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Role of three bird species in the life cycle of two *Sarcocystis* spp. (Apicomplexa, Sarcocystidae) in the Czech Republic

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ABSTRACT

Birds are one of the groups involved in the development of *Sarcocystis* Lankester (1882), serving either as intermediate or definitive hosts. The white-tailed sea eagle *Haliaeetus albicilla* (Linnaeus, 1758), red kite *Milvus milvus* (Linnaeus, 1758) (both Accipitriformes) and common starlings *Sturnus vulgaris* Linnaeus, 1758 (Passeriformes) were examined to elucidate their participation in the development of *Sarcocystis*, as well as to determine the specific identity of the parasites based on morphological and especially molecular analyses. In 2020–2021, one white-tailed eagle, one red kite and five common starlings were parasitologically examined for the presence of *Sarcocystis* using flotation centrifugation coprological method and by wet mounts of intestinal mucosa scrapings and/or muscle samples. Positive samples were processed by light microscopy, histologically and followed molecularly at four genetic markers (*18S* rRNA, *28S* rRNA, ITS1 and *cox1*). The white-tailed eagle harboured oocysts/sporocysts of *S. arctica* Gjerde et Schulze, 2014 in the intestinal mucosa, while the intestinal mucosa of the red kite and breasts and leg muscles of one common starling were positive to *S. halieti* Gjerde, Vikøren et Hamnes, 2018. Sequences from eagle shared 99.6–100% identity with each other and *S. arctica* in the red fox (*V. vulpes* Linnaeus, 1758) from the Czech Republic. Sequences from the common starling and red kite shared 100% identity with each other and with *S. halieti* in the great cormorant (*P. carbo* [Linnaeus, 1758]) from Lithuania and *H. albicilla* from Norway. The white-tailed sea eagle might act as definitive host of *S. arctica*, whereas the common starling and red kite represent intermediate and potential definitive hosts, respectively, for *S. halieti*.

1. Introduction

Species of *Sarcocystis* Lankester (1882) parasitize a wide host range of wild and domestic animals around the world and use two obligate hosts in the life cycle, where omnivores/carnivores (e.g., owls, eagles, foxes, racoon dogs) act as definitive hosts and herbivores, omnivores and carnivores (e.g., rodents, wild boars, badgers, racoons, foxes) as intermediate hosts (see Dubey et al., 2016). Birds are one of the groups involved in the development of *Sarcocystis*, serving either as intermediate or definitive hosts.

Dead specimens of the white-tailed sea eagle *Haliaeetus albicilla* (Linnaeus, 1758), red kite *Milvus milvus* (Linnaeus, 1758) (both Accipitriformes) and common starlings *Sturnus vulgaris* Linnaeus, 1758 (Passeriformes) were delivered to the State Veterinary Institute Prague, Czech Republic to determine their cause of death. The white-tailed sea eagle is a large diurnal raptor in Eurasia that acts as intermediate host for *S. wobeseri* Kutkienė, Prakas, Sruoga et Butkauskas, 2010 in the United Kingdom (Shadbolt et al., 2021) and as definitive host for *Sarcocystis halieti* Gjerde, Vikøren et Hamnes, 2018 (Gjerde et al., 2018) and *Sarcocystis lari* Prakas, Kutkienė, Butkaukas, Sruoga et Žalakevičius, 2014 (Prakas et al., 2014). The intermediate hosts of *S. halieti* are the great cormorant *Phalacrocorax carbo* (Linnaeus, 1758), the little owl (*Athene noctua* Scopoli, 1769), the western marsh harrier (*Circus aeruginosus* [Linnaeus, 1758]) and the black kite (*Milvus migrans* [Boddaert, 1783]) (see Gjerde et al., 2018; Prakas et al., 2018, 2020, 2021; Maier-Sam et al., 2021); whereas that in *S. lari* is the great black-backed gull (*Larus marinus* Linnaeus, 1758) (Gjerde et al., 2018; Prakas et al., 2014, 2020). The red kite is also a diurnal raptor mostly distributed in western Europe, northwestern Africa and northern Iran and reported as a

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Abbreviations

bp:	base pairs
cox1	Cytochrome *c* oxidase subunit 1
DNA	Deoxyribonucleic Acid
ITS1	Internal Transcribed Spacer 1
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid

possible definitive host of unnamed *Sarcocystis* spp. in Germany and Spain (Krone, 2000; Sánchez-Andrade et al., 2002), although their morphological and molecular analyses are missing. The common starling is resident in western and southern Europe and southwestern Asia, and it has not been reported as host of *Sarcocystis*.

As above mentioned, the white-tailed sea eagle has been more studied than the other two bird species and, along with other birds, considered as a potential definitive host of *S. arctica* Gjerde et Schulze, 2014 and *S. lutrae* Gjerde et Josefsen, 2014 due to their predation activity on the arctic fox *Vulpes lagopus* (Linnaeus, 1758) (see Gjerde and Schulze, 2014; Gjerde and Josefsen, 2015; Ye et al., 2018), although this assumption is still uncertain. On the other hand, the role of the red kite and common starling in the life cycle and the identity of *Sarcocystis* species in these two hosts are still incomplete. Therefore, the goal of this study was to elucidate the participation of these three avian species in the development of *Sarcocystis*, as well as to determine the specific identity of the parasites based on morphological and especially molecular analyses.

2. Materials and methods

One white-tailed sea eagle, one red kite and five juvenile common starlings were sent to the State Veterinary Institute Prague for necropsy in 2020–2021. These birds were parasitologically examined through wet mounts of muscle samples (breast, heart, legs) for the presence of sarcocysts using a Leica DM2500 LED optical microscope equipped with a digital camera Leica DFC420 with microscope software Leica Application Suite X (Leica Microsystems, Wetzlar, Germany). Faecal and intestinal mucosa samples were examined by flotation-centrifugation coprological method and by wet mounts with water or glycerine. Isolated oocysts/sporocysts or sarcocysts were transferred to an Eppendorf tube for DNA extraction. For histological study, tissue portions (breast, leg) were fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin and eosin staining.

Total genomic DNA was extracted by glass bead disruption from two and four isolates from the oocysts/sporocysts collected from small intestine mucosa scrapings of the white-tailed sea eagle and red kite, respectively, as well as two isolates from sarcocysts of the common starling. These isolates were extracted by using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer, and stored at −20 °C until use in polymerase chain reaction (PCR). PCR was carried out by using primers for *18S* rRNA (nested PCR Fext/Rext; Fint/Rint, ERIB1/A2R, A1F/S2r, A2F/Primer BSarc) (Barta et al., 1997; Fischer and Odening, 1998; Gjerde, 2014; Dubey et al., 2015; Gjerde et al., 2018), *28S* rRNA (KL_P1R/KL_P1F, nested PCR A2F/KL3 and KL1/KL3 and KL1/LS2R; LS1F/KL3) (Mugridge et al., 1999; Kutkienė et al., 2010; Gjerde, 2013; Gjerde et al., 2018), ITS1 region (ITS-F/ITS-R) (Kutkienė et al., 2010) and *cox1* (SF1/SR5) (Gjerde, 2013). Reaction was performed in a total volume of 25 µl containing GoTaq® G2 Green Master Mix (Promega, Madison, Wisconsin, USA), 0.4 µM of each primer, DNA template and nuclease-free water. The amplification cycles were as follows: 95 °C for 3 min, 5 cycles of 94 °C for 45 s, 64 °C for 60 s, 72 °C for 90 s; followed by 30 cycles of 95 °C for 30 s, 52–60 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min. The PCR products were visualized on a UV transilluminator after electrophoresis in 1% agarose gels and staining with ethidium bromide. Amplicons were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) or the ExoSAP-IT™ Express PCR Product Cleanup Reagent kit (Thermo Fisher Scientific) according to the manufacturer's recommendations and were subjected to sequencing by Eurofins Genomics (Ebersberg, Germany) in both directions. Nucleotide sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com), compared to reference sequences from GenBank using BLAST (Basic Local Alignment Search Tool). The sequences obtained from *18S* rRNA, *28S* rRNA, ITS1 and *cox1* loci in this study have been deposited in GenBank under accession numbers: MZ329343, MZ329344, MZ333536 and MZ332967 from the white-tailed sea eagle (S. arctica); MZ329386, MZ329403, MZ333537, MZ332968 from the red kite (S. halieti); and MZ329690, MZ329777, MZ333538 and MZ332969 from the common starling (*S. halieti*).

The evolutionary history was inferred at ITS1 region by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model with invariable sites (HKY + I) (Hasegawa et al., 1985). There was a total of 871 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [Kumar et al., 2018]. The numbers beside the branches represent bootstrap values based on 1000 replications.

3. Results

White-tailed sea eagle harboured oocysts/sporocysts in the intestinal mucosa. Oocysts measured 18.5–18.8 × 11.6–14.0 µm (n = 5) and sporocysts 10.6–12.7 × 8.7–10.6 µm (n = 40); sausage-shaped sporozoites were 7.9–9.3 × 1.7–2.4 µm (n = 10) in size. The intestinal mucosa of the red kite was parasitized by oocysts with two sporoblasts, 14.5–15.3 × 11.9–13.0 µm (n = 5) in size; oocysts without sporoblasts were 17.4–20.4 × 10.9–13.3 µm (n = 5), and sporocysts were 12.8–15.8 × 8.6–10.9 µm (n = 15). Muscle samples of heart, breast and legs of these two hosts were negative to sarcocysts. Breast and leg muscles of one out of the five common starlings were positive to *S. halieti* (Fig. 1a). The longest sarcocyst was 792.1 × 54.7 µm long, without visible villar protrusions under light microscopy (Fig. 1b) and with a wall 0.7 µm thick. Released bradyzoites were 6.1–8.8 × 1.6–1.9 µm (n = 30) in size. Heart muscles and coprological examination of common starlings were

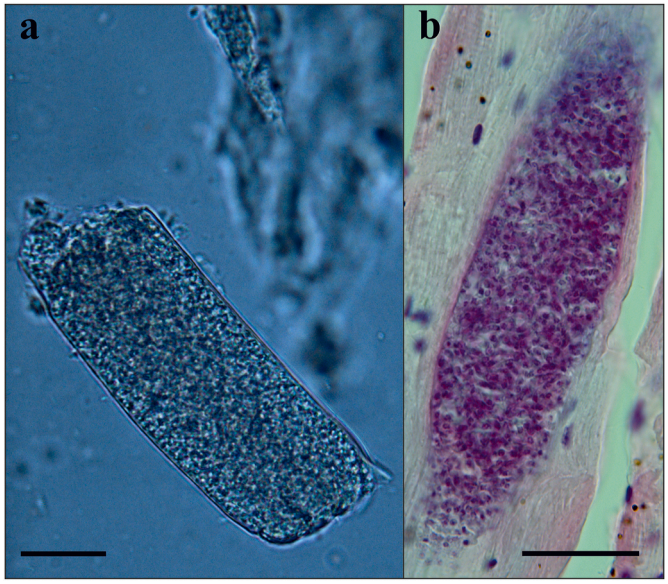

Fig. 1. *Sarcocystis halieti* from *Sturnus vulgaris*. (**a**) Free thin-walled sarcocyst from skeletal muscle, wet mount. (b) Haematoxylin and eosin-stained histological sections of breast muscle with sarcocyst. Scale bars = 25 µm.

negative to *Sarcocystis*. No inflammatory response in bird was found.

Most PCR primers worked properly, excepting KL3 primer for *28S* rRNA gene which failed or produced short sequences in the common starling and white-tailed sea eagle. However, length of produced sequences was correct and useful to distinguish species. The two isolates from the white-tailed sea eagle at *18S* rRNA (1772 bp), *28S* rRNA (1081 bp), ITS1 (872 bp) and *cox1* (1032 bp) loci shared 100% identity with each other (Genbank accession number: MZ329343, MZ329344, MZ333536 and MZ332967, respectively). These sequences were 100% similar at *18S* rRNA, 99.9–100% at *28S* rRNA, 99.6–100% at ITS1 and 99.8–100% at *cox1* with *S. arctica* in the red fox (*V. vulpes* Linnaeus, 1758) from the Czech Republic (KX156837–KX156839, KY609323, KY609324) and other published sequences from Latvia, Lithuania and Spain (MF596217–MF596237, MF596240–MF596260, MF596262–MF596282, MF596286–MF596306), as well as from other intermediate hosts as the arctic fox (*V. lagopus*) from Norway (KF601301, KF601306, KF601312, KF601320, KF601321) and USA (KY947304–KY947311) and the Alaskan wolf (*Canis lupus pambasileus* Elliot, 1905) (KX022100–KX022111) from USA (Table S1).

The sequences at *18S* rRNA (1568 bp), *28S* rRNA (883 bp) and *cox1* (1019 bp) genes from the common starling (MZ329690, MZ329777, MZ333538, MZ332969), as well as those at *18S* rRNA (1774 bp), *28S* rRNA (1456 bp) and *cox1* (1045 bp) genes from the red kite (MZ329386, MZ329403, MZ333537, MZ332968) shared 100% identity with each other and with S. halieti in the great cormorant (*P. carbo*) from Lithuania (JQ733511, JQ733512, MH130210, MH130211, MH138308, MH138309) and 99.9–100% in H. albicilla from Norway (MF946583, MF946587, MF946610). Sequences at ITS1 region of common starling (992 bp) and red kite (974 bp) were 99.6% similar to *S. halieti* in *P. carbo* from Lithuania (JQ733513, MH130209) and in the western marsh harrier (*Circus aeruginosus* [Linnaeus, 1758]) from Spain (MW929599), 98.6–99.6% in the herring gull (*Larus argentatus* Pontoppidan, 1763) from Lithuania (MN450340–MN450356), 98.4–98.8% in *H. albicilla* from Norway (MF946589–MF946596) and 97.4–98.3% in the black kite (*M. migrans*) from Spain (MW929600, MW929601) (Table S1, Fig. 2).

4. Discussion

The oocyst/sporocysts molecularly identified as *S. arctica* in the white-tailed sea eagle might represent the first report of its definitive host and the first morphological and molecular characterization of these developmental stages. *Sarcocystis arctica* was originally described from the arctic fox in Norway (Gjerde and Schulze, 2014) and later in the same host from Alaska, USA (see Cerqueira-Cézar et al., 2017), in the wolf *Canis lupus* Linnaeus, 1758 from Alaska, USA (Calero-Bernal et al., 2016), in *V. vulpes* from the Czech Republic (see Pavlásek and Máca, 2017), Latvia, Lithuania and Spain (see Kirillova et al., 2018), as well as in corvid birds from Lithuania (Juozaityté-Ngugu et al., 2021). In all these reports, carnivores might act as intermediate hosts. Apparently, the white-tailed sea eagle gets infected after feeding on intermediate hosts as red foxes, which have been reported to harbour sarcocysts of *S. arctica* (see Pavlásek and Máca, 2017; Kirillova et al., 2018) and constitutes one of the feeding items of the eagle (see Nadjafzadeh et al., 2016), or on other still unknown possible intermediate hosts. The white-tailed sea eagle has also been reported as definitive host of *S. halieti* and *S. lari*, which showed molecular differences with *S. arctica* to be considered as separated species (see Gjerde et al., 2018). Interestingly, developmental stages (oocysts, sporocysts, sarcocysts) of *S. halieti* and *S. lari* were not molecularly identified in the white-tailed sea eagle, even though they were reported in the same host from Norway (see Gjerde et al., 2018). This might be related to the availability and susceptibility of those prey use as intermediate hosts by the parasite in

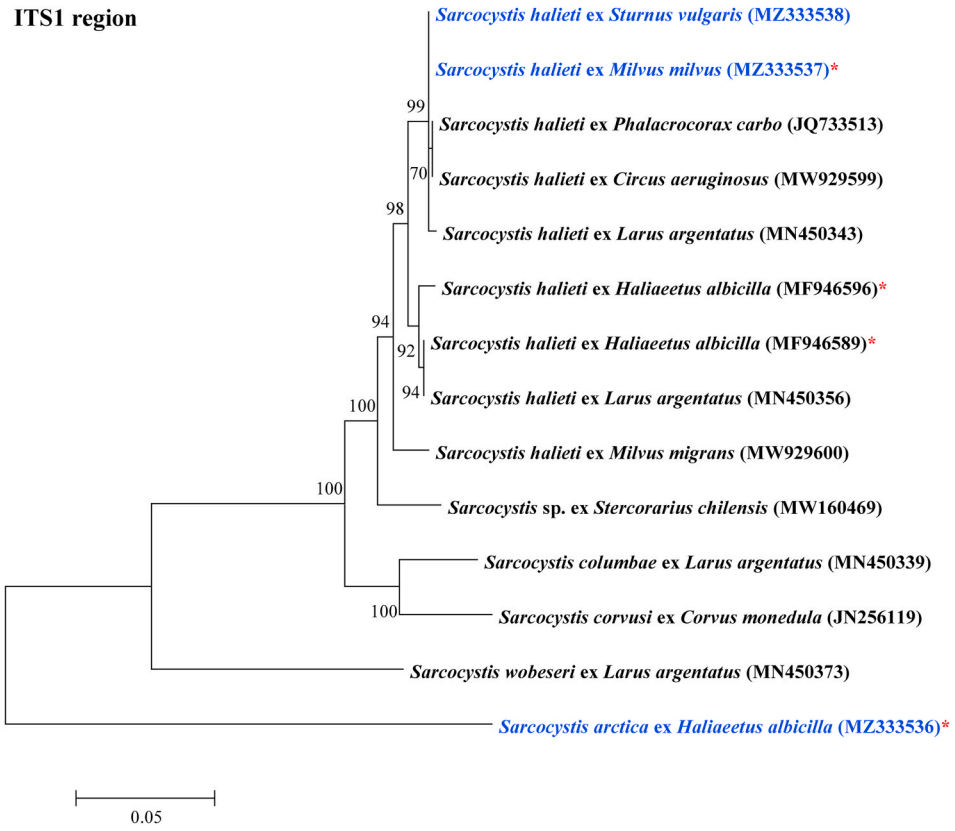

Fig. 2. Maximum likelihood tree for *Sarcocystis halieti* isolates from intermediate and definitive hosts (red asterisk) based on internal transcribed spacer sequences (HKY + I model). Sequences of the present study in blue. The tree was rooted on *Sarcocystis arctica*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

different geographical regions.

Muscle samples (heart, breast, legs) of the white-tailed sea eagle were negative to sarcocysts in the present study. However, after examining the same host from Norway and UK, Gjerde et al. (2018) reported sarcocysts of an unnamed *Sarcocystis* sp. in the cardiac muscle, whereas Shadbolt et al. (2021) found *S. wobeseri*-like sarcocysts in the pectoral and cardiac muscles, respectively. Apparently, the white-tailed sea eagle plays a role as intermediate host for these parasites, but more samples should be examined to determine its possible involvement in the life cycle.

This is the first record of a named species of Sarcocystis (S. halieti) in intestinal samples of the red kite, even though Krone (2000) and Sánchez-Andrade et al. (2002) previously reported Sarcocystis spp. in either one red kite from Germany and Spain, respectively. Unfortunately, molecular and morphological analyses missed in both studies, thus making impossible the comparison with the present finding. Gjerde et al. (2018) officially described *S. halieti* in its definitive hosts, the Eurasian sparrowhawk *Accipiter nisus* (Linnaeus, 1758) and *H. albicilla* in Norway. The sarcocysts of this protozoan were reported in 2012 as *Sarcocystis* sp. in the intermediate host (*P. carbo*) from Lithuania, as mentioned by Prakas et al. (2018). The same unnamed species was found in *A. nisus* from Germany and identified as *S. halieti* (Mayr et al., 2016). More recently, the herring gull from Lithuania (Prakas et al., 2020), the black kite and the western marsh harrier from Spain (Prakas et al., 2021), and the little owl (*Athene noctua* [Scopoli, 1769]) from Germany (Maier-Sam et al., 2021), were all reported as intermediate hosts of *S. halieti*; whereas oocysts were found in the intestinal mucosa of corvid birds from Lithuania (Juozaityté-Ngugu et al., 2021), as well as in the Cooper's hawk (*Accipitter cooperi* [Bonaparte, 1828], the red-shouldered hawk (*Buteo lineatus* [Gmelin, 1788]) and the red-tailed hawk (*B. jamaicensis* [Gmelin, 1788]) from the USA (Rogers et al., 2022). It is worth noting that the red kite acts as definitive host for this protozoan and get infected after scavenging on any of the above mentioned intermediate hosts. However, more studies are required to elucidate the spectrum of intermediate hosts and to complete the life cycle of the parasite. For a simple comparison, the oocyst and sporocysts from the red kite were smaller than those of *S. halieti* in the white-tailed sea eagle from Norway (14.5–20.4 × 10.9–13.3 μm and 12.8–15.8 × 8.6–10.9 μm vs. 21.8–22.8 × 16.0–17.0 μm and 16.0–17.0 × 10.5–11.2 μm). This fact corroborates the unreliability of using morphometrics of developmental stages to identify species of *Sarcocystis*.

Like the white-tailed sea eagle, muscle samples of the red kite were also negative to sarcocysts. In Spain, after examining a congeneric bird species, the black kite *M. migrans*, Prakas et al. (2021) found muscular sarcocysts in this host and considered it as *S. halieti*, which formed a sister branch to other sequences of the same species. Similarly, *Sarcocystis* sp. in the Chilean skua (*Stercorarius chilensis* [Bonaparte, 1857]) from Chile (see Acosta et al., 2021; Prakas et al., 2021) also formed a sister branch. Our phylogenetic analysis showed similar branching, so it is difficult to state whether these sequences belong to the same species, until more samples are molecularly obtained. Since oocysts and sporocysts were herein found in the red kite it apparently indicates that birds of *Milvus* (Accipitridae) could be either intermediate or definitive hosts for *S. halieti*.

In the present case, the molecular diagnosis helped to clearly identified the parasite species at each host. As already mentioned in several investigations (e.g., Prakas et al., 2020), the effective molecular identification of *Sarcocystis* species should always be included, since the morphology of the developmental stages, under light microscopy, is rather similar among them and indistinguishable at species level. Particularly, the ITS1 region was the most conclusive marker to distinguish species and it has been mentioned that this region vary considerably more than sequences of *18S* and *28S* rRNA genes among different species with avian intermediate hosts (see Prakas et al., 2014).

Molecular analyses need to be applied with caution, especially when oocysts/sporocysts are isolated from content of small intestine, faecal samples and/or passaged developmental stages in the intestinal content. Sometimes, these findings could be accidental because the hosts get the parasite after feeding in an infected prey, as in the case of *S. truncata* Gjerde, 2914 in *H. albicilla* from Norway (see Gjerde et al., 2018). Evidently, more data on birds of prey and their prey spectrum are needed to elucidate the real role of hosts in the life cycle of *Sarcocystis* species.

Even though the three bird species looked healthy, neurologic diseases caused by *Sarcocystis* should be monitored, since protozoal meningoencephalitis involving the cerebrum and cerebellum in USA (see Olson et al., 2007) and mortality in North America (Wünschmann et al., 2010) have been reported in the bald eagle (*Haliaeetus leucocephalus* Linnaeus, 1766). This disease is rare in raptors, but its presence could cause paralisis of wings and mild motor incoordination, as in other birds (see Konradt et al., 2017).

5. Conclusions

The possible natural role of three bird host species in the life cycle of *S. arctica* and *S. halieti* was determined by using a molecular characterisation of 4 loci (*18S* rRNA, *28S* rRNA, ITS1 and *cox1*). Apparently, the complete life-cycle of *S. arctica* in the Czech Republic is finally known, where the white-tailed sea eagle acts as natural definitive host and could get infected after probably preying on red foxes. The common starling and red kite represent new intermediate and potential definitive hosts, respectively, for *S. halieti*.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.01.002.

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