The morphological discrimination of microfilariae of *Onchocerca volvulus* from *Mansonella ozzardi*

R. J. POST[1]*, Z. ADAMS[1], A. J. SHELLEY[1], M. MAIA-HERZOG[2], A. P. A. LUNA DIAS[2] *and* S. COSCARÓN[3]

[1] *Department of Entomology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
[2] *Departamento de Entomologia, FIOCRUZ, Ministério da Saúde, Avenida Brasil 4365, 21045-900 Rio de Janeiro, Brazil*
[3] *Museo La Plata, Paseo del Bosque, La Plata, Argentina*

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SUMMARY

There is no published account which allows the morphological discrimination of microfilariae of *Onchocerca volvulus* and *M. ozzardi* from each other. However, they occur together in parts of Brazil and Venezuela, and presumably there is always the possibility that migration could establish new sympatric populations in the future. The objective of this study was to evaluate simple morphological characters that might be used for species-diagnosis of microfilariae. The conclusions were that the location of microfilariae in the blood or skin, the body size and the nucleation of the nerve ring are expected to be useful first indications of species identity, but cannot be used for confident diagnosis. The structure of the cephalic armature (stained with alcian blue) seems to be species specific, but is of limited application because it is often difficult to see. However, the pattern of nucleation of the tail (as expressed by the ratio of the length of the terminal nucleus compared with the length of the tail space) is distinctive and is expected to be diagnostic.

Key words: onchocerciasis, mansonelliasis, diagnosis, identification, Brazil.

INTRODUCTION

Microfilariae of *Mansonella ozzardi* (Manson, 1897) have been reported to occur in the skin as well as in the peripheral blood (Moraes, 1976; Ewert, Smith & Corredor, 1981; Moraes *et al*. 1983). However, most modern and historical textbooks of parasitology or tropical medicine do not seem to recognize this, and often describe the occurrence in blood as a part of the diagnostic process (e.g. Coombs & Crompton, 1991; Garcia, 1999, 2001; Maegraith, 1980; Muller, 2002; Peters & Pasvol, 2002; WHO, 1997). There are a few exceptions which do mention the occurrence of *M. ozzardi* microfilariae in skin, but for various reasons they still do not contain sufficient other information for identification (e.g. Chiodini, Moody & Manser, 2001; Cook, 1996; Crewe, 2002). Hence there is a real risk of misidentification of *M. ozzardi* during clinical examination of patients for the better known skin-dwelling microfilariae such as *Onchocerca volvulus* (Leuckart, 1893). This sort of problem led to the initial misidentification of *M. streptocerca* for *O. volvulus* in Uganda where *M. streptocerca* was not expected (Fischer *et al*. 1998). *Mansonella ozzardi* and *O. volvulus* are only known to be sympatric in the Amazonian focus of onchocerciasis which straddles the Brazil-Venezuela frontier (Fig. 1), but in view of the apparent potential for the spread of onchocerciasis through human migration, and the actual discovery of a new (but possibly temporary) focus of the disease in central Brazil (Maia-Herzog *et al*. 1999), it should not be assumed that outside the Amazonian focus there will be no problems. Hence, when Chamon *et al*. (1999) reported corneal eye lesions associated with *M. ozzardi* in a different part of the Brazilian Amazon it was necessary to confirm that *O. volvulus* was not implicated, and this was achieved partly through the development of a new molecular (DNA) assay (Morales-Hojas *et al*. 2001). Another complication for diagnosis is that *O. volvulus* can be found in the peripheral blood of up to one-third of infected people in hyperendemic communities (WHO, 1987), and this further increases the possibility of not detecting mixed infections.

Of course the morphology of the adults and microfilariae of *M. ozzardi* has been described (Orihel & Eberhard, 1982; Kozek, Eberhard & Raccurt, 1983), but in view of the lack of recognition that *M. ozzardi* commonly occurs in the skin there is no account that specifically compares the morphology of microfilariae of *M. ozzardi* and *O. volvulus* for the purposes of distinguishing these two species when they might occur together in a skin biopsy (or peripheral blood sample). However, most standard texts refer to the body size (length and sometimes width) and the pattern of nucleation of the tail. There is no doubt that the microfilariae of *M. ozzardi* are smaller than those of *O. volvulus*, but most texts also

* Corresponding author: Department of Entomology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK. Tel: +44 (0)20 7942 5593. Fax: +44 (0)20 7942 5229. E-mail: r.post@nhm.ac.uk

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Fig. 1. Geographical distribution of *Onchocerca volvulus* and *Mansonella ozzardi* in America.

indicate that their ranges overlap. With regards to nucleation of the tail most standard texts simply indicate that there is an anucleate tail space in both species (unlike other human unsheathed microfilariae such as *M. perstans*). However, the drawings (but not the text) of Faust, Russell & Jung (1970) seem to indicate that the length of the tail space and the last nucleus might be different. Kozek *et al.* (1983) described the terminal nucleus of *M. ozzardi* from both Haiti and Colombia as elongated and possibly species specific. Another interesting feature of the illustrations of the standard textbooks is that they often show *M. ozzardi* with little or no anucleate nerve ring, whereas in *O. volvulus* the gap is always well illustrated. Both *M. ozzardi* and *O. volvulus* have a finely striated cuticle (Bain, 1969; Kozek & Raccurt, 1983) but there have also been reports of larger annular rings in the cuticle of *M. ozzardi*. However, Chadee *et al.* (1994) came to the conclusion that these annular rings were artifacts of the method of preparation. Laurence & Simpson (1968) described different shapes of the cephalic armature for *M. ozzardi* and *O. volvulus* when stained with alcian blue. Finally, the movement of microfilariae emerging from skin snips has been found to be useful in distinguishing between *O. volvulus* and *M. streptocerca* in Africa (Muller, 2002), with *O. volvulus* being much more active and faster moving than *M. streptocerca*. However, Shelley, Maia-Herzog & Calvão-Brito (2001) found no difference between the movement of microfilariae of *O. volvulus* and *M. ozzardi* in Brazil.

Molecular identification represents an alternative to morphological examination, but immunodiagnostic methods which have been used for the successful detection of *O. volvulus* elsewhere have either failed when tested against *M. ozzardi* in infected human populations (Shelley *et al.* 2001; Cabrera *et al.* 1989), or they have yet to be extensively tested (e.g. Ngu *et al.* 1998). Morales-Hojas *et al.* (2001) have developed and tested a molecular assay based on species-specific PCR amplification of part of the internal transcribed spacer region (ITS2) of the nuclear ribosomal DNA. This seems to be specific and at least as sensitive as microscopical examination of capillary blood. This high sensitivity was surprising because Raccurt *et al.* (1982) had estimated that examination of skin biopsies was only 35% as sensitive as examination of blood. The difference was most likely due to the different ways in which the skin biopsies were examined. The traditional method (as used by Raccurt *et al.* 1982) involves incubating the skin biopsy in a suitable fluid and then counting the number of microfilariae which emerge, but it has been established for *O. volvulus* that many microfilariae remain inside the biopsy and are therefore undetected (Schulz-Key, 1978; Taylor *et al.* 1989). The molecular method on the other hand involves the extraction of DNA from the whole (unincubated) skin biopsy and the subsequent detection of parasite DNA. Thus all microfilariae existing within a skin biopsy are available to be detected.

The objective of this study was to evaluate simple morphological characters that might be used for species-diagnosis of microfilariae of *O. volvulus* and *M. ozzardi*. We have examined a mixture of new characters and characters which appear to be potentially species specific from examination of the literature (see above). *Mansonella perstans* (Manson, 1891) has also been reported from peripheral blood samples from Mexico, Panama, Colombia, Venezuela, Guyana, Surinam, French Guiana, Trinidad and some of the other Caribbean islands (Hawking, 1979). A single infection has been reported from Roraima state in Brazil outside the Amazonian onchocerciasis focus, but this was probably not autochthonous (Oliveira, 1963). In any case, *M. perstans* does not present an identification problem because the unsheathed microfilariae (which unlike *M. ozzardi* really are only found in blood) are easily identified by their tails which end bluntly and have nuclei to the tip (see for example Muller, 2002; WHO, 1997).

MATERIALS AND METHODS

A nodule of *O. volvulus* was supplied by Dr Renerys from a Yanomami Indian at the Indian Hospital in Boa Vista in Roraima State Brazil (near the

Table 1. Measurements (in μm) of microfilariae of *Onchocerca volvulus* and *Mansonella ozzardi*

Sample	Tissue	Body length		Sample size	Cephalic space	Nerve ring	Tail width				Terminal nucleus width	Terminal nucleus length	Tail space	ts/tnl*
		Sample size	Mean (range)				1	2	3	4				
O. volvulus														
Yanomami	Skin	5	259 (252–267)	8	8·1 (6·7–9·6)	5·9 (5·3–6·7)	2·0 (1·4–2·4)	2·1 (1·4–2·4)	2·7 (2·4–2·9)	4·3 (3·9–4·8)	1·0 (0·9–1·3)	2·6 (2·2–3·5)	11·9 (10·5–13·2)	4·6 (3·3–5·0)
Boa Vista	Nodule	29	254 (232–277)	50	7·0 (5·8–8·2)	5·9 (5·8–6·2)	2·0 (1·9–2·4)	2·4 (1·9–2·9)	3·0 (2·4–3·4)	4·4 (3·8–4·8)	1·0 (0·9–1·3)	3·4 (2·2–5·2)	13·1 (9·1–16·5)	4·2 (2·0–6·3)
Xitei	Skin	33	251 (186–286)	16	8·1 (6·7–9·6)	5·6 (4·8–6·7)	1·9 (1·4–1·9)	2·0 (1·9–2·4)	2·6 (2·4–3·4)	4·1 (3·1–4·1)	1·0 (0·9–1·3)	2·7 (2·2–4·4)	11·8 (8·3–14·0)	4·5 (2·9–5·6)
Total		88	253 (186–286)	53	7·5 (5·8–9·6)	5·8 (4·8–6·7)	1·9 (1·4–2·4)	2·3 (1·4–2·9)	2·8 (2·4–3·4)	4·4 (3·1–4·8)	1·0 (0·9–1·3)	3·1 (2·2–5·2)	12·6 (8·3–16·5)	4·3 (2·0–6·3)
M. ozzardi														
Normenta†	Blood smear	50	195 (176–211)	0	—	—	—	—	—	—	—	—	—	—
Normenta	Haemoglobin-free blood	50	193 (165–228)	6	4·2 (3·8–4·8)	4·6 (4·3–4·8)	1·0 (1·0–1·4)	1·4 (1·4–1·4)	1·8 (1·4–1·9)	3·2 (2·9–3·4)	1·0 (0·9–1·3)	6·7 (4·8–7·9)	5·4 (4·4–7·9)	0·9 (0·7–1·6)
Acre†	Skin	36	171 (149–202)	0	—	—	—	—	—	—	—	—	—	—
Pauini and Labrea	Skin	33	195 (168–224)	19	5·2 (3·8–6·7)	3·9 (2·4–5·3)	1·0 (0·5–1·4)	1·3 (1·0–1·4)	1·6 (1·4–1·9)	2·9 (2·4–3·4)	0·8 (0·4–1·3)	5·5 (4·4–7·0)	5·7 (3·1–8·4)	1·0 (0·5–1·4)
Total		169	189 (149–228)	25	5·0 (3·8–6·7)	4·1 (2·4–5·3)	1·0 (0·5–1·4)	1·3 (1·0–1·4)	1·7 (1·4–1·9)	2·9 (2·4–3·4)	0·9 (0·4–1·3)	5·8 (4·4–7·9)	5·6 (3·1–8·4)	1·0 (0·5–1·6)

* ts/tnl is the ratio of the length of the tail space and the terminal nucleus.
† The microfilariae from the blood smear from Normenta and the skin biopsies from Acre were not well enough preserved to enable measurements other than total body length.

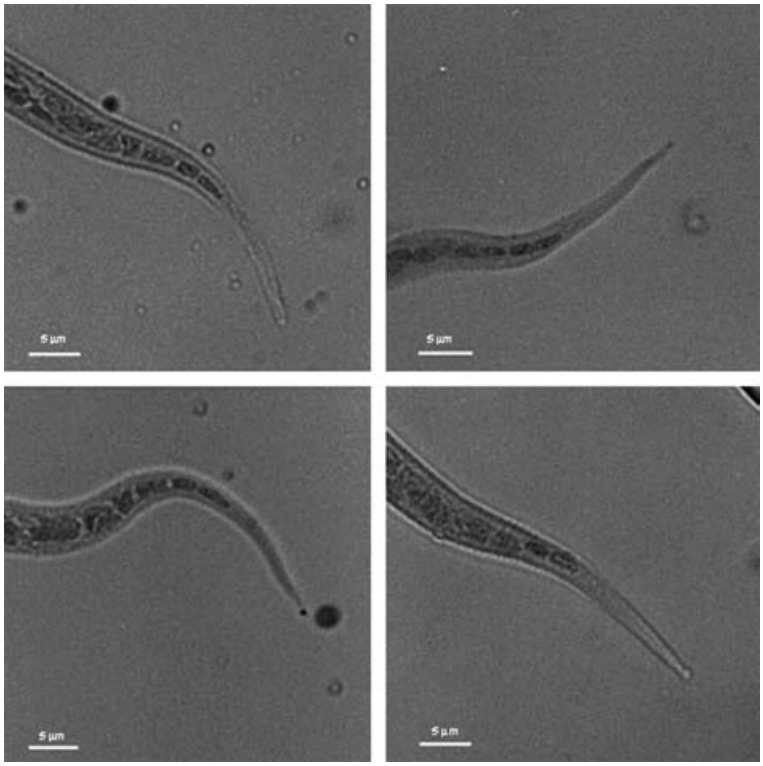


Fig. 2. Tails of four microfilariae of *Onchocerca volvulus* stained with lactopropionic orcein to show nucleation.

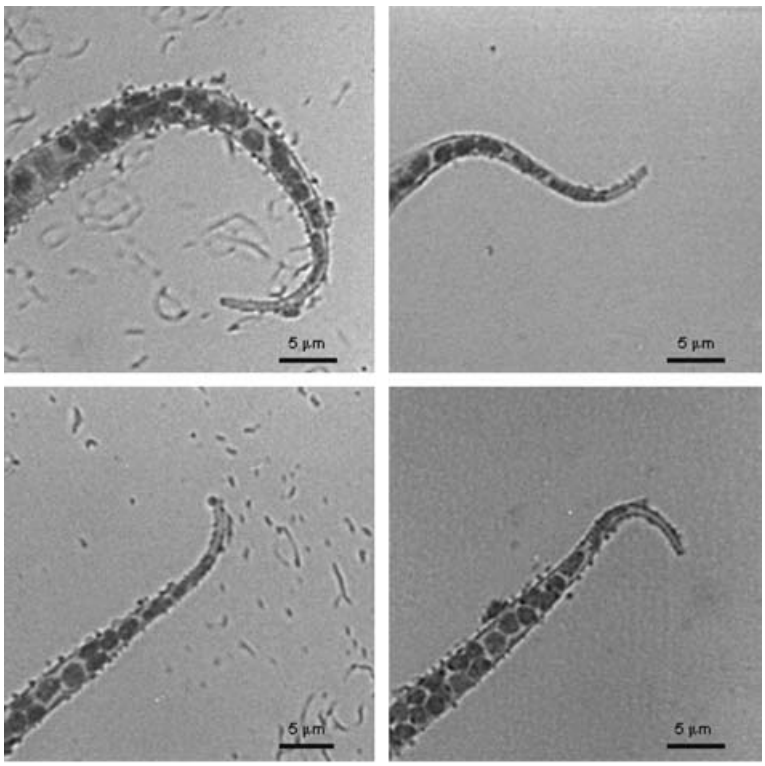


Fig. 3. Tails of four microfilariae of *Mansonella ozzardi* stained with lactopropionic orcein to show nucleation.

Amazonian onchocerciasis focus) on 26 April 1999. Nodules were preserved in ethanol and microfilariae obtained by pulling out broken lengths of adult female worms and squeezing out their body contents into ethanol which could then be dropped onto a microscope slide. Thus, mature (stretched) microfilariae would have come from the uterus of the adult females, but they could be easily separated from earlier immature (unhatched) stages (Schulz-Key, Jean & Albiez, 1980).

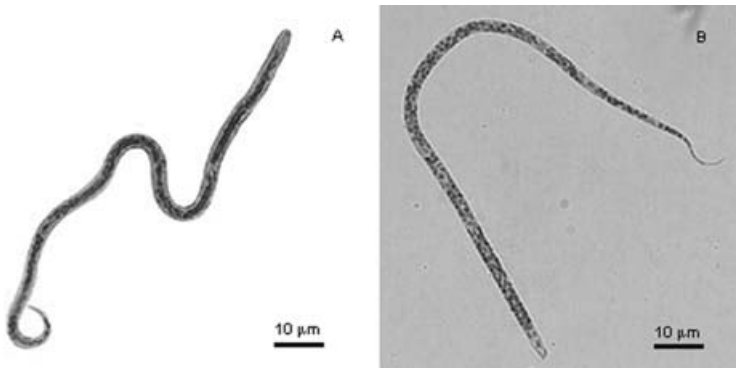


Fig. 4. Whole microfilariae of *Onchocerca volvulus* (A) and *Mansonella ozzardi* (B) stained with lactopropionic orcein.

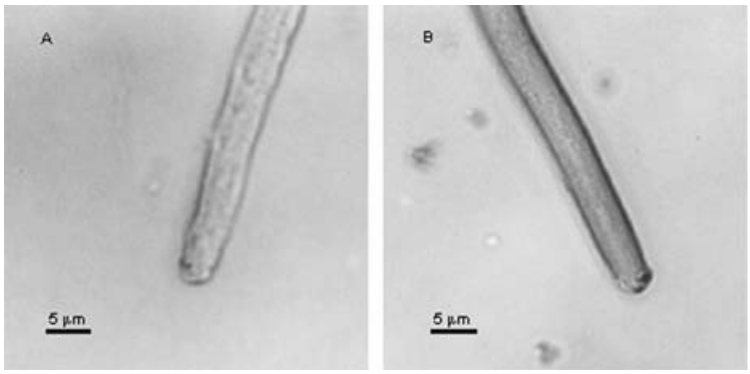


Fig. 5. Cephalic armature of *Mansonella ozzardi* (A) and *Onchocerca volvulus* (B) stained with alcian blue.

Two samples of *M. ozzardi* microfilariae were obtained from blood of a heavily infected person from Normenta in Jujuy Province in northern Argentina on 26 January 1998. Firstly, a pool of microfilariae was obtained from 20 ml of blood removed by syringe. Haemoglobin was removed and the blood centrifuged to lightly pellet the microfilariae. These were then resuspended and preserved in absolute ethanol, and then dropped onto a microscope slide. Secondly, a thick blood film was prepared and dried for later examination.

Skin biopsies were obtained using a corneoscleral punch from within the Brazilian Amazonian onchocerciasis focus by J. C. M. Schuertz from Xitei (Roraima State) in November 1999, and outside the onchocerciasis focus from Antimari (River Acre, Acre State), Labrea and Pauini (both on the River Purus in Amazonas State), a region where Chamon *et al*. (1999) had reported eye lesions associated with *M. ozzardi*. Skin biopsies from Antimari (October 1998), Labrea and Pauini (both August 1999) were incubated for 30 min in distilled water (for microscopical examination for emergent microfilariae) and subsequently preserved in ethanol. Skin biopsies from Xitei were preserved directly into ethanol without prior incubation. In the laboratory, microfilariae were extracted from preserved skin biopsies by collagenase digestion according to Schulz-Key (1978).

Microfilariae in a drop of alcohol or collagenase digestion solution were normally mixed with a drop of lacto-acetic orcein and allowed to stain for approximately 10 min before adding the cover-slip and examining under the microscope at $\times 400-\times 1000$ magnification with bright-field illumination.

Orcein-stained microfilariae were measured to determine total length, length of cephalic space, width at nerve ring, width of the terminal nucleus, length of the terminal nucleus, length of the tail space and shape of the tail. The shape of the tail was initially compared between 2 microfilariae of *O. volvulus* from skin snips from different infected persons from the Amazonian onchocerciasis focus, and 2 microfilariae of *M. ozzardi* from the pool from Argentina. The width of the tail was measured at $12 \times 0{\cdot}96\ \mu$m intervals from the tip to estimate the point of maximum width difference (which was $2{\cdot}16\ \mu$m at a point $6{\cdot}71\ \mu$m from the tip of the tail). Subsequently, regular width measurements were made at the distal (tw1) and proximal (tw2) ends of the last nucleus, the proximal end of the penultimate nucleus (tw3) and $6{\cdot}71\ \mu$m from the tip of the tail (tw4). As well as the measurements the distribution and shape of the nucleii in the head, nerve ring and tail was observed and illustrated.

Microfilariae of *O. volvulus* from Boa Vista (nodule) and *M. ozzardi* from Argentina were stained with alcian blue to show the cephalic armature

according to methods described by Laurence & Simpson (1968) and Garms (1985). These small structures were observed under the microscope at $\times 1000$ magnification bright field illumination.

RESULTS

The measurements of *O. volvulus* and *M. ozzardi* are presented in Table 1. The body length, length of the cephalic space and width at the nerve ring indicate that *M. ozzardi* is generally smaller than *O. volvulus*, but there are overlaps in the ranges of all these characters. The 4 tail width measurements also indicate that *M. ozzardi* is simply smaller, but the data for tw1, tw2 and tw3 show no overlap between the species, and of these tw3 (width of body at proximal end of penultimate nucleus) is most distinctive. The average lengths of the terminal nucleus and the tail space are very different, although they overlap between the species. To examine the differences in nucleation of the tail the ratio of the length of the terminal nucleus to its width was compared, and found to have overlapping ranges. However, the ratio of the length of the tail space (which was on average longer in *O. volvulus*) to the length of the terminal nucleus (which was on average shorter in *O. volvulus*) was found to be very distinctive and the ranges did not overlap (*O. volvulus* mean = 4·3, range 2·00–6·33; *M. ozzardi* mean = 1·00, range 0·53–1·64). The appearance of these differences is illustrated in Figs 2 and 3.

Examination of the cephalic space revealed no differences in the pattern of nucleation, but there was some difference in the pattern of nucleation of the nerve ring. In all *O. volvulus* examined ($n = 56$) the nerve ring consisted of a distinctive anucleate gap. In a few specimens the gap was quite narrow, and in one it was narrow and bridged by a single nucleus. By contrast, in *M. ozzardi* ($n = 21$) the nerve ring was always narrow to such an extent that there was often no distinct anucleate area (Fig. 4).

The cephalic armature did not always stain well with alcian blue, but in the best specimens it was clear that there was a difference between the two species (Fig. 5), and that this was the same as that originally described and illustrated by Laurence & Simpson (1968).

DISCUSSION

The method of specimen preparation is known to have a large effect on microfilariae body-size measurements (Bain, 1969; WHO, 1997), but our length measurements for *O. volvulus* (range 186–286 μm) and *M. ozzardi* (range 149–228 μm) are comparable with those published by previous authors. For example, for *O. volvulus* Bain (1969) gave a range of 230–270 μm and Muller (2002) gave 280–330 μm. For *M. ozzardi* Crewe (2002) gave 200–225 μm and Muller (2002) gave 175–240 μm. Our results confirm that *O. volvulus* is usually larger than *M. ozzardi* for a number of body measurements, but that the ranges overlap (or are likely to overlap with larger samples). Body size of microfilariae is therefore a useful indication of species identity, but is not sufficiently different for definitive diagnosis.

In 98% of microfilariae of *O. volvulus* there was a distinctive anucleate nerve ring, whereas 43% of *M. ozzardi* showed no distinctive anucleated gap at the nerve ring. Hence the nucleation of the nerve ring is a useful first indication of species identity, but it is not sufficiently different for definitive diagnosis.

The structure of the cephalic armature as revealed by alcian blue was clearly different between the species (as described by Laurence & Simpson, 1968), and might also be useful for identification of L1 larvae from the vector because it persists into that stage (Garms, 1985). However, it did not stain clearly in many specimens, and even when it does stain well it is a very small structure at the limits of visibility using standard laboratory microscopes at $\times 1000$ magnification.

The nucleation of the tail was found to be species-specific and should be diagnostic. The average length of the terminal nucleus (longer in *M. ozzardi*) and tail space (longer in *O. volvulus*) were quite different, but the ranges overlapped, whereas the ratio of the two measures showed a 4-fold mean difference with distinctive non-overlapping ranges. This character can be expected to be diagnostic.

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