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Parasitology Research

Filarial infection caused by Onchocerca boehmi (Supperer, 1953) in a horse from Italy

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Abstract:	Equids may be infected by a range of skin-dwelling filarial nematodes, including four species of the genus Onchocerca. Current literature on equine onchocercosis is fragmentary, and often limited to isolated case reports. The present study describes a clinical case caused by Oncocerca boehmi (syn. Elaeophora boehmi) in an 8-years old horse from northern Italy. The animal presented a firm and painless mass on the proximal third of the right metacarpal region. Ultrasound examination showed a peritendinous enlargement around the palmaro-lateral part of the tendons, characterized by an elongated hypoechoic and well-defined structure, embedding a coiled hyperechoic line. The metacarpal nodule was resected and histologically examined. Fragments of a parasitic nematode were detected, isolated and examined. Total genomic DNA was extracted from individual fragments using a commercial kit and the nematode was identified as O. boehmi. Comparative sequence analysis of the nematode cytochrome oxidase subunit 1 (cox1) sequence with data available in the GenBankTM database revealed high sequence similarity (i.e., 91%) with that of Onchocerca lupi. Thus far, O. boehmi has only been described in two isolated reports in Austria and Iran, and information about its life-cycle and vectors is lacking. The systematic position of this species within the genus Onchocerca is in concordance with our morphological and molecular results. In this article, we describe the first autochthonous case of equine onchocerciasis in Italy caused by O. boehmi, and discuss novel parasitological, clinical and pathological data on these neglected pathogens of horses.
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Author Comments:	TTo the Editor of Parasitology International Dear Editor, Please find herein enclosed the manuscript entitled "Infection by Onchocerca boehmi Supperer, 1953 in a horse in Italy" by Yasen Mutafchiev, Vincenzo Veneziano, Alessio Giannelli, Francesca Abramo, Mario Santoro, Maria Stefania Latrofa, Cinzia Cantacessi, Coralie Martin, Domenico Otranto, Andrea Bertuglia, Barbara Riccio, and myself, to be considered for publication in Parasitology International. As you know, horses may be infected by a range of skin-dwelling onchocercid nematodes, including Onchocerca reticulata or O. cervicalis. Unfortunately, the current literature on equine onchocerciasis is fragmentary, and often limited to isolated case reports. In addition, no information is available for other species infecting horses, as in the case of O. boehmi. In the present study, we provide the full description of a clinical case of equine onchocerciasis caused by O. boehmi in a 8-years old gelding Belgian show jumper from northern Italy. The diagnosis of the infection was performed by a comprehensive diagnostic panel, which included ultrasound and histological examination. In addition, the parasite was morphologically identified and molecularly characterized by using microscopy (either light or SEM) and cytochrome oxidase subunit 1 sequencing, respectively. Thus far, O. boehmi has only been described in two isolated reports, and information about its biology is lacking. In the present study, we hypothesize that the unusual anatomical localization of this onchocercid described in this report has resulted from the migration of the parasite from the circulatory system (i.e., arteries and veins of limbs) to the subcutaneous tissues of the metacarpal region. I hope you will find the manuscript of interest for the readers of your journal. Thank you for your kind cooperation, Yours faithfully, Riccardo Paolo Lia
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Filarial infection caused by *Onchocerca boehmi* (Supperer, 1953) in a horse from Italy

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Abstract

Equids may be infected by a range of skin-dwelling filarial nematodes, including four species of the genus *Onchocerca*. Current literature on equine onchocercosis is fragmentary, and often limited to isolated case reports. The present study describes a clinical case caused by *Oncocerca boehmi* (syn. *Elaeophora boehmi*) in an 8-years old horse from northern Italy. The animal presented a firm and painless mass on the proximal third of the right metacarpal region. Ultrasound examination showed a peritendinous enlargement around the palmaro-lateral part of the tendons, characterized by an elongated hypoechoic and well-defined structure, embedding a coiled hyperechoic line. The metacarpal nodule was resected and histologically examined. Fragments of a parasitic nematode were detected, isolated and examined. Total genomic DNA was extracted from individual fragments using a commercial kit and the nematode was identified as *O. boehmi*. Comparative sequence analysis of

the nematode cytochrome oxidase subunit 1 (*cox*1) sequence with data available in the GenBank™ database revealed high sequence similarity (i.e., 91%) with that of *Onchocerca lupi*. Thus far, *O. boehmi* has only been described in two isolated reports in Austria and Iran, and information about its life-cycle and vectors is lacking. The systematic position of this species within the genus *Onchocerca* is in concordance with our morphological and molecular results. In this article, we describe the first autochthonous case of equine onchocerciasis in Italy caused by *O. boehmi*, and discuss novel parasitological, clinical and pathological data on these neglected pathogens of horses.

Keywords: equine onchocerciasis, *Onchocerca boehmi*, horse, limb nodules.

Introduction

The genus *Onchocerca* (Spirurida, Onchocercidae) includes more than 30 species of nodule-inducing nematodes inhabiting different anatomical regions of the subcutaneous tissues, ligaments, and aponeuroses of domestic mammals (Anderson 2000, Uni et al. 2015). The microfilariae released by the female worms migrate through the dermis of specific body areas, and they are ingested by blood feeding intermediate hosts (e.g., blackflies). In the vector, larvae moult twice, reaching the infective third larval stage (L3) in about 3-4 weeks. The L3s are then transmitted to a susceptible vertebrate host via the blood meal (Onmaz et al. 2013). The infection is patent in about 12-16 months (Taylor et al. 2007). *Onchocerca reticulata* Diesing, 1841, and *Onchocerca cervicalis* Railliet and Henry, 1910 are the best-known filarial worms of equids due to their wide geographical distribution and potential clinical relevance (Muller 1979). In particular, *O. cervicalis* infection was firstly described in Australia as "Queensland itch" (Riek 1953), and is characterised by the occurrence of an allergic dermatitis, likely induced by the skin dwelling microfilariae (Lees et al. 1983). Microfilariae may also invade the eyes, causing ocular signs (Cello 1971; Munger 1983), while *O. cervicalis* adults may cause inflammatory reactions in the nuchal ligament, which range from acute oedematous necrosis to chronic granulomatous changes. Conversely, the infection by *O. reticulata* is usually characterised by the presence of subcutaneous nodules over or within the flexor tendons and suspensory ligaments, where it is potentially associated with swelling and lameness (Scott and Miller 2003). Equids may also be infected by *Onchocerca raillieti* Bain, Muller, Khamis, Guilhon & Schillhorn van Veen, 1976, a species detected in subdermal masses in the withers or penis and in the perimuscular conjunctive tissue of domestic donkeys from Africa (Bain et al. 1976). Another species of the genus, *Onchocerca boehmi* (Supperer, 1953) (syn *Elaeophora boehmi*), was described in the arteries and veins of the limbs of Austrian horses. In most cases, horses infected by *O. boehmi* are asymptomatic (Supperer, 1953).

The scientific literature on equine onchocerciasis is fragmentary and often dated. For example, *O. cervicalis* has been long considered a synonymous of *O. reticulata*, until Bain (1975) morphological differences between these two species. Similarly, epidemiological data on onchocercid species infecting horses are scarce. For instance, infection by *O. cervicalis* has been diagnosed in the United States (Stannard and Cello 1975), Canada (Marcoux et al. 1977), Australia (Riek 1954), and South America (Marques and Scrofernecker 2004). In Europe, few studies have been performed (Anderson 2000), and onchocercids have seldom been identified at species level. In this article, we describe the

first autochthonous case of equine onchocerciasis in Italy caused by *O. boehmi*, and discuss novel parasitological, clinical and pathological data on these neglected pathogens of horses.

Materials and Methods

Case presentation

An 8-years-old 570 kg gelding Belgian horse, used in show-jump competitions, housed in northern Italy (Genoa, Liguria region, Italy), was presented in July 2013 at the Veterinary Teaching Hospital of the University of Turin (Piedmont, Italy) with an evident lump in the right metacarpal region. This lesion had appeared six months prior to presentation as a diffuse swelling, during the spring season, that had progressively increased in size. The owner sought the advice of clinicians in order to investigate the presence of a tendinitis in the mid-metacarpal region. During the clinical examination, the horse presented a firm and painless mass located palmaro-laterally on the proximal third of the right metacarpal region and a mild swelling in correspondence of the medial aspect of the left metacarpal region (**Figure 1**). Numerous firm and small subcutaneous nodules were observed on the back of the animal, along the epiaxial muscles. The horse was mildly lame only at the beginning of the clinical presentation. Palpation did not allow defining the relationship of the mass with the superficial digital flexor tendon (SDFT). Previous treatments included DMSO (dimethyl sulfoxide) Gel 99.9% as a topical application, twice daily over 3 weeks, to reduce the swelling. An oral administration of ivermectin paste was previously recommended by the practitioner, at double label dose (400 µg/kg body weight), on the basis of previous experience with similar subcutaneous nodules of suspected parasitic aetiology.

Ultrasonographic examination

An ultrasonographic examination was conducted using a mobile Logiq E Vet Ultrasound machine (General Electric Company Fairfield, CT, USA) with a linear multifrequency transducer (8-12 MHz). The examination was carried out on site, with the horse in standing position. No sedatives were administered. Prior to the ultrasound examination, both palmar metacarpal regions were prepared using standard procedures. Images were obtained using a standoff pad coupled to the transducer. The examination showed the presence of a peritendinous enlargement around the palmaro-lateral aspect of the SDFT, on the right forelimb, exerting a mass-effect on the whole soft tissues. The abnormal peritendinous mass was characterized by an elongated hypoechoic and well-defined structure, including a coiled hyperechoic line. On the left forelimb, the ultrasound examination revealed the same ultrasonographic pattern on the medial aspect of the mid metacarpal region, but with a more

echogenic structure and lacking the hyperechoic linear structure. Ultrasonographic findings of both structures were consistent with a peritendineous localization of a verminous nodule (**Figure 2**).

Surgical removal of the nodule

Surgical removal of the peritendineous mass was performed, with the horse standing and sedated using a constant infusion rate. In particular, the infusion rate was prepared by adding 2 mg of medetomidine to a 0.5 litre bag of saline (4 μg/mL) and this volume was administered at a rate of 1 drop/sec (10 drops/mL infusion set drip rate), which provides approximately 80 min of infusion. A local analgesia was administered using a high metacarpal nerve block, with a 2% solution of mepivacaine. The nodule was resected from the SDFT peritenon and the deep metacarpal fascia. Haemorrhage was controlled using an Esmark bandage, applied proximally to the carpal region. The skin was closed using routine procedures and a half-limb bandage was applied post-operatively. Post-surgery standard anti-inflammatory and antibiotic therapies were administered over 3 days following the procedure, and the horse was not trained for two weeks post-surgery, and it had a complete functional outcome.

Histopathological analysis

Histopathological examination of the excised metacarpal nodule was performed; the tissue was fixed in a 10% formalin solution (pH 7.4) and processed using standard procedures (Mutafchiev et al. 2013).

Parasitological and molecular analyses

A sub-section of the nodule was fixed and preserved in 70% ethanol, and dissected under a stereomicroscope. For light-microscopy, nematode fragments were cleared and examined as temporary mounts in lactophenol, while those used for scanning electron microscopy observations were prepared and studied, as described elsewhere (Mutafchiev et al. 2013). A female of *O. boehmi* (one microscopic slide) from the collection of Supperer deposited in the University of Veterinary Medicine Vienna (UVMV) was used as comparative material. In addition, total genomic DNA was extracted from parasite fragments recovered from an individual specimen using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany) in accordance with the manufacturer's instructions; a partial region of cytochrome c oxidase subunit 1 mitochondrial gene (*cox*1; ~689 bp) was amplified as previously described (Otranto et al. 2011). The amplicon obtained was purified using Ultrafree-DA columns (Amicon, Millipore; Bedford, USA) and sequenced directly using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an

automated sequencer (ABI-PRISM 377). Sequences were determined from both strands (using the same primers individually as for the PCR) and the electropherograms were verified by eye. The nucleotide sequence of the *cox*1 fragment was conceptually translated into amino acid sequences using the invertebrate mitochondrial code by MEGA 6.0 software (Tamura et al. 2013). Finally, the nucleotide sequence was compared with those available in the GenBank™ database by BLAST analysis.

Results

Histopathological analysis

Both haematoxylin and eosin and trichromic stains revealed a number of multifocal coalescing parasitic and necrotic granulomas. Each granuloma was characterised by a central cavity containing one or more parasitic sections (possibly due to coiled bodies); the cavity was lined by necrotic material and eosinophilic products of degranulation, surrounded by macrophages and by an external layer of dense collagen. Parasitic granulomas were separated by a dense interstitial eosinophilic and macrophage infiltrate on a background of fibroplasia. Rare collagenolitic granulomas were scattered around the nodule. A visible body wall with an outer cuticule with subcuticular striations and an inner hypodermal layer could be visualised for some of the parasites. Small intestine and empty uteri were also observed. Based on their morphological features, the parasites were identified as nematodes (**Figure 3**).

Morphological and molecular identification

Nematode fragments (n=83) recovered from the nodule varied in length from 0.25 to 8.83 mm, amounting to 18.6 mm total length and a diameter ranging from 127 to 320 µm. The fragments contained only empty ovaries and were considered as belonging to unknown number of unfertilized female worms (**Figure 4A, 4B**). The anterior and posterior extremities were not detected. The cuticle was 16–25 µm thick with three distinct layers: an external layer 3–4 µm thick with transverse striations 7–12 µm apart interrupted along the medial lateral linings (**Figure 4C, 4D, 5A**) and ornate with fine irregularly anastomosing crests (**Figure 4D, 4E, 5B**); a median layer 10–18 µm thick, with annular striae with length corresponding to the distance between the external transverse striations (**Figure 4E**), and an internal hyaline layer 3–5 µm thick.

The morphological identification was confirmed by comparing samples with the voucher material collected by Supperer, which consisted of a single developing young and unfertilised female measuring 54.5 mm in length, without a posterior extremity. The specimen had a maximum body

width of 170 µm at about mid-body and a width, measured at the level of vulva and oesophago-intestinal junction, of 104 µm; the oesophagus was 1,259 µm long and vulva was situated at 575 µm from anterior body end. The cuticle at mid-body was 15–22 µm thick (thicker on lateral sides) with three distinct layers: an external layer 2 µm thick with fine transverse striations 3–5 µm apart, median layers 10–15 thick with annular striae with length coinciding with distance between external transverse striations, and internal layer without specific detailed structure with regular thickness of 4–5 µm (**Figure 6**).

A fragment of 950 bp of the c*ox*1 gene was amplified. BLAST analysis of this sequence revealed the highest nucleotide similarity (i.e., 91%) with that of *Onchocerca lupi* Rodonaja, 1967 available from GenBank™ (Accession Number EF521410).

Discussion

The present study describes a case of *O. boehmi* infection in a horse from Italy, where equine onchocerciasis had never been reported and it is therefore unknown to veterinary practitioners. In equine practice, the appearance of skin nodules is often asymptomatic, and it goes therefore unnoticed by owners (B. Riccio, personal communication). However, in the present report, the clinical presentation was accompanied by an impaired function of the suspensory ligament and occurrence of mild lameness. Interestingly, prior to this case, no clinical symptoms associated to infestation by *O. boehmi* had been described. Given the anatomical localisation of the worms and the nodules, we therefore hypothesize that the nematode might have undertaken an erratic migration from the in circulatory system (i.e., the arteries and veins of limbs) to the subcutaneous tissues of the metacarpal region. *Onchocerca boehmi* had only been diagnosed in two isolated reports, and information about its biology is lacking. According to the original report by Supperer (1953), adults were detected in the medial or external layer of tissues within the artery wall 6.7% of Austrian horses, while a second survey from Iran indicated that 8.69% of horses examined had microfilariae in the blood (Mirzayans and Maghsoodloo 1977).

The presence of numerous parasites in the nodule allowed to assess the histopathological lesions caused by the nematode. Eosinophils were the main inflammatory cells found in the nodule as already reported (Scott and Miller 2003); indeed these cells are typically detected in parasitic cutaneous nodules, and they play a direct role in the defence against the parasites via their main basic protein. Remarkably, eosinophils also play pivotal roles in hypersensitivity disorders (Scott and Miller 2003), and they are seen in eosinophilic granuloma of horses, which are characterised by the presence of cutaneous nodules and by the histopathological occurrence of collagen flame figures

(Scott and Miller 2003). Flame figures, albeit rare, were observed in the case herein described. Onchocerciasis in horses is characterised by both parasitic encystment (adult parasites) and hypersensitivity, the latter usually caused by microfilariae; nevertheless, dead or dying microfilariae were not observed in the tissue examined and the lesions were not pruritic.

The morphology of the cuticle of the nematode fragments collected resembled to that of the voucher material from Austria; this allowed to consider both samples conspecific. In particular, while *O. boehmi* is characterized by cuticle without external ridges and three distinct layers with specific morphology, other *Onchocerca* parasitizing equids, i.e. *O. cervicalis* and *O. reticulata* have a cuticle with well-distinct external annular ridges (Bain 1981). Conversely, the cuticle of *O. raillieti*, which is smooth and does not bear any external ridges, is thicker that than of *O. boehmi* (up to 50–55 µm vs 22–25 µm) and has longer striae (up to 16–20 µm vs 6–12 µm) (Bain et al. 1976; present study). Data reported in the present study indicate that the systematic position of *O. boehmi* should be ranked within the genus *Onchocerca*, as suggested by Bain et al. (1967), and not in the genus *Elaeophora*, as corroborated by morphological and molecular results here produced.

Equine onchocerciasis has been reported worldwide, but most epidemiological information date back from the 70s'. For instance, *Onchocerca* sp. has been diagnosed in horses from the United States, where Stannard and Cello (1975) reported a mean 48% prevalence, whereas Lloyd and Soulsby (1978) recovered microfilariae in 61% of examined animals from the eastern part of the country. Schmidt and colleagues (1982) examined the nuchal ligament of 83 horses from Midwestern U.S., and 37% of were positive for adult parasites. Klei et al. (1984) detected microfilariae in 76% (out of 84) of ponies from the Gulf Coast area and in 82.4% of horses (out of 51) from the Louisiana State. Of 664 horses from southeaster and midwestern US, 341 (51.4%) were positive for cutaneous microfilariasis by *O. cervicalis* (Cummings and James, 1985). Monahan et al. (1985) diagnosed *O. cervicalis* infection in 30.5% (25/82) ponies in USA. Finally, Lyons and colleagues (2000) reported *O. cervicalis* in 24% of horses (out of 157) examined for several species of internal parasites at necropsy in Kentucky. Infection by *O. cervicalis* infection has reported also in Canada (Marcoux et al. 1977; Lees et al. 1983). Indeed, during a survey on 383 slaughtered horses from the western Canadian provinces, *O. cervicalis* microfilariae were detected in 11.8% of umbilical samples (Polley 1984). Riek (1954) examined the nuchal ligaments of 282 Australian horses from Queensland and found that 79.8% were infected with *Onchocerca* (erroneously reported as *O. reticulata*), whereas Ottley et al. (1983) sampled a small group of horses and ponies from Queensland and the Northern Territory, and diagnosed *O. cervicalis*, *O. gutturosa* and *O. reticulata* in those animals. In South America, Mancebo et al. (1997) found *O. cervicalis* microfilariae in 24% of the 257 adult work

horses examined in Argentina. A similar result was reported in Brazil by Marques and Scrofernecker (2004), who described *O. cervicalis* microfilariae in the midventral skin samples of 17.9% (215/1,200) horses examined, while adult worms were recovered from the nuchal ligaments of 200 (16.6%) animals. In Europe, a few studies have been performed thus far. In England, Mellor (1973) detected adult *Onchocerca* sp. in nuchal ligaments of 15.8% (33/209) British horses; in France, Moignoux (1954) reported that 6% of horses living in Camargue were infected by subcutaneous *Onchocerca* microfilariae. However, Collobert et al. (1995) found that only 1% (out of 368 horses) were positive for *Onchocerca* at post mortem examinations in Normandy. In other European countries, out of 160 horse skin biopsies examined on horses showed a very low prevalence 3.7% of *Onchocerca* microfilariae from Spain and Poland (Franck et al. 2006). Finally, in Finland, skin biopsies from 42 horses were all negative for microfilariae (Solismaa et al. 2008). These data indicate that equine onchocerciasis is common in horse populations; however, as a consequence of the non-specific clinical presentation and challenging diagnosis, the infection prevalence is most likely underestimated. Additional large-scale studies are required to ascertain the presence and diffusion of *O. boehmi* and other onchocercid species in Italian and European horse populations.

Based on these observations, we therefore argue that veterinary practitioners should be aware of the presence and of the clinical issues associated with infection by these equine parasites, whose occurrence is very often underestimated whose identification may require the application of non-conventional diagnostic methods. Finally, the life cycle of *O. boehmi* remain unknown and further studies should be performed to elucidate the biology of this poorly-knowm onchocercid.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Figure captions

Figure 1. Right forelimb of the horse, showing a subcutaneous firm nodule in the palmaro-lateral aspect of the right metacarpal region. Palmar (A) and lateral (B) view of the limb.

Figure 2. Transversal (A, B) and longitudinal (C, D) ultrasound scans of both mid metacarpal regions, showing a verminous nodule on the palmaro-lateral aspect of the right forelimb. The parasite appears as a coiled hyperechoic line within a hypoechoic nodule, surrounding the superficial digital flexor tendon (B: red arrows). In the longitudinal scan (D) it is evident the localization at the level of the deep metacarpal fascia.

Figure 3. Histopathology of the nodule. A) Granulomatous reaction around a parasite: the cavity is lined by necrotic material with products of eosinophil degranulation (*), macrophages and giant cells (>), collagen bundles, eosinophils and lymphocytes (trichrom stain); B) Morphological details of a coiled parasite within a granuloma: small intestine, uteri (>) and lateral chord (*)(HE stain); C) Subcuticular striations (HE stain); D) Collagenolitic granuloma at the periphery of the nodule (HE stain).

Figure 4. *Onchocerca boehmi*, light microscopy, horse from Italy. A) Body fragment with intestine (arrow) and two uteri (arrowheads); B) Transverse section through body, note two uteri (arrowheads); C) Surface of cuticle, note the interrupted externals transverse striations along median lateral line (C2); D) Surface of cuticle exhibited when studied without coverslip, note internal striae (arrowheads), transverse striations (arrows) and ornamentation of fine irregularly anastomosing crests; E) Detail of cuticle of two body fragments, note fine external crests on the surface (arrowheads) and internal annual striae of the median layer (arrows).

Figure 5. *Onchocerca boehmi* scanning electron microscopy, horse from Italy. A) Transverse striations (arrows) of cuticle surface; B) Cuticle ornamentation, note transverse striations (arrows) and fine external crests (arrowheads).

Figure 6. *Onchocerca boehmi*, cuticle of young female, horse from Austria. Note the fine external crests (arrowheads) and the internal striae (arrows).

Figure 1

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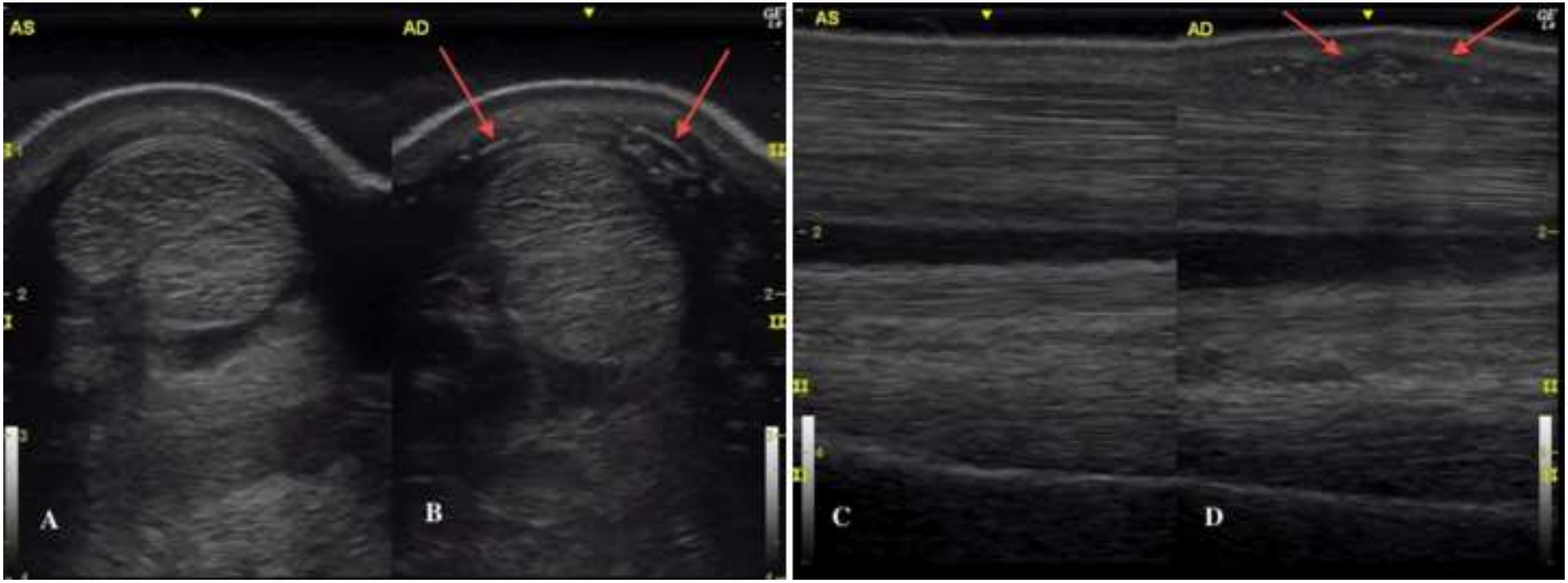

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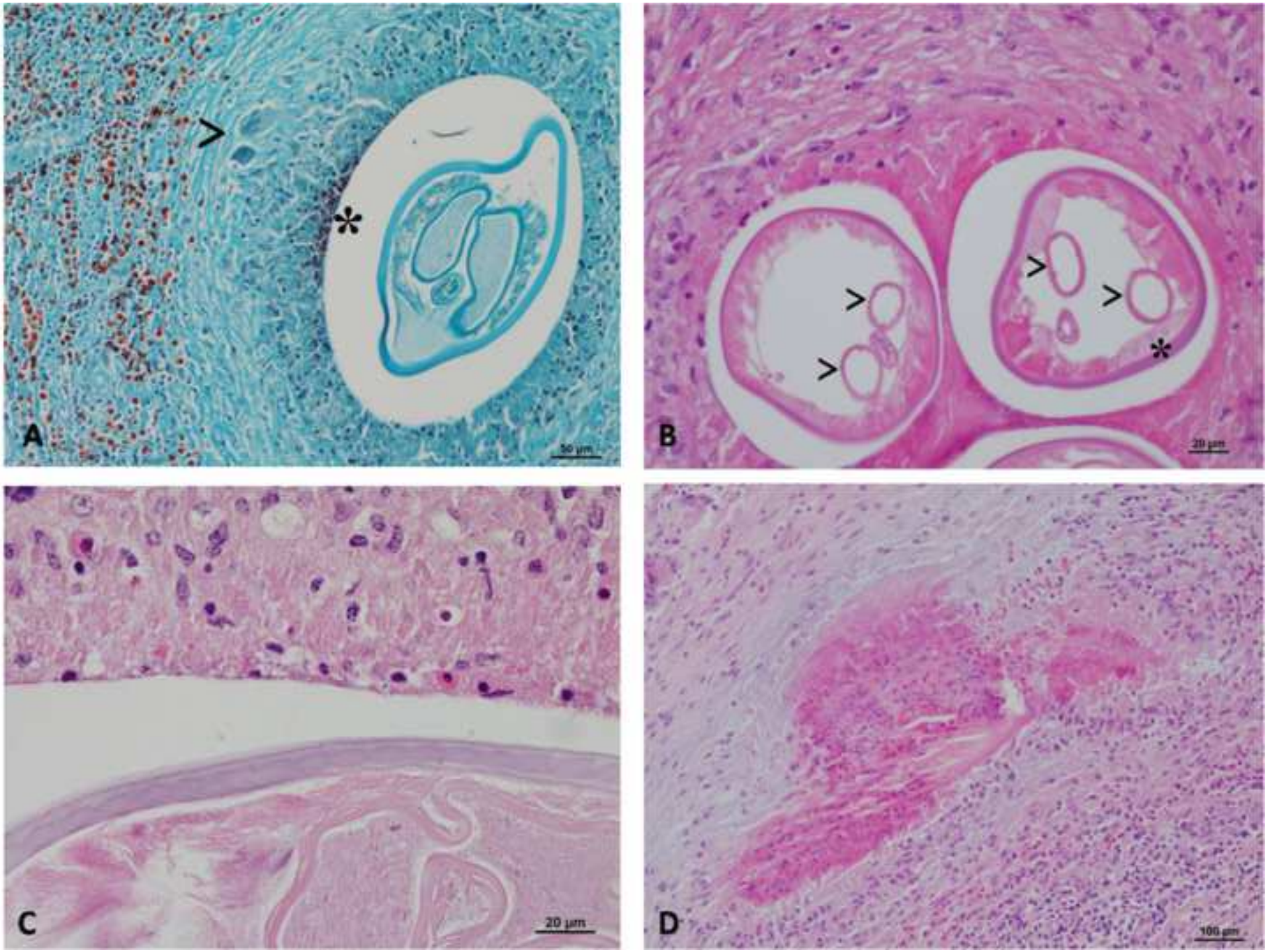

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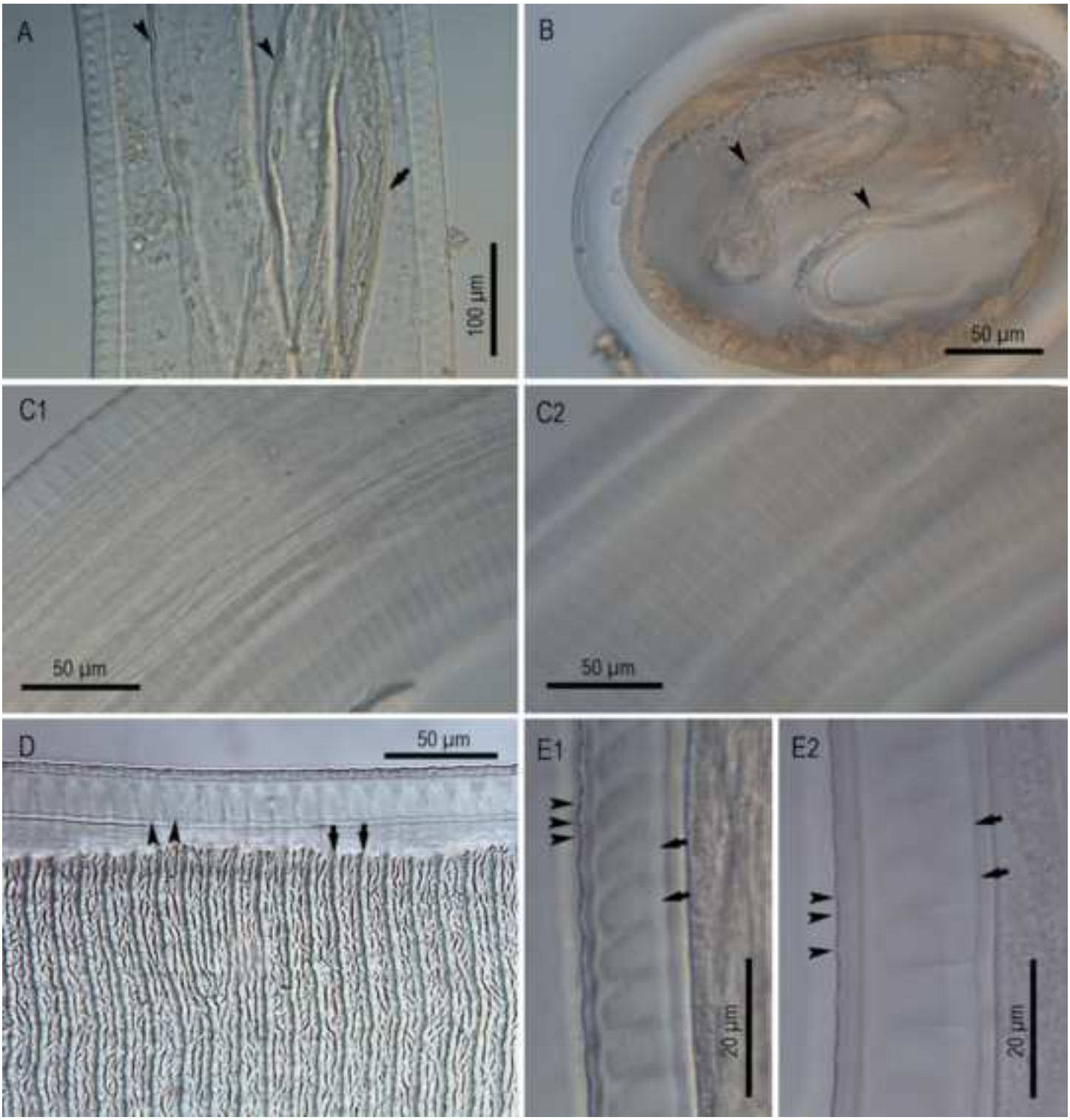

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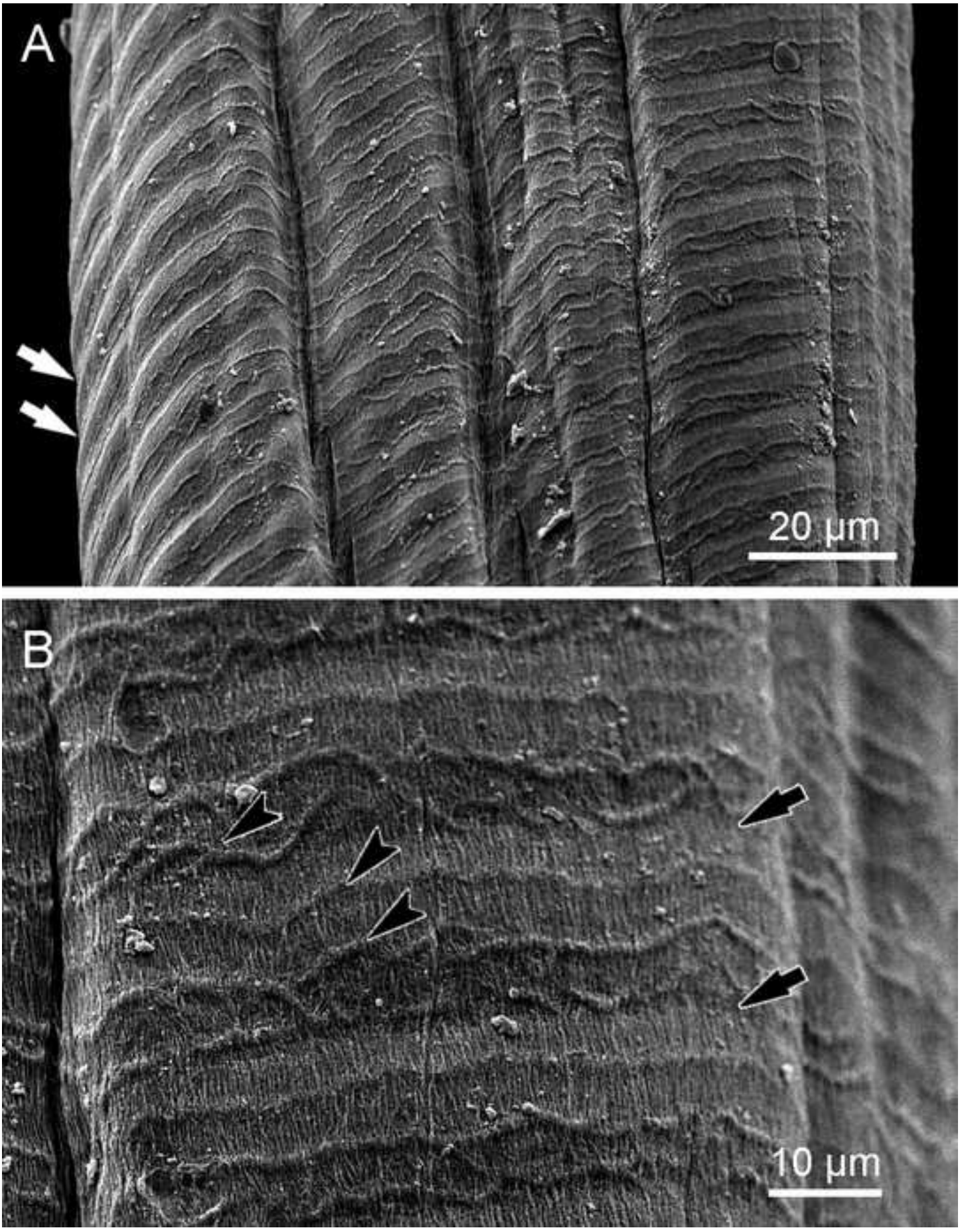

Figure 6

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