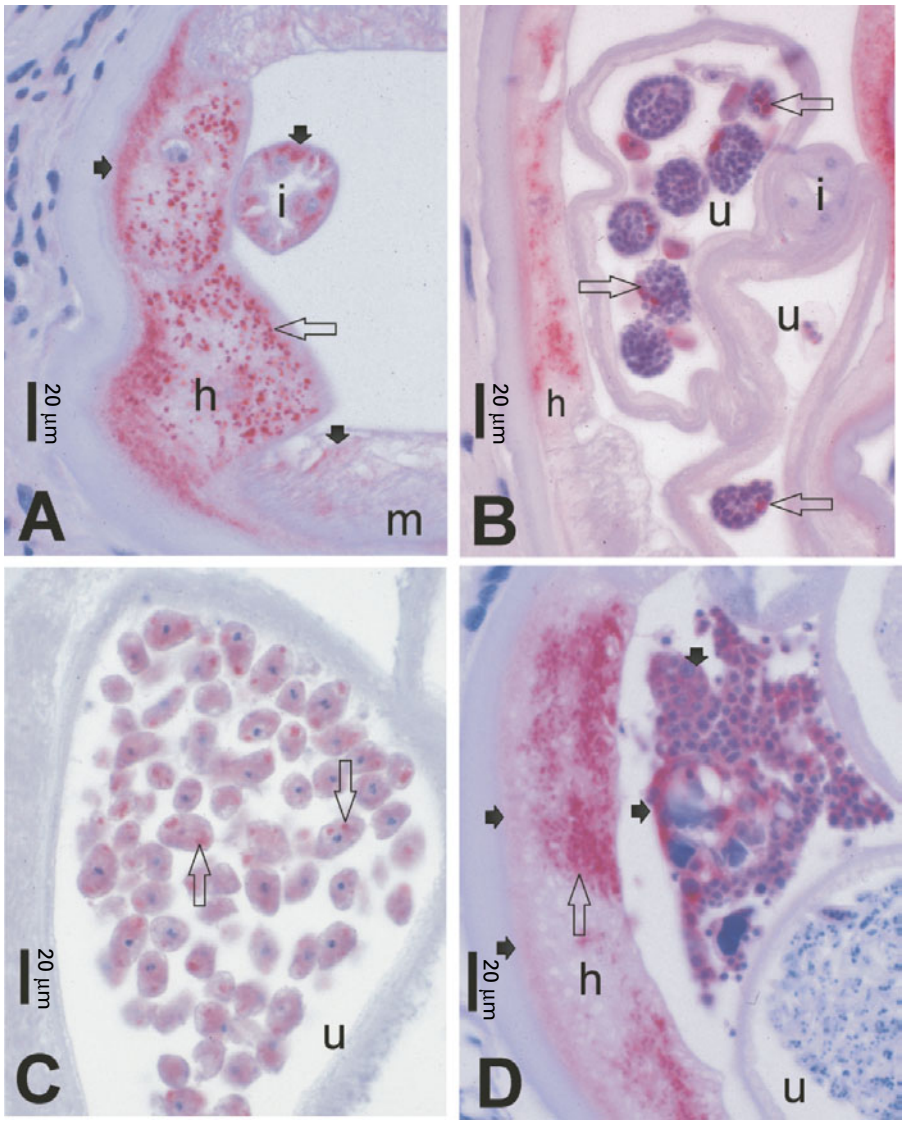

Fig. 3. Immunohistochemical labelling of HSP60 in *Wolbachia* endobacteria and in the tissues of female *Onchocerca volvulus* after treatment with ivermectin (A, 20 months; B, 5 months) or untreated. Granular endobacteria in the hypodermis (open arrows in A, B and D), in morulae (B) and in oocytes in the uterus (C) are strongly labelled. The mitochondria of the filaria are weakly labelled in the outer zone of the hypodermis (closed arrows in A), in the intestine (A), and in neoplastic cells in the pseudocoeloma (D). Stronger labelling of the intestinal mitochondria after ivermectin (A) is often seen. h, Hypodermis; i, intestine; m, muscle; u, uterus.

also located in the middle layer of the hypodermis, or the cytoplasm of the hypodermis and the epithelia of *O. volvulus* (Fig. 1C). There was weak labelling of the folded outer cell membrane of the hypodermis and the cuticle (Fig. 1A and 2C). The specific localization of both *Wolbachia* and mitochondria was even more pronounced when oocytes were examined. *Wolbachia* were detected near one or both ends of the oocytes, whereas the mitochondria were localized to the rest of the *Wolbachia*-free cytoplasm surrounding the chromosomes (Fig. 1D). Mitochondria were detected using HSP60 antiserum but not with the antiserum Ov-GST on sections from the same block (*cf.* Fig. 2A and B). Also, the cuticle and the outer membrane of the hypodermis were stained for HSP60 but not for glutathione S-transferase (Fig. 2C and D), although both antisera were detected with the same secondary antibody, showing the specificity of the anti-Y-HSP60 antibody for *Wolbachia*.

Anti-Y-HSP60 labelling of O. volvulus *mitochondrial HSP60 increases when* Wolbachia *are depleted*

Using the specific anti-Y-HSP60 serum, granular staining of *Wolbachia* was seen in the hypodermis, oocytes, as well as in embryos of filariae not exposed to doxycycline (Fig. 3A–D, open arrows). Weak staining, representing mitochondrial HSP60, was also seen in worms not exposed or exposed to ivermectin. This staining was diffuse in the outer zone of the hypodermis (Fig. 3, panels A and D, closed

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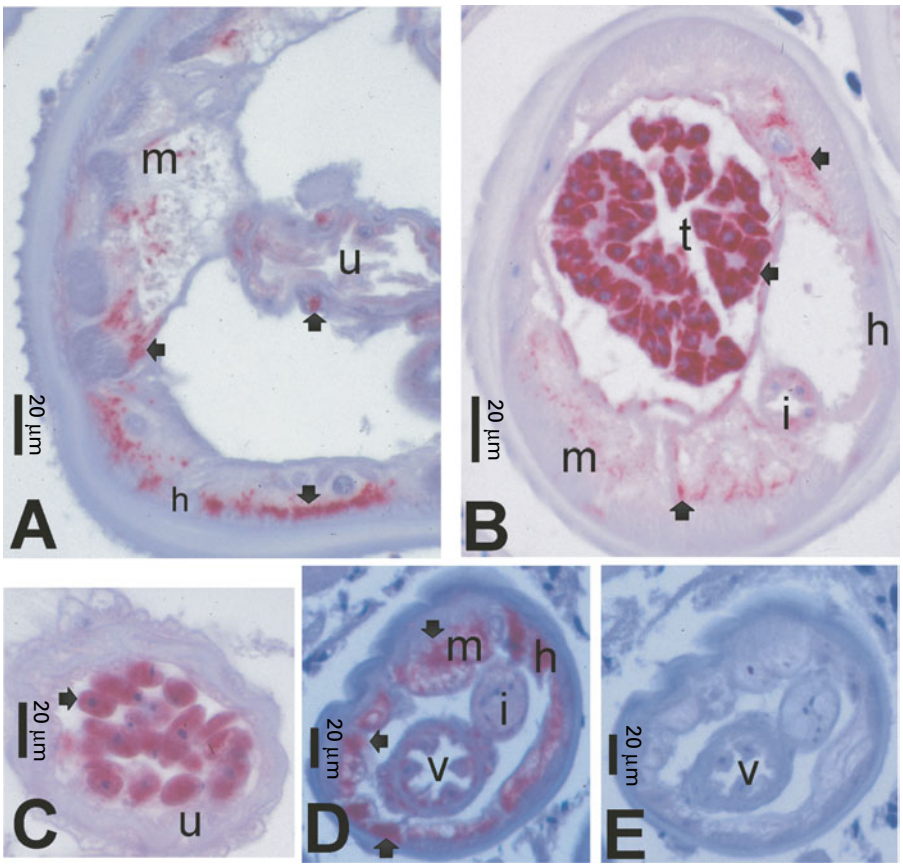

Fig. 4. Female and male *Onchocerca volvulus* collected 5 (A, C) or 18 months (B, D, E) after doxycycline treatment showing increased labelling of filarial mitochondrial HSP60 in the hypodermis (closed arrows in A and D), muscles of the body wall (A, B, D), muscle cells of uterus (A) or vagina (D), the entire oocytes (C, in contrast to the granular bacteria in Fig. 3C), and developing spermatozoa in the testis (B). (D + E) Serial sections of a female worm demonstrated that endobacteria were absent by staining with antiserum against *Wolbachia* surface protein (E), and is also a negative control for the specificity of the HSP60 antiserum in (D). h, Hypodermis; i, intestine; m, muscles; t, testis; u, uterus, v, vagina.

arrows), the epithelia of the intestine (Fig. 3A) and genital tracts, muscles of the body wall (Fig. 3A) and uterus, oocytes (Fig. 3C), embryos (Fig. 3B), and the developing spermatozoa, but not in elongated, mature spermatozoa. The intensity of staining varied greatly, being strongest in portions of the filaria in which no bacteria are present and often in moribund worms, such as those with neoplastic cells in the pseudocoeloma (Fig. 3D; *cf*. Duke *et al*. 2002). Also, ivermectin-exposed worms more frequently showed an increase of mitochondrial HSP60 (intestine in Fig. 3A) compared with untreated worms (Fig. 3, panels C and D).

The examination of sections from onchocerciasis patients treated for 6 weeks with doxycycline showed that the hypodermis, oocytes and embryos were devoid of *Wolbachia* (Fig. 4, panels A–E). These worms showed a noticeable increase in mitochondrial HSP60 staining in the outer zone of the hypodermis, the oocytes and embryos. The labelled mitochondria appeared as small dots or as a diffuse staining that were distinct from the granular or ring-like structures of labelled *Wolbachia*. The diffuse staining was probably due to the labelled outer membrane folding of the hypodermis (lamellae). Tissues that were unstained or only lightly stained in untreated worms also had increased mitochondrial HSP60 expression (Fig. 4A–D, closed arrows). The staining seen in doxycycline-treated worms was not due to *Wolbachia*. Anti-Y-HSP60 serum specifically labelled the muscles in worms from patients who had received doxycycline (Fig. 4, panels A, B and D). However, serial sections labelled with anti-WSP serum, specific for bacteria, showed clearly that the *Wolbachia* had been depleted from these worms (Fig. 4E), indicating that the staining detected using the anti-Y-HSP60 serum represented the filarial mitochondria. The comparison of mitochondria in consecutive sections labelled using the anti-Y-HSP60 but not the WSP antiserum demonstrated the specificity of the antibody probes (*cf*. Fig. 4, panels D and E).

We hypothesize that the increase in mitochondrial HSP60 was not a direct effect of the antibiotic on the filariae but due to the depletion of the *Wolbachia* from the nematodes. After the administration of

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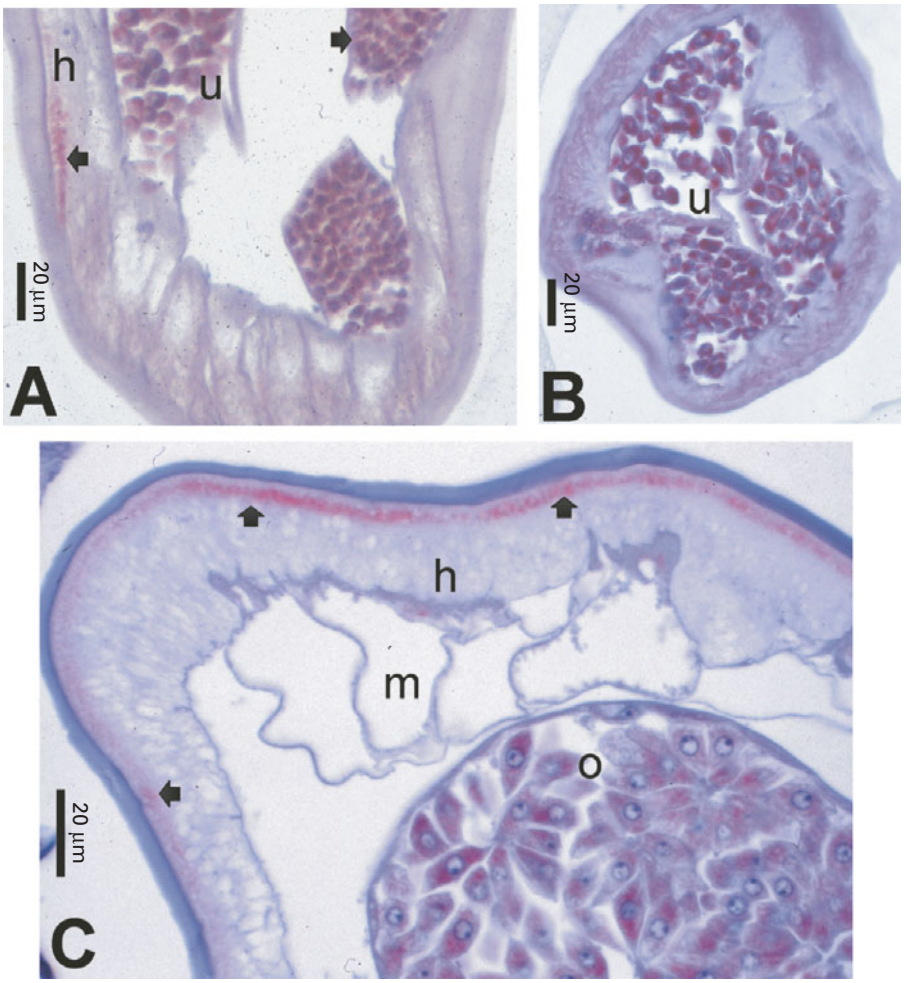

Fig. 5. (A and B) No difference is seen in the labelling of filarial HSP60 in untreated (closed arrow, A) and treated (B) female *Acanthocheilonemea viteae*, a species without *Wolbachia* (after 70 days treatment with 25 mg/kg/day tetracycline). (C) In an untreated female *Onchocerca flexuosa*, another species without endobacteria, only the outer zone of the hypodermis (closed arrow) and the entire cytoplasm of oocytes in the ovary are labelled for filarial HSP60. h, Hypodermis; m, muscles; o, ovary; u, uterus.

doxycycline for 2 weeks, a treatment that does not deplete *Wolbachia*, increased staining of the mitochondria was not detected at any point of time up to 30 months after treatment. In contrast, after 6 weeks of doxycycline administration, a regimen that depletes *Wolbachia*, labelling increased at 18 and 24 months. We cannot exclude damage to the worms from the 2 weeks of doxycycline treatment, but the worms did not show more damage than that seen in untreated worms (data not shown).

Acanthocheilonemea viteae (subfamily Onchocercinae) does not contain endobacteria (Hoerauf *et al*. 1999). Comparing sections from untreated (Fig. 5A) and tetracycline-treated *A. viteae* (Fig. 5B), there was no increase in HSP60 staining in worms. The staining in the oocytes, muscles and gut was the same between both groups.

Onchocerca flexuosa, is another species that does not harbour *Wolbachia* (see Plenge-Bönig *et al*. 1995). Untreated female *O. flexuosa* often showed strong labelling in the entire cell of the oocytes and some staining in the outer hypodermis using anti-Y-HSP60 serum, but no labelling in regions of the hypodermis where *Wolbachia* are known to be found (Fig. 5C, compare with Fig. 2). The experiments using *A. viteae* and *O. flexuosa* support the hypothesis that the increased labelling of HSP60 in the mitochondria of *O. volvulus* is seen after depletion of *Wolbachia*.

Quantification of hsp60 transcription following Wolbachia *depletion*

One argument for the increase in mitochondrial HSP60-staining could be that the specific antiserum has a higher affinity for the bacterial HSP60 and thus only stains weakly the mitochondrial HSP60 until the *Wolbachia* are depleted. To address this, we measured *hsp60* transcription from onchocercomas extirpated from untreated and doxycycline-treated patients 5 months after the commencement of doxycycline treatment to demonstrate that the increase in mitochondrial HSP60 is also reflected at the level of transcription. The primers to the gene

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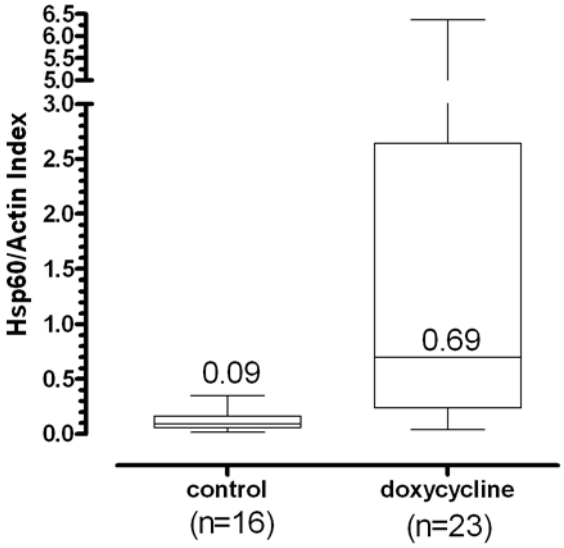

Fig. 6. Filarial mitochondrial *hsp60* transcription is up-regulated after depletion of *Wolbachia* endosymbionts from *O. volvulus*. Up-regulation of mitochondrial *hsp60* at the mRNA level was demonstrated by quantitative PCR using the ratio of filarial *hsp60* to actin. The line in the boxes represents the median value (the exact value represented by the number in each column); the bottom and top of the boxes represent the 25th and 75th percentiles, respectively; the lower and upper lines represent the minimum and maximum values, respectively. $P = 0{\cdot}0002$, as determined by the Mann-Whitney test.

hsp60 had no similarity to the sequence of the *hsp60* gene of *Wolbachia*, as determined by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). The actin primers were tested on human cDNA and did not amplify the human gene (data not shown). The *hsp60* index generated by quantitative PCR clearly showed a significant increase (7·7 fold; $P = 0{\cdot}0002$) in *hsp60* transcription after *Wolbachia* had been depleted by doxycycline treatment (Fig. 6).

DISCUSSION

Using the specific anti-Y-HSP60 serum, we were able to show that mainly HSP60 from *Wolbachia*, the filarial mitochondria, the outer folding of the hypodermis, and the cuticle are labelled, but not other organelles. Using electron microscopy, we confirmed that the mitochondria and *Wolbachia* localized to discrete regions of the hypodermis, the oocytes, zygotes, and embryos, thus allowing the differentiation of *Wolbachia* from mitochondria based on their location in the worm (*cf.* Franz and Büttner, 1983; Franz, 1988).

Strong mitochondrial HSP60-labelling was also detected in some worms from untreated persons. These latter worms were classified moribund, as the worms contained neoplastic cells in the pseudocoeloma (*cf.* Duke *et al.* 2002). Increased HSP60 expression in such worms is probably related to a normal stress response to the uncontrolled growth of the neoplastic cells. Increased HSP60 labelling was also detected in developing (but not mature) spermatozoa within the testis of male worms depleted of *Wolbachia*. This result was unexpected because *Wolbachia* in filariae had not been reported to occur in the male genital tract (Franz, 1988; Wu *et al.* 2000; Hoerauf *et al.* 2003). The higher levels of HSP60 in the testis may reflect 'general stress' in the male worms after *Wolbachia* have been depleted.

In the present study, we demonstrated that the up-regulation of mitochondrial HSP60 in worms that had been depleted of *Wolbachia* was also reflected in transcription levels, with 7·7-fold higher levels in worms from nodules from patients treated with doxycycline but not ivermectin compared with those from untreated patients. The quantitative PCR used to measure mitochondrial *hsp60* was specific for filarial mitochondria. This was inferred from *in silico* analysis of the primer sequences. This was also inferred empirically as human *hsp60* transcription would be higher than mitochondrial *hsp60* due to the larger amount of human tissue in the onchocercomas, making detection of the mitochondrial signal impossible.

The broad range of the *hsp60*/actin ratio in the onchocercomas from doxycycline treated patients, while undesirable, is difficult to reduce because of the nature of the samples. Frozen onchocercomas were used in this study rather than worms from collagenase-treated onchocercomas to avoid inducing HSP60 during overnight incubation with an enzyme that is also active against the worms. Thus, the exact nature and status of the worms is unknown. Since whole onchocercomas were used for the RNA extraction, we were unable to undertake histological examinations to establish the relative age and status of individual worms in each nodule, as we have done before (see Hoerauf *et al.* 2007). This limitation could affect the *hsp60*/actin ratio in 2 ways. (1) These patients live in a hyperendemic region and are therefore constantly exposed to new infections. Newly acquired worms in the same onchocercomas with doxycycline treated worms would result in a lower ratio. (2) Another possibility is that different aged worms may regulate HSP60 differently, resulting in onchocercomas with worms that only have up-regulated HSP60, onchocercomas with worms that are too old to react to the loss of their endosymbionts or onchocercomas with a mixture of the previous worms.

We believe that the up-regulation of filarial mitochondrial HSP60 is not merely a reaction to the stress of accumulated dead bacteria, but due to a long-term disruption of the homeostasis in the worms following the depletion of their essential endobacteria. This is reflected clearly in Fig. 4, panels

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A, B, and D, in which HSP60 staining is greater in musculature and spermatozoa usually lacking *Wolbachia*. Since *Wolbachia* and mitochondria are located in different tissues or zones of the hypodermis and oocytes of the worms and stain differently by immunohistochemistry (granular/ring-like *vs* diffuse), the increased staining of mitochondrial HSP60 infers increased protein expression.

The up-regulation of the mitochondrial protein HSP60 may indicate efforts by the filarial cells to compensate for an interruption in some cell processes requiring *Wolbachia*. This interruption may lead to an accumulation of proteins which require HSP60 for proper folding, as has been described in the free-living nematode *Caenorhabditis elegans* (see Yoneda *et al*. 2004). In the latter nematode, the accumulation of unfolded proteins leads to an up-regulation of HSP60 specifically in the mitochondria and is associated with proteins imported from the cytoplasm into the mitochondria which subsequently form multi-protein complexes. HSP60 is not up-regulated in the mitochondria due to other stresses, such as reactive oxygen species (Zhao *et al*. 2002; Yoneda *et al*. 2004). Whether the up-regulation of the expression of mitochondrial HSP60 is due to a direct interaction between *Wolbachia* and mitochondria has not yet been shown. However, it is more likely to be the result of a long-term stress induced by the disruption of the homeostasis of metabolic pathway(s) for which *Wolbachia* may be needed by the nematode (Foster *et al*. 2005; Pfarr and Hoerauf, 2005).

In conclusion, the depletion of *Wolbachia* from the filarial worms examined herein leads to both an increase in transcription of the *hsp60* gene and the translated protein. This increase is probably not a direct effect of the antibiotic treatment, but a response to the loss of the essential *Wolbachia*. Since this up-regulation lasts for up to 18 months or longer, doxycycline treatment has a long-lasting effect on the metabolism of *O. volvulus*, and eventually results in detrimental effects on the adult worm (Taylor *et al*. 2005*b*; Hoerauf *et al*. 2007). It would be interesting to establish whether HSP60 is up-regulated in a similar manner in arthropods that are cured of their *Wolbachia* infections. If results are specific to filarial *Wolbachia*, it may provide insight into proteins involved in this mutualistic symbiosis.

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