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First finding of *Trichinella pseudospiralis* in two Tawny Owls (*Strix aluco*) from Sweden

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Summary

The worldwide distribution of *Trichinella pseudospiralis,* the first discovered non-encapsulated *Trichinella* species infecting both mammals and avian hosts, has been suggested to be attributed to bird migration. At present, the knowledge on the role of carnivorous avian species as a reservoir hosts in Europe is still limited. Thus, the aim of this research was to screen for *T. pseudospiralis* in raptorial, carrion-feeding, and scavenging birds in Sweden and Slovakia, where the parasite has been previously documented in wildlife. In total, 212 pectoral muscle samples of carnivorous birds from Slovakia (n = 153) and Sweden (n = 59) were examined for the presence of *Trichinella* larvae using standard artificial digestion method. Out of 12 Accipitridae species, 4 Falconidae species, 2 Strigidae species, 1 Tytonidae species, and 4 Corvidae species examined within our study, muscle larvae were found in two non-migratory tawny owls (*Strix aluco*) from one geographical region of Sweden. Histological and molecular methods confirmed the presence of *T. pseudospiralis*. This is the first report of this parasite in an avian species in Sweden and the second report in European birds.

Keywords: *Trichinella pseudospiralis*; epidemiology; carnivorous birds; birds of prey; tawny owl; *Strix aluco*; Sweden; Slovakia

Introduction

Nematode parasites of the genus *Trichinella* are the agents of worldwide disease in both sylvatic and domestic epidemiological cycles and also cause an important foodborne zoonosis. The parasite has low host specificity and is able to infect a broad spectrum of mammalian hosts. *Trichinella pseudospiralis* is the first discovered non-encapsulated *Trichinella* species (Garkavi, 1972), unique among other species by infectivity not only for mammals, but also for avian species. *Trichinella pseudospiralis* is known to have a cosmopolitan distribution and world-wide, 63 isolates of the species have been detected in mammals and birds to date (Pozio & Zarlenga, 2013). In Europe, so far two cases of *T. pseudospiralis* infection have been documented in sedentary night-birds of prey in central Italy – in a tawny owl (*Strix aluco*) and a little owl (*Athene noctua*) by Pozio *et al*. (1999). It has been suggested that carnivorous birds might play a role in maintaining the parasite in the sylvatic cycle and are probably related to the spread of the parasite (Pozio & Murrell, 2006). Pozio *et al*. (2004) suggested that the few cases of *T. pseudospiralis* that have been recorded recently in the European region have originated from migratory birds of temperate regions such as Scandinavia.

To support this hypothesis, monitoring of *Trichinella* spp. in birds from other European countries is required. There is lack of data on the prevalence of *T. pseudospiralis* in raptorial, carrion-feeding, and scavenging birds in Sweden and Slovakia, where the parasite has been documented in wildlife (Pozio *et al*., 2004; Hurníková & Dubinský, 2009). In Sweden, *T. pseudospiralis* has been detected in three wild boars from mid-Sweden (Stockholm County) and in a lynx from northern Sweden (Jämtlands County) in the years 1985 – 2003. This suggests that *T. pseudospiralis* is present sporadically and multifocally in Sweden (Pozio *et al*., 2004) and also throughout the Scandinavian Peninsula (Oivanen *et al*. 2002; Oivanen & Oksanen 2009; Airas *et al*., 2010). In Slovakia, the first focus of *T. pseudospiralis* was documented from a pig breeding farm in Eastern Slovakia in 2004 (Hurníková *et al*., 2005) and was later found in co-infection with *T. britovi* in a wild boar and in red foxes from the same region suggesting the establishment of wildlife circulation of the parasite (Hurníková & Dubinský, 2009).

The aims of our work were to study the presence of *T. pseudospiralis* in carnivorous birds from Slovakia and Sweden using parasitological methods as well as molecular and histological identification of nematode isolates, and to

determine the possible role of raptorial and carrion-feeding birds in the epidemiology of *T. pseudospiralis.*

Material and methods

Parasitological investigation

Pectoral muscle samples from a total 212 carnivorous birds, belonging to 5 families – Accipitridae (12 species), Falconidae (4 species), Strigidae (2 species), Tytonidae (1 species), and Corvidae (4 species) as shown in table 1 were examined within our study. Investigated samples were collected during 2007 – 2013 from carcasses found by workers of the Research Station and Museum of the Tatra National Park, or from birds that died in raptor rescue centers and bird clinics in Slovakia (n = 153). From Sweden, samples were donated from the National Veterinary Institute's (SVA) biobank for the purpose of our study (n = 59). These samples had previously been cryopreserved and stored in SVA's biobank after necropsies within the national wildlife disease surveillance program. Samples were taken from 1994 – 2011 and in 2012 they were sent to the University of Veterinary Medicine and Pharmacy in Košice for investigation. Individual pectoral muscle samples (5 – 20 g) were examined for the presence of *Trichinella* larvae by artificial digestion according to standard methods (Gamble *et al*., 2000) within 2012 and 2013. The number of larvae per gram of muscle tissue (lpg) was determined according to Kapel and Gamble (2000). Larvae from infected birds were preserved in 70 % ethanol until molecular identification.

Isolation of DNA and polymerase chain reactions

Firstly, DNA was isolated from single larvae using 10 µl of extraction mixture according to the protocol described by Pozio and La Rosa (2003). Samples of isolated larvae were incubated overnight at 54 °C following an inactivation step at 90 °C for 10 min. To identify *Trichinella* species, the universal primer set (forward: 5´-GTTCCATGT GAACAGCAGT-3´) and (reverse: 5´-CGAAAACATACG ACAACTGC-3´) amplifying lsr DNA-derived expansion segment V (ESV) sequences described previously by Zarlenga and Dame (1992) was used in PCR reactions. The PCR mixture (total volume of 25 µl) consisted of 2.5 µl of 10x PCR buffer, 1 µl 10 mM MgCl2, 1 µl of 10 mM dNTPs mix, 2 µl of each 10 mM primers, 3 µl of isolated genomic DNA and 1U of Go Taq polymerase (Promega, USA). Reactions were carried out in BioRad C1000 Thermal Cycler and after initial denaturation at 95 °C for 4 min, the following cycling conditions in total 40 cycles were: 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min. Total genomic DNA was also isolated from *T. pseudospiralis* (code ISS13)*, T. spiralis* (ISS004) and *T. britovi* (ISS1088) reference strains, maintained in laboratory mice. In addition, samples of DNA from four geographically related *T. pseudospiralis* isolates previously used in the study of Wu *et al*. (2007) were kindly provided by Dr. Vilam Šnábel. Of these, three isolates were stated as reference samples characterized by code number and locality, namely ISS1348 (Sweden), ISS1432 (Slovakia), ISS141 (Australia-Tasmania) and one non-reference isolate from Finland. Amplification of corresponding ESV gene sequence was performed under the same reaction conditions and amplicons were electrophoresed on 1.5 % agarose gel and stained with ethidium bromide.

Amplification of a 419 bp region of the mitochondrial cytochrome c-oxidase subunit COI gene was performed on total DNA isolated from a higher number of muscle larvae with DNA Tissue isolation Kit (Machery Nagel, Germany) due to the lack of signal in PCR reactions on DNA from 3 larvae.

Trichinella spp. specific primers L6625 (5´-TTYTGRTTY TTYGGNCAYCC-3´) and H7005 (5´-ACNACRTARTAN GTRTCRTG-3´) originally reported by Hafner *et al*. (1994) were used in PCR reactions and amplification was performed under the reaction conditions described by Nagano *et al*. (1999).

RFLP analysis of COI gene

After electrophoresis, an approximately 419 bp band, previously characterized by a high variability among *T. pseudospiralis* isolates (La Rosa *et al*. 2001; Wu *et al*. 2007) was subjected to RFLP analysis (restriction fragment length polymorphism) with restriction endonuclease RsaI Fast digest (Fermentas) according to the manufacturer´s instructions. PCR products for above mentioned isolates were purified using NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Germany). Digested products were electrophoresed in 4 % agarose gel and visualized after addition of ethidium bromide.

Histological examination

Muscle tissue from two positive tawny owls, stored in 10 % formaline, was delivered from SVA for the histological examination. After an intensive washing in tap water, tissue blocks were processed by standard procedures and embedded in paraffin. Tissue sections (5 – 8 µm thick) were stained with Mayer´s haematoxylin/eosin, dehydrated, cleared in the Histochoice clearing solution and mounted in Histochoice mounting fluid (Amresco, USA).

Results

Parasitological investigation

Out of 212 carnivorous bird samples recovered in Slovakia and Sweden (Table 1), *Trichinella pseudospiralis* larvae were found in two tawny owls (*Strix aluco*) from Sweden (Fig. 1). In tawny owl V1997-0431 from Stockholm County, the intensity of infection was 0.7 lpg. Tawny owl V2011-0076 from Uppsala County was infected with 1.6 lpg. Out of the the total 59 samples originating from Sweden, 38 were from tawny owls. The prevalence of *T. pseudospiralis* in tawny owls from Sweden was 5.26 %. *Trichinella pseudospiralis* was not confirmed in any other avian species included in the study, neither from Slovakia nor from Sweden.

Table 1. Raptorial and carrion-feeding birds examined for *Trichinella pseudospiralis* in Slovakia and Sweden

Family	Species	Migratory status	No. of examined/positive	
			Slovakia	Sweden
Accipitridae	Montagu´s harrier (*Cicrus pygagrus*)	FM	2/0	-
	Western marsh-harrier (*Circus aeruginosus*) *	P	4/0	-
	Northern harrier (*Circus cyaneus*)	P	1/0	-
	Eurasian sparrowhawk (*Accipiter nisus*)	P	8/0	-
	Goshawk (*Accipitergentilis*)	P	4/0	-
	Golden eagle (*Aquila chrisaetos*)	R	1/0	-
	Imperial eagle (*Aquila heliaca*)	P	1/0	-
	Lesser spotted eagle (*Aquila pomarina*)	M	2/0	-
	Red kite (*Milvus milvus*)	P	1/0	-
	Common buzzard (*Buteo buteo*)	M	41/0	12/0
	Rough-legged buzzard (*Buteo lagopus*)	FM	1/0	1/0
	Long-legged buzzard (*Buteo rufinus*)	R	1/0	-
Falconidae	Common kestrel (*Falco tinnunculus*)	P	19/0	-
	Peregrine falcon (*Falco peregrinus*)	R	5/0	1/0
	Merlin (*Falco columbarius*)	P	1/0	2/0
	Saker falcon (*Falco cherrug*)	P	1/0	-
Strigidae	Tawny owl (*Strix aluco*) †	R	6/0	38/2 (5.26 %)
	Long-eared owl (*Asio otus*)	P	5/0	-
Tytonidae	Barn owl (*Tyto alba*)	P	1/0	-
Corvidae	Carrion crow (*Corvus corone*)	R	5/0	-
	Common raven (*Corvus corax*)	R	18/0	5/0
	Rook (*Corvus frugilegus*) ‡	M	14/0	-
	Eurasian magpie (*Pica pica*)	R	11/0	-

FM – full migrants moving over long distances; M – full migrants concentrated at specific areas; P – partial migrants or mostly so; R – resident or mainly resident (www.birdguides.com)
**T. pseudospiralis* infection reported by Obendorf and Clark 1992
†*T. pseudospiralis* infection reported by Pozio et al. 1999
‡*T. pseudospiralis* infection reported by Shaikenov 1980

PCR analysis of ESV and COI gene

PCR amplification of expansion segment five (ESV) of the genomic large ribosomal subunit on DNA from larvae isolated from the tawny owls showed identical size with band for *T. pseudospiralis* reference strain (ISS1432) circulating in Slovakia (Fig. 2). Obtained isolates and the reference isolate were represented by a single dominant band migrating at approximately 300 bp, and were clearly distinguished from *T. spiralis* (170 bp fragment) and *T. britovi* (127 bp fragment). The size of the amplicons from PCR on three reference DNA isolates from the Palearctic region (approximately 300 – 310 bp) and one from the Australian region (approximately 330 – 340 bp), indicates that our isolates are genetically related to the Palearctic isolates (Fig. 3).

DNA from pooled larvae isolated from bird muscles was used in PCR with primers specific for mitochondrial COI gene loci for *T. pseudospiralis*, resulting in fragmented DNA banding profile. Consequently, the specific product of COI gene migrating at approximately 419 bp was generated in PCR reactions with *Trichinella* spp. – specific primers, only. The band with the same size was amplified in PCR for all *T. pseudospiralis* reference isolates (Fig. 4.).

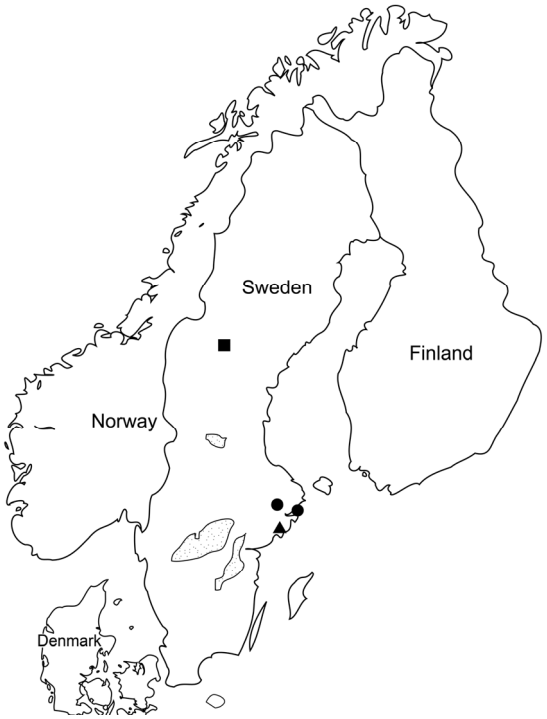

Fig. 1. Map of Sweden indicating findings of animals infected with *T. pseudospiralis*
■ Wild boars (Pozio *et al*., 2004); ▲ lynx (Pozio *et al*., 2004); ● tawny owls (presented findings)

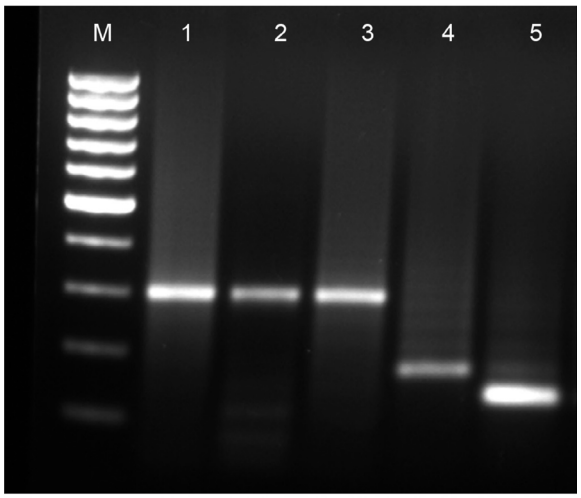

Fig. 2. Agarose gel separation of PCR products on DNA from 3 larvae amplified with primers specific for *Trichinella* spp. ESV gene. Lanes: 1-2, *Trichinella* isolates from tawny owls, 3, *T. pseudospiralis* reference isolate (code ISS13); 4, *T. spiralis* (ISS004); 5, *T. britovi* (ISS1088). 100 bp marker

Polymerase chain reaction-restriction fragment length polymorphism

RFLP analysis of COI gene fragment was used to further characterize the new *T. pseudospiralis* isolates. Specific profile obtained with restriction enzyme RsaI was identical among isolates from the Palearctic region, but was different among isolates from three different zoogeographic regions. The banding profile after RFLP analysis of COI gene of our isolates and reference isolates is shown on Fig. 5. All examined isolates from Palearctic region showed identical pattern formed of approximately 300 bp and 100 bp fragments and one isolate from Australian region produced RFLP profile of approximately 330 and 100 bp. Interestingly, the results obtained for our isolates showed bands migrating at approximately 280 bp and 100 bp.

Histological observations

The muscle tissues obtained from the two positive tawny owls was moderately to markedly autolytic indicated by the loose of indistinct morphology of muscle fibers. After H&E staining of longitudinal and transversal sections, infection with nematode larvae was demonstrated as basophilic elongated cigar-shaped smooth organisms with a diameter of 20 – 25 µm and filled with multiple nuclei in host muscle cells (Fig. 6a). The remnants of the larvae without the intact cuticule were seen inside infected muscle cells, which were transformed to nurse cells. These cells could be recognized as a weakly stained material without striated morphology. There was no indication of the presence of collagen capsules, typical for species such as *Trichinella spiralis* (Fig. 6b). Occasionally, cross sections of larvae were observed in the remnants of nurse cells (Fig. 6c) and nucleated larval-like material was observed between muscles (Fig. 6d).

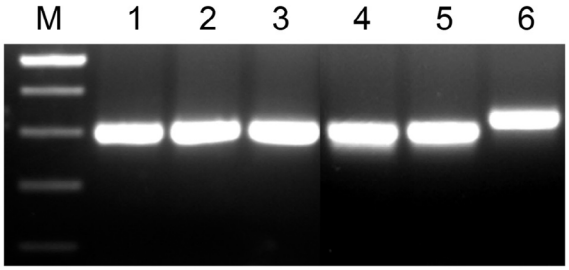

Fig. 3. Results from agarose gel electrophoresis of PCR products of ESV gene for *Trichinella pseudospiralis* genotypes representing Palearctic isolates. Lanes 1-3, Sweden (ISS1348), Finland, Slovakia (ISS1432); Lanes 4 – 5, new isolates from tawny owls; Lane 6, isolate (ISS141) from Tasmania. Reactions were performed on DNA isolated from multiple larvae. 100 bp marker

Discussion

The increasing number of findings of *T. pseudospiralis* in mammals throughout Europe within the last decades (Nöckler *et al*., 2006; Pozio, 2009), may indicate that this parasite is prevalent in all potential natural hosts including birds. Since there are few studies on birds as hosts of *Trichinella*, there is not much data available to give an accurate picture of the role of carnivorous avian species as reservoir hosts in Europe. Furthermore, as raptorial species are protected by governmental bodies of most European countries, it is difficult to acquire an adequate number of samples for extensive surveillance studies. In the past and even presently, most studies on *Trichinella* in animals have primarily been focused on animals hunted for sport or meat, such as wild boars, while many other putative host species are unaccounted for (Pozio, 2005).

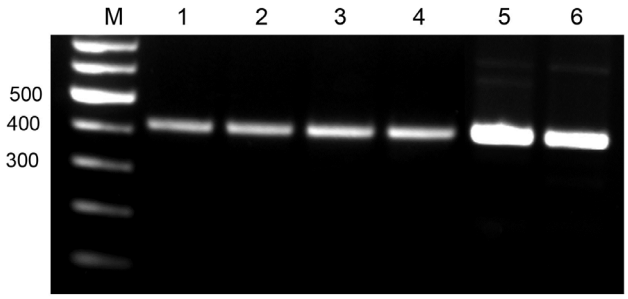

Fig. 4. Agarose gel showing a 419 bp products of COI gene generated in PCR on DNA for *T. pseudospiralis* isolates from Palearctic region (Lanes 1 – 3), isolate from Australian region (Lane 4) and DNA of new isolates from tawny owls from Sweden. 100 bp marker

Our study revealed two findings of *Trichinella pseudospiralis* in non-migratory tawny owls from one geographical region of Sweden. One tawny owl was sampled in Stockholm county in 1997 and the second in Uppsala county in 2011. These findings are the second report of cases of nocturnal birds of prey with *T. pseudospiralis* infection in Europe. The first finding of *T. pseudospiralis* in birds in Europe was documented in central Italy by Pozio *et al*. (1999), The only other similar finding of *Trichinella*-like larvae in birds within Europe was reported by Calero *et al*. in 1978 in a buzzard (*Buteo buteo*) in Spain, however, the larvae found

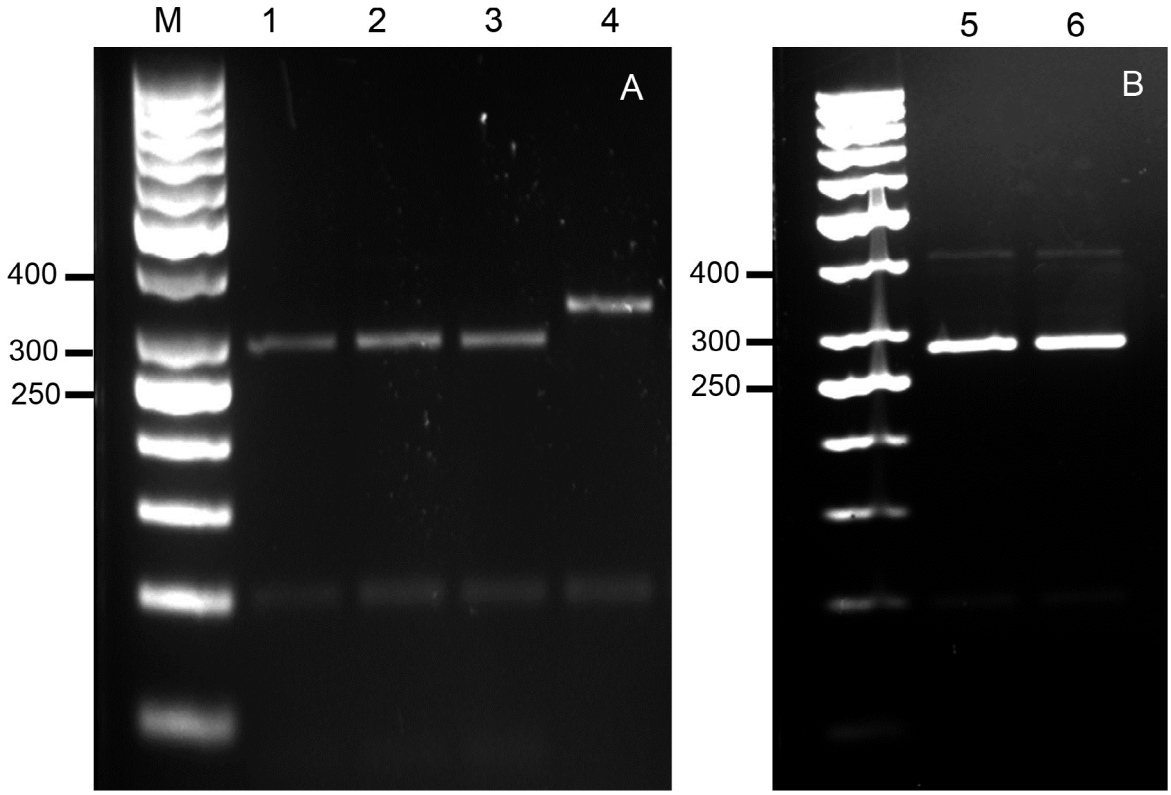

Fig. 5 a, b Banding profile of RFLP analysis of COI gene fragment obtained with RsaI restriction enzyme.
A, *T. pseudospiralis* reference isolates from Palearctic region (Lanes 1-3), isolate from Australian region (Lane 4).
B, restriction enzyme profile for COI of the new isolates from Sweden. 50 bp marker

in this specimen were not confirmed as *T. pseudospiralis*.

In our study, identification of the larvae isolated from muscles of two owls was performed by both molecular analysis and histological examination of formalin-fixed tissue. Firstly, in PCR on DNA from single intact larvae, *Trichinella pseudospiralis* was confirmed based on the length of the amplicon of lsr DNA-derived expansion segment V (ESV) using a primer set described by Zarlenga and Dame (1992). Zarlenga *et al*. (1999) showed that size variability within this region allows differentiation among zoogeographical isolates of *T. pseudospiralis*. The length of approximately 300 bp indicates that our two Swedish isolates belong to the group of Palearctic genotypes and shows a genetic similarity with isolates previously obtained from Sweden, Finland, and Slovakia. The RFLP profile of COI gene for our isolates from Sweden differed moderately from banding profiles for other isolates from Palearctic region, which might indicate a higher level of polymorphism between our isolates and others from this zoogeographic region. Histological analysis of muscle sections revealed the presence of unencapsulated larval-like material localized in the remnants of nurse cells. The diameter and the morphology of larvae localized in experimentally infected mouse and bird's muscle were very similar (data not shown). These observations supported our molecular findings.

There was a very low prevalence of *T. pseudospiralis* found in the birds sampled in our study (2/212, 0.94 %) as well as a low intensity of infection (0.7 lpg and 1.6 lpg, respectively). The only two birds in the study with *Trichinella* infection were non-migratory tawny owls from Sweden. This is one of two most abundant owl species in Sweden with 20,000 – 40,000 individuals ranging in woodlands and also rural habitats from the southern tip at 55° N to between 60 – 61° N. The prey that tawny owls feed on in Sweden are primarily the small rodent species *Arvicola terrestris* and *Microtus agrestis* (Lundberg 1980), although their diet may also consist of earthworms, insects, birds, frogs, fish, lizards, molluscs, and crustaceans. Previous studies have observed parasitic nematode communities of owls and birds of prey in Europe and recognized that both intrinsic and extrinsic factors including diet, habitat, behavior, migration, sex, age, geographic range, and distribution may influence the richness of nematodes within this category of birds (Santoro *et al*., 2012).

Up to date, all confirmed cases of *T. pseudospiralis* in birds within Europe have only been from the family Strigidae. Nocturnal owls hunt during dusk and night-time when only a specific range of prey animals are present, whereas diurnal birds of prey have a wider range of prey species to feed on (Jaksic, 1982). Therefore, the findings of *T. pseudospiralis* in tawny owls in Sweden may be due to the population's selective feeding habits on a narrow range of rodent species (i.e. *Arvicola terrestris* and *Microtus agrestis*) that may be more commonly infected with the nematode. An extensive surveillance study on the prevalence of

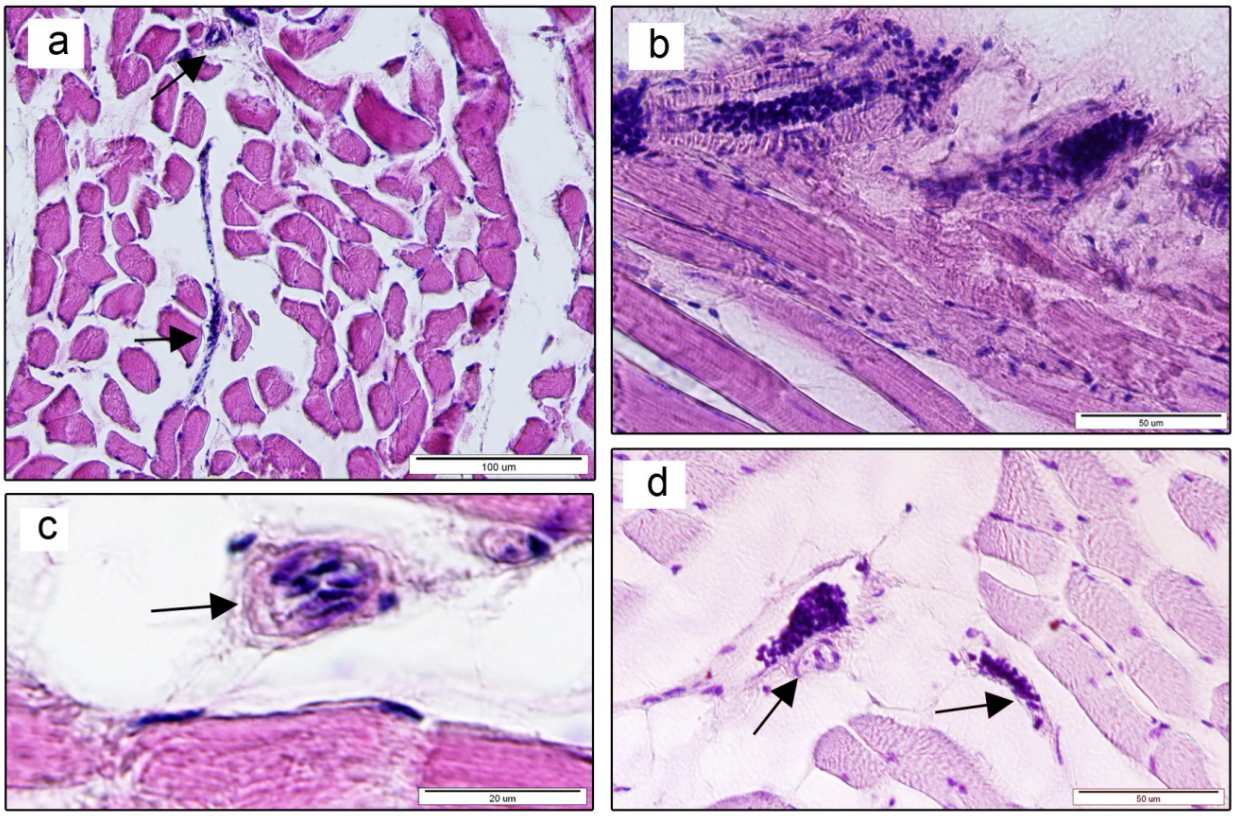

Fig. 6 a-d. Histological images of the muscles obtained from examined tawny owls suspected for *T. pseudospiralis* infection. (a-d), Hematoxylin and eosin staining. Longitudinal (a) and transversal (b) sections of the larval-like material (arrows) localized inside remnants of the nurse cells seen as accumulation of dark blue staining; (c), cross- section of the partly damaged larva from infected bird´s muscle; (d) presence of larval-like material (arrows) localized between infected muscles

T. pseudospiralis in the rodent species that share a common habitat with *Strix aluco* is therefore relevant to our study and recommended in the future.

Another factor other than feeding habit that may influence the prevalence of *T. pseudospiralis* in owls is body size. Kinsella *et al*. (2001) suggest that body size is often related to other aspects of parasite transmission such as dietary range, rate of food intake, and population density. The fact that several confirmed cases of *T. pseudospiralis* in owls have been reported in different parts of the world including Europe, USA, and Tasmania suggest the possibility that this nematode may be an „owl specialist" (Santoro *et al*., 2012) and has a higher host specificity for these species than diurnal raptors and other carnivorous birds.

Tawny owls are highly territorial, which would suggest that they are not directly involved in spreading of *T. pseudospiralis* parasite into new geographical regions. Our findings of this nematode in two tawny owls (5.26 % study prevalence in this species) indicate that this parasite is endemic to the area of Stockholm and Uppsala County. This is similar to the findings of Pozio *et al*. (2004) who detected the parasite in a lynx (*Lynx lynx*) and three wild boars (*Sus scrofa*) in Sweden. *Trichinella pseudospiralis* has also been detected in Finland (Oivanen *et al*. 2005; Airas *et al*., 2010) and Baltic countries (Malakauskas *et al*., 2007). Since several decades, various reports have been made of *T. pseudospiralis* findings in avian species. The birds that have been documented as naturally infected and positive for *T. pseudospiralis* all fit the profile of being carnivorous, omnivorous, scavenging, or carrion-feeding. In 1980, two omnivorous rooks (*Corvus frugilegus)* were documented as being naturally infected for the first time (Shaikenov, 1980). A black vulture (*Coragyps atratus*) was documented as the first host of *T. pseudospiralis* in North America, found in 1995 in the state of Alabama (Lindsay *et al*., 1995). According to mentioned authors, three other cases were previously reported in North America; one in a great horned owl (*Bubo virginianus*) in Iowa, one in a Cooper's hawk (*Accipiter cooperi*) in California, and one pomarine jaeger (*Stercorarius pomariunus*) in Alaska. However, these three cases were unverified, making the positive finding of *T. pseudospiralis* in the black vulture the only documented case in birds in North America to date. *T. pseudospiralis* was also reported by Obendorf and Clarke (1992) in a masked owl (*Tyto novaehollandiae*) in Tasmania. The wide geographical range of positive findings in birds suggests that *T. pseudospiralis* is established in its ecological niche worldwide.

When investigating the specific role of carnivorous or omnivorous birds as a primary host of *T. pseudospiralis*, it is important also to consider the natural prey of these animals and how they may interplay in the vast epidemiologi-

cal picture of this parasite. Therefore, it is recommended to include corvid bird species in future monitoring programs of *T. pseudospiralis* (Nöckler *et al*., 2006). Focusing primarily on migratory avian species in a surveillance study may be particularly relevant to detecting positive *T. pseudospiralis* transmission in the European region.

Our study confirmed the participation of birds-of-prey in circulation of *Trichinella pseudospiralis* in the wildlife of Sweden and also introduced new questions regarding the epidemiological cycle of the parasite. Since the only birds that have been found infected with this *Trichinella* species in Europe are owls, further study is necessary to determine if there are specific factors that correlate with a higher prevalence of *T. pseudospiralis* in nocturnal owl species than those of diurnal birds of prey and corvids.

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