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Tetracycline treatment and sex-ratio distortion: a role for *Wolbachia* in the moulting of filarial nematodes?

M. Casiraghi[a], J.W. McCall[b], L. Simoncini[a], L.H. Kramer[c], L. Sacchi[d], C. Genchi[a], J.H. Werren[e], C. Bandi[a,*]

[a]*Dipartimento di Patologia Animale, Università di Milano, Milan, Italy*
[b]*College of Veterinary Medicine, The University of Georgia, Athens, GA, USA*
[c]*Dipartimento di Produzione Animale, Università di Parma, Parma, Italy*
[d]*Dipartimento di Biologia Animale, Università di Pavia, Pavia, Italy*
[e]*Department of Biology, University of Rochester, Rochester, NY, USA*

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Abstract

Filarial nematodes harbour intracellular bacteria of the genus *Wolbachia*. These bacteria are thought to be beneficial to the host nematode. Indeed, tetracycline treatments reduce the population of *Wolbachia* in filarial worms and have detrimental effects on the nematode. Even though various antibiotic-curing experiments have been performed on filariae, the actual role of *Wolbachia* in the biology of these nematodes is not yet clear. To address this issue, we designed a first experiment on a model filaria (*Brugia pahangi*), maintained in the gerbil (*Meriones unguiculatus*). In this experiment, timing of tetracycline treatment was set on the basis of the larval stage of the nematode. This first experiment showed that 2 weeks of treatment started after the L_4–L_5 moult of males, but before the moult of females, led to significant sex-ratio distortion of the nematodes. We thus hypothesised that tetracycline interferes with the moult in *B. pahangi*. To test this hypothesis, we designed a second experiment in which antibiotic treatments were started (1) before the moult of both sexes, (2) after the moult of males but before the moult of females, or (3) after the moult of both sexes. Treatment 1 determined a reduction of worm recovery with no sex bias. Treatment 2 led to a male-biased sex-ratio. Treatment 3 had no effect on either worm recovery or sex-ratio. These results thus support the hypothesis that tetracycline treatment interferes with the L_4–L_5 moult of *B. pahangi*. The nematodes recovered from the treated and control animals were examined for the presence of *Wolbachia* using both immunohistochemistry and real-time PCR. In general, nematodes from treated animals showed a dramatic reduction in *Wolbachia* content. In one group, *Wolbachia* depletion, as observed at the end of the treatment, was followed by a rebound to 'normal' values 160 days later. Prospects for antifilarial therapy using *Wolbachia*-targeted tetracycline treatments should thus take into account the possibility of *Wolbachia* rebound.

Keywords: Nematode moult; Filariasis; Therapy; Symbiosis; *Wolbachia*; Real-time PCR

1. Introduction

Wolbachia is an intracellular bacterium found in arthropods and in filarial nematodes. Several studies have been published on the *Wolbachia* of filarial nematodes (for references, see Bandi et al., 2001a). However, the nature of the relationship between *Wolbachia* and its nematode host has yet to be clearly defined. *Wolbachia* is vertically transmitted to offspring in both arthropods and nematodes. There is evidence that horizontal transmission also occurs in arthropods (Werren, 1997; Stouthamer et al., 1999; Bandi et al., 2001b). In nematodes there is no evidence for horizontal transmission of *Wolbachia*. Vertical transmission of a symbiont suggests a close link between the fitness of host and symbiont and implies the development of a mutualistic interaction (Yamamura, 1993). Reproductive parasitism is another evolutionary option where, through the manipulation of host reproduction, the symbiont can reduce the fitness of those individuals that do not harbour the symbiont or are not involved in its transmission (Werren, 1997; Werren and O'Neill, 1997; Bandi et al., 2001b).

Tetracycline treatment experiments have provided interesting clues for the possible role of *Wolbachia* in the biology of filarial nematodes. The results so far obtained suggest that *Wolbachia* is needed by filarial nematodes for their develop-

* Corresponding author. Università di Milano, Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Patologia Generale e Parassitologia, via Celoria, 10-20133, Milano, Italy. Tel.: +39-02-5031-8093; fax: +39-02-5031-8095.
E-mail address: claudio.bandi@unimi.it (C. Bandi).

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ment, reproduction and, possibly, for their long-term survival (Bandi et al., 1999; Hoerauf et al., 1999; McCall et al., 1999; Langworthy et al., 2000). However, the results of experiments with tetracycline (and other antibiotics) must be interpreted carefully (Bandi et al., 1999, 2001c; Smith and Rajan, 2000). It is indeed possible that the *Wolbachia*–filaria relationship is a mutualistic system (and that tetracycline interferes with this system), but it is also possible that tetracycline has a direct effect on the nematode. Finally, if *Wolbachia* in filarial nematodes behaves as a reproductive parasite, some of the effects of tetracycline might derive from an interference with the manipulations determined by *Wolbachia* (Bandi et al., 1999). We emphasise that the three hypotheses are not mutually exclusive (see Bandi et al., 2001b).

Despite growing interest and intense research focused on the antifilarial effect of tetracycline and its application in the therapy of human onchocerciasis (Hoerauf et al., 2000a), the results of the first paper published on this subject (Bosshardt et al., 1993) have not yet been fully evaluated and discussed. In this study, tetracycline treatments were applied at different larval stages (L) of the filaria *Brugia pahangi* in its experimental host (the gerbil *Meriones unguiculatus*). The antifilarial effects recorded in Bosshardt et al.'s paper can be summarised as follows: (1) treatments throughout the L_3 (and early L_4) or L_3–L_4 (and early L_5 of males) determined an arrest in development (prophylactic effect); (2) treatment started at late L_4 of females/early L_5 of males and continued throughout the L_5–adult stage did not inhibit development, but inhibited microfilaria production. However, Bosshardt et al. also reported a treatment schedule which had apparently no antifilarial effect: treatment during the L_4 (and early L_5 of males) had no effect either on development or microfilarial production. We believe that Bosshardt et al.'s approach of designing the treatment schedules on the basis of larval developmental stages could be very useful for investigating the biological role of *Wolbachia* in filarial nematodes.

This paper reports a series of tetracycline treatment experiments on gerbils infected with *B. pahangi*, in which antibiotics were applied at different stages of nematode development. A first experiment was designed to time treatments at the larval stage. A second experiment was then designed to apply antibiotics more precisely around moulting times. The effects of antibiotic treatment on worm development, sex-ratio and production of microfilariae were evaluated, as were the effects on the *Wolbachia* abundance. The study suggests that *Wolbachia* is implicated in the moult of the nematode.

2. Materials and methods

2.1. Drug and dose level, animal care and necropsy

The drug used was tetracycline dihydrochloride (tetracycline–HCl powder). For all treated groups, tetracycline was offered in the drinking water at a concentration of 1.2% or 0.3% w/v, depending on the experiment and/or the experimental group of gerbils. The medicated, drinking water solutions were prepared daily. After treatment was started, weekly weight data were monitored carefully to ascertain that the gerbils were not losing weight. At the appropriate times, the gerbils were humanely euthanised by carbon dioxide inhalation. The gerbils were maintained with due regard for their welfare and in accordance with applicable legislation and guidelines.

2.2. Experimental groups and treatment schedules

2.2.1. Experiment I

The treatment schedule for Experiment I was based on larval stages and is summarised in Fig. 1a. Briefly, 40 gerbils were intra-peritoneally infected with 100 L_3 of *B. pahangi.* Thirty-two gerbils were divided into five experimental groups (A–E) and treated with 1.2% tetracycline–HCl in drinking water according to the following treatment schedules: the eight animals in group A were treated from 13 to 26 days post infection (p.i.) during the L_4 (and the very beginning of male L_5); the six animals in group B were treated from 26 to 59 days p.i., starting from late female L_4/early male L_5 and continuing to the adult stage; the eight gerbils in group C were treated from 13 to 59 days p.i. (early L_4–adult); the six gerbils in group D were treated from 26 to 82 days p.i. (late female L_4/early male L_5, continuing into the adult stage); the four animals in group E were treated from 26 to 40 days p.i. (late female L_4/early male L_5 continuing to the beginning of the adult stage). The remaining eight gerbils were not treated and were used as controls (Co1–Co4). Since for most groups in Experiment I we had two worm recoveries, we will refer to the gerbils necropsied at the first and the second recoveries, respectively, as subgroup *1 and *2 (e.g. group B includes subgroups B1 and B2).

2.2.2. Experiment II

In Experiment II (Fig. 1b), where antibiotic treatment was more precisely applied during larval moults (Fig. 1c), 38 gerbils were i.p. infected with 150 L_3 of *B. pahangi.* Twenty-eight gerbils were divided into four experimental groups (F–I): the six animals in group F were treated with 0.3% tetracycline–HCl in drinking water from 13 to 41 days p.i. Animals in groups G–I were treated with 1.2% tetracycline–HCl in drinking water according to the following schedules: group G (10 animals) was treated from 26 to 41 days p.i. (precedes the L_4–L_5 moult of the females, see Fig. 1c); group H (six animals) was treated from 22 to 36 days p.i. (precedes the L_4–L_5 moult of both sexes); group I (six animals) was treated from 32 to 46 days p.i. (following the L_4–L_5 moult of both sexes, even though some females could have still been in the moulting phase). The remaining 10 gerbils were not treated and were used as controls (Co5).

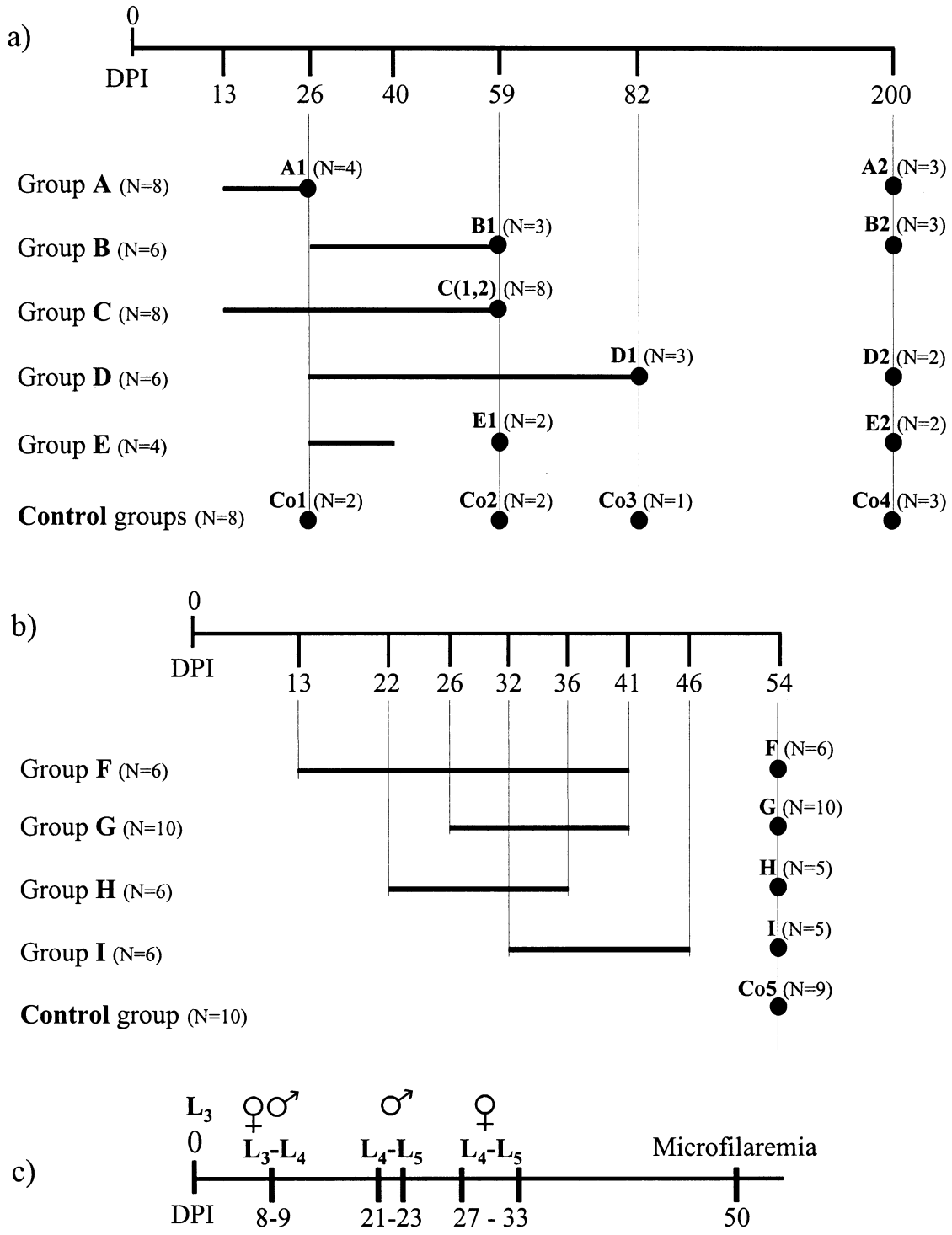

Fig. 1. Experimental groups, control groups and treatment schedules of the two experiments (I and II). The upper bars in (a,b) show the days p.i., and the bars below these indicate the duration of the antibiotic treatments in each group (A–I); dots indicate the timings of worm recoveries in each group; the number of gerbils for each group (and subgroup) is indicated in brackets (differences between the initial number of infected gerbils and the final number at necropsy are due to the natural death of animals). (a) Experiment I: five experimental groups (A–E) are divided into two subgroups each (with the exception of group C, see text for details); an additional group of infected untreated gerbils was used as a control (Co1–Co4); necropsy was done in four different times: 26 days p.i. (A1 and Co1); 59 days p.i. (B1, C1,2, E1 and Co2); 82 days p.i. (D1 and Co3); 200 days p.i. (A2, B2, D2, E2 and Co4). (b) Experiment II: four experimental groups (F–I) of gerbils and an additional group of infected untreated gerbils used as a control (Co5) were necropsied at 54 days p.i. (c) Schematic representation of the development of *Brugia pahangi* in *Meriones unguiculatus*; L_3 is the infective stage larva; L_3–L_4 moult takes place around 8–9 days p.i. for both sexes; L_4–L_5 moult takes place around 21–23 days p.i. in the males, and around 27–33 days p.i. in the females; microfilariae in the peritoneal cavity and/or microfilaremia are detectable in the host starting from 50 days p.i.

2.3. Worm recovery and storage

Worms were recovered from the gerbils at necropsy (see Fig. 1 for details). In Experiment I (Fig. 1a) worms were recovered twice: at the end of antibiotic treatment (with the exception of subgroup E1) and at the end of the experiment at 200 days p.i. (with the exception of group C). For subgroup E1, worm recovery was postponed to 59 days p.i. in order to allow eventual microfilaria production to start (see Fig. 1c); for group C, during necropsy of the first half of the gerbils we did not observe nematodes and we thus decided to examine all the gerbils. In Experiment II (Fig. 1b) worms were recovered once, at 54 days p.i. Worms were enumerated and sexed. Statistical analyses for determination of sex-ratio distortions and comparison of worm recoveries were performed using the software package STATISTICA 5.1 (StatSoft Italia, 1997. STATISTICA versione 5.1 per Windows, StatSoft Italia S.r.l.). For histology and immunohistochemistry, worms were fixed in 2.5% paraformaldehyde in PBS. For determination of the uterine content, worms were fixed in 0.5% paraformaldehyde in PBS. For PCR examination, individual nematodes were put into 28 μl of 0.01 M Tris–HCl (pH 8) and immediately frozen at −20 °C.

2.4. Real-time PCR

Real-time PCR was performed using a Bio-Rad iCycler apparatus. Primers, TaqMan® probe, method development and PCR conditions are described in Simoncini et al. (2001). As reported, the target of this real-time PCR is a 98 nucleotide portion of the *fts*Z gene from the *Wolbachia* of *B. pahangi*. Real-time PCR was effected on crude DNA preparations obtained through proteinase K treatment. Briefly, the samples stored in the freezer were quickly transferred from −20 °C to a thermal cycler pre-heated at 90 °C. After incubation at 90 °C for 10 min, a Tris–HCl–proteinase K solution was added to the samples (final volume: 30 μl; final proteinase K concentration: 200 μg/ml). Further processing is as described in Simoncini et al. (2001). Dilution of the samples was optimised on the basis of the standard curve: PCR was effective in a dilution range corresponding to 1/30–1/30,000,000 of crude DNA preparations of single nematodes (Simoncini et al., 2001). Default PCR was effected on the dilution corresponding to the mid-point of the standard curve (1/600). To confirm the results of default PCRs, we also performed the PCRs on more concentrated samples (1/30; 'enhanced' conditions). Briefly, the 30 μl volumes containing single nematodes, after proteinase K treatment, were diluted 100 times (default) or five times (enhanced); 5 μl, corresponding to 1/600 or 1/30 of the above dilutions of single nematode preparations, was then used as templates in PCR reactions. Each sample was tested in three replicates. The number of amplification cycles was 50.

In Tables 2 and 3 and Sections 3 and 4, results of real-time PCR for *Wolbachia* are expressed in terms of the threshold cycle (TC). The difference between the average TC from treated and control nematodes is indicated as ΔTC (ΔTC = TC treated − TC control). ΔTC is proportional, with an exponential relationship, to the difference in *Wolbachia* content. For example, a ΔTC of 10 suggests 2^{10}-fold (i.e. 1,024-fold) less *Wolbachia* in treated compared with control nematodes.

2.5. Microscopy and immunohistochemistry

The worms used in histological and immunohistochemical examinations were processed and embedded in paraffin. Where available, up to five nematodes for each sex for each group were embedded in one block to guarantee, where possible, at least 10 sections per slide. At least five slides were prepared for each group. The number of nematodes examined for each group is reported in Table 3. For histology, 6 μm thick sections were stained with H & E. For immunostaining of *Wolbachia*, 5 μm thick sections were stained with a rabbit polyclonal antiserum against the surface protein of *Wolbachia* (WSP) from *B. pahangi/Brugia malayi*. Production of the recombinant WSP and raised antiserum are described in Kramer et al. (2002). Worm sections were pretreated in a microwave oven in 0.1 M citrate buffer for three 5 min cycles at 400 W. Sections were firstly incubated in 1% H_2O_2, and then for 10 min with 10% rabbit serum and for 30 min with anti-WSP antiserum diluted at 1:200 in 10% PBS. The samples were then treated with a secondary anti-rabbit IgG–streptavidin complex, rinsed and incubated with a biotin–horse radish peroxidase complex (LSAB kit, DAKO). Reactivity was revealed by 5 min incubation with the red chromogen 3-3-aminoethylcarbazine (AEC, Sigma) and sections were counterstained with haematoxylin. Immunohistochemical staining was also performed with a polyclonal antibody against the heat shock protein 60 (HSP60) of the gamma-proteobacterium *Escherichia coli* (#G6532 Sigma) with the same method as above (antiserum raised against HSP60 of a gamma proteobacterium has been previously shown to stain both *Wolbachia* and filarial mitochondria; Hoerauf et al., 2000a,b). Two slides for each sex and for each group were stained using the anti-HSP60 antibody. Negative controls were performed by omitting the primary antibody. For determination of the uterine content, female nematodes were put onto a microscope slide with a few microlitres of PBS, divided into five segments of approximately the same length, slightly compressed using a cover glass and observed at 50–100 ×. In normal embryogenesis developing embryos are expected (Bandi et al., 1999), from oocytes/morulae (at the caudal end) to stretched microfilariae (at the cephalic end).

3. Results

3.1. Worm recovery, sex-ratio and patency

Fig. 1 reports all treatment groups (A–I), treatment sche-

dules and corresponding developmental stages of *B. pahangi*. In this section, each group name will be followed by a summary of the information reported in Fig. 1, according to this scheme: treatment period: TP; male developmental stage: mS; female developmental stage: fS; necropsy: N. Table 1 summarises the results of the two experiments, including the number of patent gerbils, the mean number of recovered worms from the treated and untreated gerbils, and the mean numbers of male and female worms collected from each group of gerbils.

3.1.1. Experiment I

Compared with controls (Co1), worms from group A (TP: 13–26 days p.i.; mS: L_{4-5}; fS: L_4; N: 26 and 200 days p.i.) showed no significant difference in worm number at first recovery (subgroup A1; *t*-test not significant). However, no worms were found at the second recovery (subgroup A2; one gerbil in this group died before the final recovery). Worms were very small at first recovery and could not be sexed by microscopic examination. In group B (TP: 26–59 days p.i.; mS: L_5–adult; fS: L_{4-5}–adult; N: 59 and 200 days p.i.) all gerbils were patent at both worm recoveries (subgroups B1 and B2). There was a decrease in the number of worms recovered at both times when compared with controls (Co2 and Co4, respectively), but the difference was not statistically significant (*t*-tests not significant). No significant sex-ratio distortion was observed at either recoveries (χ^2 not significant). All eight gerbils in group C (TP: 13–59 days p.i.; mS: L_{4-5}–adult; fS: L_{4-5}–adult; N: 59 days p.i.) were necropsied at the end of antibiotic treatment. Six gerbils did not harbour any worms and the remaining two gerbils harboured a single worm each. In group D (TP: 26–82 days p.i.; mS: L_5–adult; fS: L_{4-5}–adult; N: 82 and 200 days p.i.) no significant difference was observed at both worm recoveries (subgroups D1 and D2) when compared with the control (Co3 and Co4, respectively; *t*-tests not significant). However, at 200 days p.i. only one gerbil harboured worms (one gerbil had no worms and another one died before the end of the experiment). No significant sex-ratio distortion was observed (χ^2 not significant). In group E (TP: 26–40 days p.i.; mS: L_5; fS: L_{4-5}; N: 59 and 200 days p.i.) no significant reduction in worm recovery was observed at the two recoveries when compared with the control (Co2 and Co4, respectively; *t*-tests not significant), however a significant sex-ratio distortion was observed in both subgroups E1 and E2, with a reduction of females: just one female recovered compared with 47 males (E1: $\chi^2 = 14.17$, degrees of freedom (d.f.) $= 1$, $P < 0.00017$; E2: $\chi^2 = 7.5$, d.f. $= 1$, $P < 0.0062$).

3.1.2. Experiment II

All gerbils were necropsied at 54 days p.i. One gerbil out of the six from group F (TP: 13–41 days p.i.; mS: L_{4-5}; fS: L_{4-5}; N: 54 days p.i.) harboured a single worm at necropsy. No worms were recovered from the remaining subjects. In group G (TP: 26–41 days p.i.; mS: L_5; fS: L_{4-5}; N: 54 days p.i.), no significant reduction in worm recovery was observed (*t*-test not significant), but a significant sex-ratio distortion was observed with a reduction of females ($\chi^2 = 32.64$, d.f. $= 9$, $P < 0.00015$). Group H (TP: 22–36

Table 1
Summary of parasitological data from tetracycline-treated groups of gerbils (A–I); the control (Co1–Co5) corresponding to each collection time follows the corresponding treated group(s)

Group	Treatment periods[a]	Number of patents[b]/total	Mean number of adult worms	SD	Mean number of adult females	SD	Mean number of adult males	SD
A1	13–26	4/4	20.25	11	n.d.[c]	n.d.	n.d.	n.d.
Co1	–	2/2	29.5	0.71	n.d.	n.d.	n.d.	n.d.
B1	26–59	3/3	12	9.54	3.67	4.73	8.33	7.02
C1,2	13–59	2/8	0.24	0.46	0.12	0.35	0.12	0.35
E1	26–40	2/2	16	5.66	0.5	0.71	15.5	6.36
Co2	–	2/2	39.5	16.26	21	11.31	18.5	4.95
D1	26–82	3/3	12	9.54	0.33	0.58	11.67	10.02
Co3	–	1/1	36	–	23	–	13	–
A2	13–26	0/3[d]	–	–	–	–	–	–
B2	26–59	3/3	3.67	2.08	1	1	2.67	1.15
D2	26–82	1/2[d]	4	5.66	1	1.41	3	4.24
E2	26–40	2/2	7.5	3.54	0	–	7.5	3.54
Co4	–	3/3	21	12.49	12	7.55	9	5.2
F	13–41	1/6	0.17	0.41	0	–	0.17	0.41
G	26–41	10/10	45.4	18.65	15.8	10.12	29.6	10.75
H	22–36	5/5[d]	14.20	16.24	7.80	8.58	6.40	8.50
I	32–46	5/5[d]	27.6	17.26	13.40	6.35	14.20	13.74
Co5	–	9/9[d]	46.89	25.62	24.56	13.59	22.33	13.82

[a] Treatment periods are indicated as the range of days p.i.
[b] Patent: gerbils harbouring adult worms at necropsy (pre-adults in the case of A1 and Co1).
[c] n.d., not determined.
[d] In each of these groups one gerbil died naturally during the experiment.

days p.i.; mS: L_{4-5}; fS: L_{4-5}; N: 54 days p.i.) showed a significant reduction in worm recovery ($t = -2.98$, d.f. = 13, $P = 0.01$), but no significant sex-ratio distortion (χ^2 not significant). In group I (TP: 32–46 days p.i.; mS: L_5; fS: L_5; N: 54 days p.i.) the reduction in worm recovery was not significant (*t*-test not significant); sex-ratio distortion was also not significant (χ^2 not significant). The results of Experiment II are summarised in Fig. 2.

All control gerbils had microfilariae in the peritoneal cavity at the moment of worm recovery (with the exception of the gerbils in the control group Co1, because the worms were still at the L_4 stage). Microfilariae were not observed in any of the treated gerbils from either experiment (I and II).

3.2. Real-time PCR

The results of real-time PCR are summarised in Table 2 (for a definition of the TC see Section 2.4). In total, 127 individual nematodes were tested in triplicate at both default and enhanced conditions, for a total of over 750 PCR reactions. All nematodes from control animals (Co1–Co5) were PCR positive for *Wolbachia*. The results obtained from each nematode were reproducible, with the three repetitions showing almost identical curves and with default and enhanced PCRs giving congruent results. For example, the samples were 20-fold more diluted in default PCR compared with enhanced PCR. As expected, TCs were always four to five times higher in default conditions, which implies 2^4–2^5-fold less *Wolbachia* (i.e. 16- to 32-fold less *Wolbachia*). In default PCRs from untreated worms, TC ranged from around 22 to 25 in females (approximate average TC: 23) and from around 26 to 34 in males (approximate average TC: 30). The average difference in the TC of males and females is thus 7, which indicates 2^7-fold (i.e. 128-fold) more *Wolbachia* in females than in males. This estimation is of course very rough and we should also consider that the volume of male nematodes is about 10 times lower than that of females (comparisons of *Wolbachia* content in males and females were not among the aims of this study; for further details on *Wolbachia* content in filariae from untreated animals, see Simoncini et al., 2001).

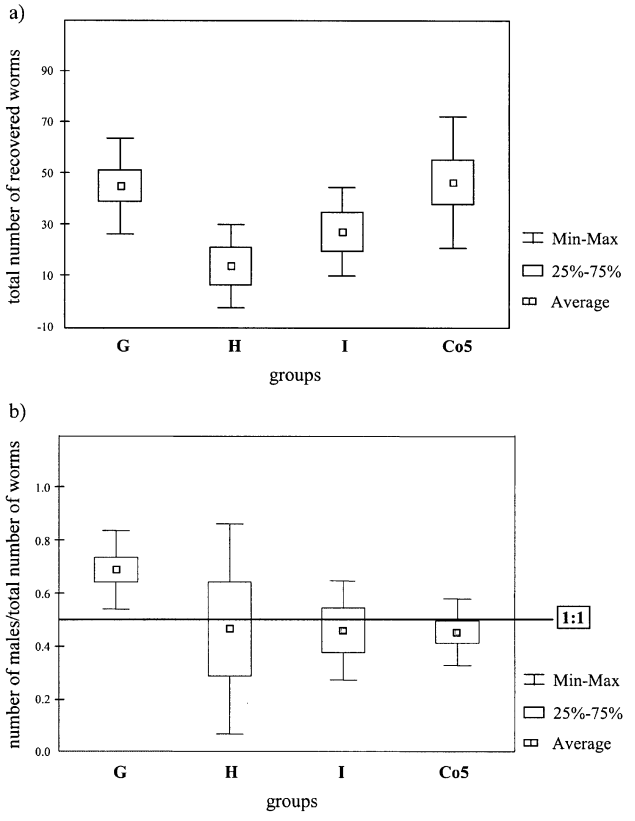

Fig. 2. Box and whiskers diagrams representing the main results of Experiment II. (a) Worm recoveries in the different groups of gerbils (*x*-axis); the *y*-axis represents the number of recovered worms; a significant reduction in the number of worms recovered was observed in nematodes from group H ($t = -2.98$, d.f. = 13, $P = 0.01$). (b) Sex-ratio in the different groups of gerbils (*x*-axis); the sex-ratio is represented by the number of males/total number of worms recovered (*y*-axis); the horizontal bar indicates the 1:1 ratio between the sexes; a significant sex-ratio bias (reduction of females) was observed in the nematodes recovered from group G ($\chi^2 = 32.64$, d.f. = 9, $P < 0.00015$).

In general, treated nematodes showed a marked reduction of *Wolbachia* content. From some groups, we collected individual nematodes that were PCR negative for *Wolbachia* using both default and enhanced conditions: six out of seven nematodes were negative in D1; two out of four in D2; three out of seven in B1; four out of five in B2. The remaining nematodes in these groups were positive only towards the last cycles of PCR (39.0–46.7). Another group that led to the collection of PCR negative nematodes was I: three out of six males in this group were negative. Positive females from this group had TCs of 34.4–41.6. In subgroups E1, C1,2 and in the females from groups G and H, *Wolbachia* was present in all individual nematodes examined, but in reduced amounts compared with the controls (approximate ΔTC: 7–13; see Table 2). Finally, in the nematodes from subgroup E2 and in the males from groups G and H *Wolbachia* content was comparable with that of the nematodes from the controls (approximate ΔTC: 1–4; see Table 2). The result of group E is thus intriguing, since TCs were higher at the first recovery (E1) compared with TCs at the second one (E2). ΔTC of E1 compared with the control suggests a *Wolbachia* reduction of 2^7-fold (128-fold), while the *Wolbachia* content appeared 'normal' in E2. In general, in nematodes from the groups treated for 2 weeks, *Wolbachia* content appeared higher than in those nematodes from groups treated for 4–8 weeks.

3.3. Histology, microscopy and immunostaining for *Wolbachia*

Histological examination of female worms from control gerbils showed normal morphology and embryogenesis (Fig. 3A), with all developmental stages from oocytes to stretched microfilariae (Table 3). Male nematodes from control animals also showed normal morphology and sper-

Table 2
Summary of the results of real-time PCR for *Wolbachia* on nematodes from treated and control groups, where sexing was possible; results are expressed in terms of the average TC

Group	TC females	nf[a]	Females TC range	TC males	nm[a]	Males TC range	ΔTC females[b]	ΔTC males[b]
B1	41.84	1	41.84	> 45.97	6	39– > 50	19.42	> 15.75
C1,2	–	0	–	37.99	1	37.99	–	7.77
E1	–	0	–	37.39	7	33.01–41	–	7.17
Co2	22.42	5	22.24–22.83	30.22	5	25.97–34.42	–	–
D1	–	0	–	> 49.41	7	45.92– > 50	–	> 19.96
Co3	22.65	5	21.94–23.56	29.45	6	27.25–31.94	–	–
B2	40.30	1	40.30	> 50	4	> 50	17.21	> 22.94
D2	> 50	1	> 50	> 46.97	3	44.20– > 50	> 26.93	> 19.91
E2	–	0	–	28.53	10	26.28–31.71	–	1.47
Co4	23.09	5	22.90–23.26	27.06	5	25.92–29.78	–	–
G	36.44	6	33.53–39.54	32.81	6	31.24–34.1	12.18	2.84
H	36.30	4	33.08–40.42	34.59	4	31.01–37.58	12.04	4.62
I	37.60	3	34.42–41.58	> 40.84	6	36.05– > 50	13.34	> 10.87
Co5	24.26	5	23.32–25.62	29.97	5	28.57–31.37	–	–

[a] Number of nematodes examined by real-time PCR in each group for females (nf) and males (nm).
[b] ΔTC is the average difference of the TC of nematodes from treated and control animals (see Section 2.4); subgroups A1 and A2, and the control Co1, are not reported in this table because sexing was not possible at the first recovery, and no worms were collected at the second recovery; average TC of unsexed nematodes in A1: 36.71 (range 34.13–38.51, $n = 11$); average TC of unsexed nematodes in Co1: 31.20 (range 29.9–33.4, $n = 5$); ΔTC A1 − Co1: 5.51; >50 means that samples were still negative at the last cycle of PCR (i.e. the 50th); averages, ranges and ΔTCs are expressed accordingly when the >50 values were included in the calculations.

matogenesis. In most female worms from treated gerbils, embryos were present in the uterus. At times, however, particularly in gerbils treated starting at 26 days p.i., embryos were often degenerated and uterine content was replaced with an amorphous material (Fig. 3B). In any case, all worms from treated groups showed evidence of a block in embryogenesis (Table 3). In groups B and D the most advanced stages were embryos/degenerated embryos. In subgroup E1 and in groups G and H we also observed developed embryos (i.e. slightly curved forms) and a few coiled microfilariae, but no stretched microfilariae (in the case of subgroup E1 we collected only one female). In the other group of gerbils with 2 weeks of antibiotic treatment (group I), the most advanced form present in the uterine content of the recovered nematodes was again that of coiled microfilariae. However, the percentage of coiled microfilariae in the uterine content of the females in group I was higher than that in groups G and H, comparable with that observed in nematodes from control animals. In male nematodes from treated groups we did not observe differences compared with the controls in terms of gonad development and spermatogenesis. We emphasise that blocking of embryogenesis/inhibition of microfilaria production has been observed in most of the studies thus far published on the effects of tetracyclines on filarial nematodes (for a review, see Bandi et al., 2001a).

The depletion of *Wolbachia* after antibiotic treatments was monitored by immunostaining using a polyclonal antiserum against the surface protein WSP. In addition, *Wolbachia* abundance was further assessed using a polyclonal antiserum against the bacterial heat shock protein HSP60. The results of anti-WSP (Fig. 3C) and anti-HSP60 (data not shown) *Wolbachia* staining were congruent. As expected (Hoerauf et al., 2000a,b), anti-HSP60 staining was also observed in filarial tissues with high mitochondrial density. This staining was anyway less intense and distinguishable from the *Wolbachia* staining, also because mitochondria have a different location (see also discussion in Hoerauf et al., 2000b). Staining of the mitochondria provided an internal control, since it was observed also after depletion of *Wolbachia* following antibiotic treatment. All female worms recovered from control gerbils showed typical staining and distribution for *Wolbachia* in hypodermal tissues (lateral cords) of both sexes and in the female reproductive tract (Fig. 3C). However, in male nematodes from control animals the distribution of *Wolbachia* in the hypodermal cells was discontinuous, with sections showing cells void of bacteria and other sections with cells full of bacteria. A reliable estimation of the presence of *Wolbachia* in male worms based on immunostaining is thus difficult since it depends on the number of sections examined. The majority of worms recovered from treatment groups showed a reduction in *Wolbachia* populations in hypodermal tissue and the almost complete elimination of *Wolbachia* from the ovaries and uteri of females (Fig. 3D). Female worms from group G appeared void of *Wolbachia* in all tissues. Table 3 summarises the results of immunostaining and real-time PCR for *Wolbachia*. Results of the two approaches for *Wolbachia* detection appear consistent.

4. Discussion

Our study was initially designed to test the idea of Bosshardt et al. (1993) that susceptibility to tetracycline was life-stage dependent: TPs were set on the life stage of the nematode (L_4; L_5; $L_4 + L_5$). Our first experiment led to

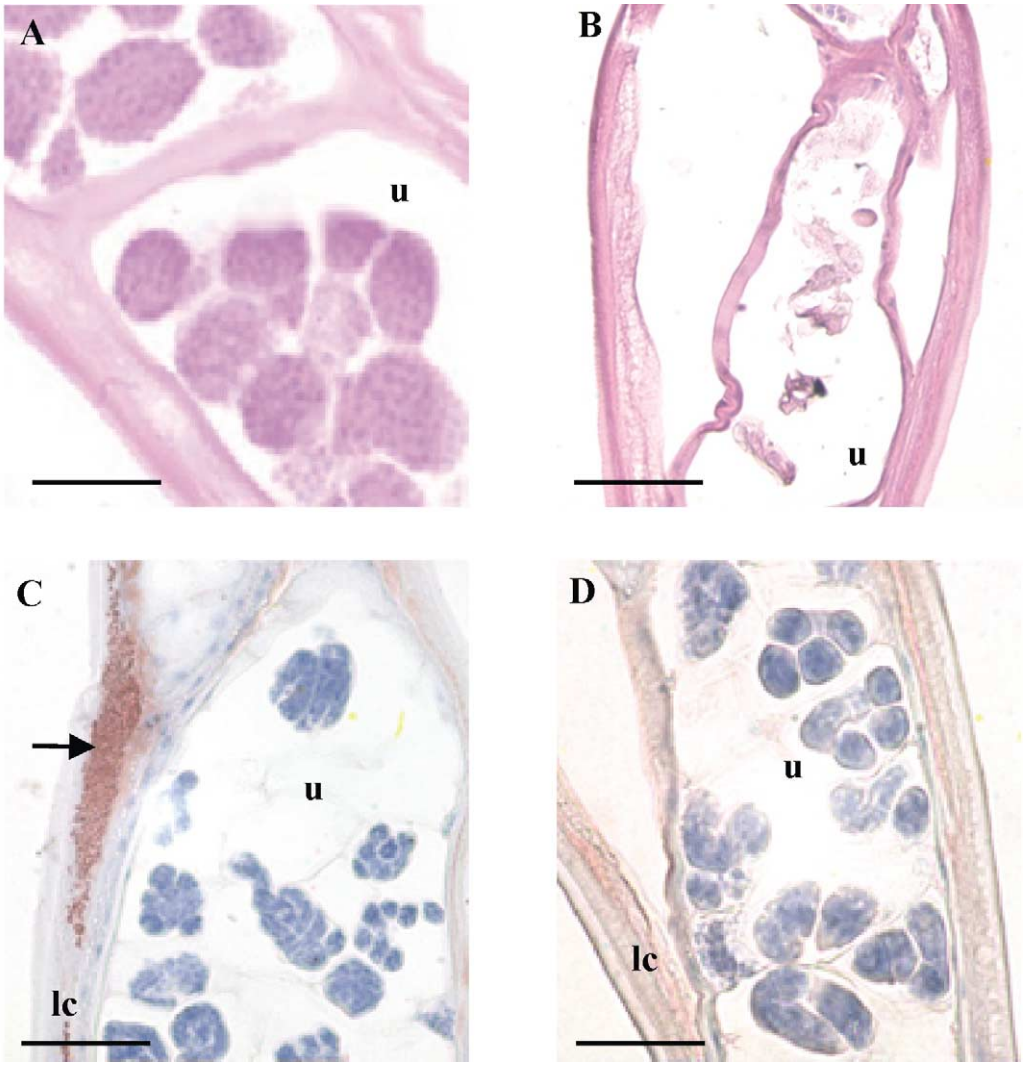

Fig. 3. Histology and anti-WSP immunohistochemical staining of *Brugia malayi* females from control (group Co2; A,C) and treated (subgroup B1; B,D) gerbils. (A) Histology showing normal developing embryos in the uterus (u) of a control female; bar, 50 μm. (B) Histology showing degenerating embryos, replaced with an amorphous material, in the uterus (u) of a treated female; bar, 200 μm. (C) Immunohistochemical staining of *Wolbachia* from a control female showing a cluster of wolbachiae (arrow) in the lateral cord (lc); bar, 100 μm. (D) Immunohistochemistry of a treated female showing a lack of staining for *Wolbachia* in the lateral cords (lc); bar, 100 μm.

unexpected results: (1) sex-ratio distortion when the treatment was during the late female L_4 and the L_5 (group E); (2) reduction of vitality when the treatment was during the L_4/early male L_5 (group A); (3) prophylactic effect (complete or almost complete absence of adult worms at recoveries) when the treatment was during the L_4, L_5 and early adult period (group C). Why did tetracycline cause sex-ratio distortion in our study? Based on the available information (Ash and Riley, 1970) and on the experience of one of the authors (J.W.McC.), the L_4–L_5 moult occurs earlier in males and later in females (see Fig. 1c). In particular, the male moult occurs around 21 days p.i. (from 18 to 24 days p.i.), while the female moult occurs with a delay of a few days (Ash and Riley, 1970). Unpublished observations by one of the authors (J.W.McC.) have confirmed that the L_4–L_5 moult of *B. pahangi* in gerbils infected via i.p. inoculation occurs earlier in males (21–23 days p.i.) and later in females (27–33 days p.i.). Therefore, treatment in groups B, D and E started at 26 days p.i., when the moult of males was likely completed and the moult of females was not yet initiated or had only just begun (see Fig. 1c). We thus hypothesised that tetracycline treatment interfered with the L_4–L_5 moult of females, leading to differential sex death and to the observed sex-ratio distortion.

Based on the above interpretation of our results, we decided to perform another experiment (II), with treatments scheduled on the basis of the L_4–L_5 moulting time. If tetracycline interferes with the L_4–L_5 moult of both sexes: (1) treatment started before the moult of both sexes (group H) should inhibit the development of both sexes; (2) treatment started after the moult of males and before the moult of females (group G) should lead to a male-biased sex-ratio;

(3) treatment started after the moult of both sexes (group I) should have no effect. The results of Experiment II were in agreement with these predictions (Table 1, Fig. 2). It has already been observed that tetracycline treatment of gerbils infected with *B. pahangi* started at 32 days p.i. (i.e. at the end of the moult of both sexes), while altering embryogenesis, does not interfere with the development of adult worms (Bandi et al., 1999) and does not cause sex-ratio distortion (C. Bandi, unpublished data, not reported in Bandi et al., 1999). The results of the present work, as well as those of Bandi et al. (1999), are thus in agreement with the idea that the timing of tetracycline treatment in relation to moulting is crucial, and that this antibiotic interferes with the L_4–L_5 moult of *B. pahangi*. Smith and Rajan (2000) have recently provided evidence that exposure to tetracycline of the L_3 of filariae of different species maintained in vitro inhibited the moult to L_4.

Another test we performed in Experiment II (group F) was a reduction in terms of TP and dosage of the treatment of group C. We were indeed surprised that group C had an almost complete prophylactic effect (two gerbils patent out of eight, with a single nematode each). Group F showed that even with a four-fold dosage reduction and a reduction of the TP from 6 to 4 weeks, a treatment that actually covers the moult of both males and females has a strong prophylactic effect (one gerbil patent out of six, harbouring a single nematode).

In addition to recording worm recoveries and sex-ratio, we examined the nematodes collected by microscopy (for determination of the uterine content), histology (to uncover possible alterations in the worms) and immunohistochemistry and real-time PCR (to monitor *Wolbachia* depletion).

Immunohistochemistry provided evidence for a strong depletion of *Wolbachia* in almost all worms from treated animals. Of course, the lack of *Wolbachia* staining does not imply that *Wolbachia* is totally cleared from these nematodes. Indeed, in one experimental group (E) we did not observe *Wolbachia* staining at the first recovery (E1), but nematodes from this group were *Wolbachia* positive at the second recovery (E2), 160 days after the end of the treatment.

Real-time PCR provided further information on *Wolbachia* abundance in the nematodes collected from the different groups. Tetracycline treatments of 4 (B) and 8 (D) weeks led to the collection of some individual nematodes that were PCR negative for *Wolbachia*. In these groups, compared with the controls, the positive nematodes showed quite high ΔTCs (see Section 3 and Table 2). We would assume that the PCR negative nematodes in these groups were actu-

Table 3
Summary of the results of immunostaining using anti-WSP antibodies (imm.) and real-time PCR for *Wolbachia* (PCR) on nematodes from treated and control groups; the results of real-time PCR are a categorisation from the difference between the average TC from treated and control nematodes recorded in Table 2 (see note below); the status of the embryogenesis in female nematodes is also recorded

Group	*Wolbachia* in females (imm.)[a]	nf[b]	*Wolbachia* in males (imm.)[a]	nm[b]	*Wolbachia* in females (PCR)[c]	*Wolbachia* in males (PCR)[c]	Uterine content[d]
B1	−−	2	−−	5	−−	−−	pl/de
C1,2	n.d.	n.d.	n.d.	n.d.	n.d.	+/−	n.d.
E1	n.d.	n.d.	−−	5	n.d.	+/−	cm
Co2	++	5	++	5	++	++	sm
D1	n.d.	n.d.	−−	5	n.d.	−−	n.d.
Co3	++	5	++	5	++	++	sm
B2	n.d.	n.d.	+/−	2	−−	−−	pl/de
D2	−−	1	−−	3	−−	−−	n.d.
E2	n.d.	n.d.	++	5	n.d.	++	n.d.
Co4	++	5	++	5	++	++	sm
F	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G	−−	5	−−	5	+/−	++	pl/de
H	+/−	1	+/−	3	+/−	++	cm
I	+/−	4	+/−	4	+/−	+/−	cm[d]
Co5	++	5	++	5	++	++	sm

[a] The categorisation of the results of immunohistochemistry is subjective: the staining level of nematodes from control groups is taken as the reference, with ++ indicating a 'normal' staining level; +/− indicates a reduced staining for *Wolbachia*; −− indicates only background staining; subgroups A1 and A2, and the control Co1, are not reported in this table because sexing was not possible at the first recovery, and no worms were collected at the second; unsexed nematodes in A1 and Co1 were +/− and ++, respectively, which agrees with the results of real-time PCR (Table 2).

[b] Number of female (nf) and male (nm) nematodes examined by immunohistochemistry: where available, up to five nematodes for each sex were examined for each group (numbers of nematodes examined by real-time PCR are reported in Table 2).

[c] Categorisation of the results of real-time PCR is based on the ΔTCs recorded in Table 2: $\Delta TC \leq 4$ is indicated as ++; $5 \leq \Delta TC \leq 13$ is indicated as +/−; $\Delta TC \geq 14$ is indicated as −−.

[d] The most advanced developmental stage observed is indicated: pl, pre-larvae; de, developed embryos; cm, coiled microfilariae; sm, stretched microfilariae; pre-larvae are forms that could be either morulae or degenerating embryos; in general, in the treated groups in which coiled microfilariae were observed, the most abundant forms were pre-larvae and developed embryos and the percentage of coiled microfilariae was very low compared with what was observed in nematodes from control animals. However, in group I the percentage of coiled microfilariae was comparable with that of nematodes from control animals; n.d., not determined. Staining using anti-HSP60 antibodies assigned the samples in the same categories as those reported in this table for anti-WSP antibodies.

ally cleared of *Wolbachia*. Another group that led to the collection of PCR negative nematodes was I. In other cases, reduction of the *Wolbachia* content was not as marked as in the groups above, particularly in nematodes from groups treated for 2 weeks, with the exception of the males from group I. In general, the reduction of *Wolbachia* content was higher in females (e.g. in groups D2, G and H). If *Wolbachia* plays a more active role in females than in males (as predicted by evolutionary hypotheses; see Werren and O'Neill, 1997; Bandi et al., 2001b), this may imply a more active bio-synthetic activity of *Wolbachia* in females and thus greater susceptibility to tetracycline.

PCR evidence for the presence of *Wolbachia* DNA after antibiotic treatment of filarial nematodes has previously been reported (Bandi et al., 1999; Hoerauf et al., 2000a,b; Smith and Rajan, 2000). Weak PCR positivity for *Wolbachia* after the end of the treatment could be due either to a residual presence of living *Wolbachia* or to the presence of *Wolbachia* DNA released from dead bacteria. In our present study, we show that 120–140 days after the end of the treatment there was still PCR evidence for *Wolbachia*; this seems to suggest that living *Wolbachia* were actually present, even though below the threshold limit needed to allow recolonisation of the nematode (see below). We should consider that tetracycline is bacteriostatic, not bactericidal. It is thus not surprising to find the presence of residual *Wolbachia* after tetracycline treatment.

The most intriguing result of real-time PCR is the evidence for *Wolbachia* rebound in group E. Indeed, the amount of *Wolbachia* in nematodes from this group at the second recovery (E2) was comparable with that of control nematodes, while at the first recovery (E1), which is closer to the end of antibiotic treatment, worms showed approximately 130 times less *Wolbachia* than the controls. *Wolbachia* thus appear capable of recolonising the nematodes even after a depletion determined by antibiotics. It should be noted that the results of real-time PCR on subgroups E1 and E2 were obtained on a total of 17 nematodes (seven in E1; 10 in E2) and were congruent with those of immunohistochemical staining for *Wolbachia*, which was negative at first recovery and positive at the second (see above and Table 3). It is unclear whether *Wolbachia* are capable of recolonising cells from which they have been eliminated, or whether these rebounds represent regrowth of *Wolbachia* that were not completely eliminated from cells by tetracycline treatment. It remains to be determined how easily *Wolbachia* can move between host cells in developing or mature individuals.

Interestingly, in groups B and D we observed reduction of the *Wolbachia* content that was not followed by rebound. In these cases, ΔTCs were very high (range 16–27), while in subgroup E1 ΔTC was quite low (around 7; see above). *Wolbachia* rebound thus appears to require a residual bacterial population that is not reduced under a given threshold value.

Our results have implications for the use of tetracycline in antifilarial therapy (e.g. see Taylor et al., 2001). In particular, any therapy should take into account the possibility of *Wolbachia* rebound in nematodes in treated patients. In our experiments we had evidence for incomplete clearance, even after treatments of 8 weeks (e.g. the two positive nematodes from group D2). It would thus be crucial to develop treatment schedules which lead to total *Wolbachia* clearance, or to perform further studies in both *B. pahangi* and other filarial species to verify the capacity of *Wolbachia* to recolonise the nematode after incomplete clearance.

Analysis of the uterine content of worms from treated gerbils revealed inhibition of embryogenesis that lasted up until the end of the experimental period (200 days p.i. in Experiment I and 54 days p.i. in Experiment II). Thus, a reduction/elimination of *Wolbachia* was associated with altered development of offspring. In the subgroup where we observed *Wolbachia* rebound (E2) we collected only males. We thus do not have any information on whether *Wolbachia* rebound in females is associated with embryogenesis resumption. In other groups, we did not observe either *Wolbachia* rebound or embryogenesis resumption in the females that we collected at 200 days p.i. There is thus an overall consistency of the results of our work with those of other studies, which show that treatment with tetracycline and derivates inhibits embryogenesis in filarial nematodes (reviewed in Bandi et al., 2001a). In particular, infertility in *B. pahangi* after tetracycline treatment has already been reported in Bosshardt et al. (1993), Bandi et al. (1999), and McCall et al. (1999). It is interesting to note that 2 weeks of treatment in groups E, G, H and I resulted in different types of embryogenetic block: in the groups where treatment started at 22 or 26 days p.i. (groups E, G and H), very few coiled microfilariae were observed. In the group where treatment started at 32 days p.i. (group I), the percentage of coiled microfilariae observed was comparable with that of control animals. It would be interesting to perform new experiments where necropsy is delayed, in order to test whether embryogenesis is really blocked at coiled microfilariae or continues to stretched microfilariae. Finally, it is notable that female nematodes from treated animals (in particular when treatment began at 26 days p.i.; groups B and D) showed remarkable alterations in uterine content, as observed by histology (see Fig. 3B).

Our study shows that it is possible to obtain adult nematodes which are free of *Wolbachia* or which, at least, harbour a significantly reduced *Wolbachia* population. Embryogenesis in these nematodes appeared to be inhibited even at 200 days p.i. Furthermore, none of the treated gerbils produced microfilariae. It was thus impossible to obtain a *Wolbachia*-free line of *B. pahangi.* However, *Wolbachia*-free adult nematodes could become useful as controls in studies on the role of *Wolbachia* in the pathogenesis and immunology of filariasis (e.g. see Bazzocchi et al., 2000; Taylor et al., 2000; Brattig et al., 2000).

We can speculate on the possible function of *Wolbachia* in larval moulting in *B. pahangi*. *Wolbachia* is present in high numbers in cells of the inner integument of filarial nematodes

(Kozek and Figueroa, 1977; Kozek, 1977). Why would they be localised in these tissues, especially since it is clear that *Wolbachia* is not required for moulting in most nematodes? Among the possibilities are that release of *Wolbachia* molecules during the moults of nematode larvae modulates the host immune response, thus reducing immunological attack to the nematodes. It would be extremely useful to perform similar experiments on tetracycline effects on the moult of a filarial nematode that does not harbour *Wolbachia*, such as *Acanthocheilonema viteae* or *Onchocerca flexuosa* (Bandi et al., 1998; Brattig et al., 2001).

The evidence that tetracycline interferes with the L_4–L_5 moulting provides new insights into the design of future studies on the role of *Wolbachia* in the biology of filarial nematodes (or at least on the effects of tetracycline). Based on our results, we cannot of course conclude that *Wolbachia* is implicated in the moult of *B. pahangi*: tetracycline could have a direct effect on the nematode. Again, future experiments should include a *Wolbachia*-free filaria such as *A. viteae* (see above). However, our results define a restricted window of time in which tetracycline has antifilarial effect (i.e. the time of the L_4–L_5 moult). Experiments could thus be designed on the basis of this information (e.g. investigation on *Wolbachia* gene expression during the moult).

Our results are also strongly suggestive of a role for *Wolbachia* in *B. pahangi* embryogenesis: that embryogenesis was still blocked 160 days after the end of the treatment implies that tetracycline determined some kind of 'permanent' alteration. Based on our results, the reduction in the *Wolbachia* population was also 'permanent' in these nematodes (the nematodes where we observed *Wolbachia* rebound were males). The fact that there is a 'chronic' depletion of *Wolbachia* does not of course imply that bacterial absence/reduction was the cause of embryogenesis inhibition. However, based on the available information, it seems reasonable to hypothesise that there is a cause–effect relationship between the two phenomena (absence/reduction of *Wolbachia* and embryogenesis block).

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