Nematode-induced pathological lesions and alterations of mucin pattern identified in abomasa of wild ruminants

Jan Magdálek [a], Pavol Makovický [b], Jaroslav Vadlejch [a],*

[a] Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamycka 129, 165 00, Prague, Suchdol, Czech Republic
[b] J. Selye University, Faculty of Education, Department of Biology, Bratislavska 3322, 945 01, Komarno, Slovak Republic

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ABSTRACT

Pathological lesions as well as mucin alterations in abomasa infected by nematodes have been thoroughly studied in livestock, but such data from wild ruminants are limited or completely lacking. Pathological data for *Ashworthius sidemi,* an invasive nematode are particularly rare. We necropsied the abomasa of 21 wild ruminants belonging to five cervid species and detected mixed nematode infections, dominated by *A. sidemi*. Samples from both gross lesions and mucous membranes without macroscopically apparent pathological alterations were subjected to standard histological procedures and histochemical staining. Histological examination found chronic abomasitis, manifested by edema, and hyperemia. Various degrees of lymphoplasmacytic infiltration were observed in all samples. Initial fibrosis (8/20, 40%) was detected in samples from both gross lesions and areas without macroscopically visible changes. Tissue from hemorrhagic lesions was superficially eroded. Generalized loss of surface polysaccharides was apparent in all samples. Only residual periodic acid-Schiff and Alcian blue (pH2.5) positivity was detected in the upper abomasal pits and in mucosal neck. This study found that nematode infections, mostly by *A. sidemi*, caused chronic inflammation and negatively affected abomasal mucin formation in wild ruminants.

1. Introduction

Wild ruminants harbor a wide spectrum of abomasal nematodes. Species that belong to the subfamily Ostertagiinae, such as *Ostertagia leptospicularis* and *Spiculopteragia spiculoptera*, were traditionally considered predominantly parasitic nematode fauna in cervids across the Holarctic (Hoberg et al., 2001; Pato et al., 2013). Recent studies, however, have reported the rapid spread of the allochthonous nematode *Ashworthius sidemi* (subfamily Haemonchiinae) amongst European cervids (Lehrter et al., 2016; Demiaszkiewicz et al., 2017; Kuznetsov et al., 2018). *A. sidemi* is a blood-feeding nematode that was originally described from Asiatic cervids. It has been introduced by sika deer into several European countries (Kotrlá and Kotrlý, 1973; Ferté et al., 2000; Dróżdż et al., 2003). In addition to the colonization of native cervids, *A. sidemi* has become a new parasite of European bison (Dróżdż et al., 1998) and poses a health risk to domestic ruminants (Kotrlá et al., 1976; Moskwa et al., 2015).

Trichostrongylid nematodes (Nematoda: Trichostrongylidae) have direct life cycles. Adult females produce eggs that leave host bodies in the feces. After the eggs hatch, larvae develop in the environment to the infective third-stage larvae that is incidentally ingested by the host during grazing. Infective larvae shed their protective cuticle and enter the predilection site of the host gastrointestinal (GI) tract. The histotrophic phase of trichostrongylids can be delayed due to arrested larval development, known as hypobiosis, in reaction to unfavorable environmental and/or host conditions (Gibbs, 1986; Belem et al., 1993; Connan, 1997). Adult nematodes emerge into the GI lumen several days after infection.

The simultaneous penetration of the mucosa by many larvae of the subfamily Ostertagiinae and their synchronous emergence after arrested development damage host epithelia that leads to type I and II ostertagiasis, respectively. This disease is grossly manifested by nodular lesions accompanied by focal ulcerations (Conti and Howetrh, 1987). Ostertagiasis is well known in farm animals, but some cases have been reported in wild cervids (Conti and Howetrh, 1987; Connan, 1991; Woodburry and Parry, 2009). The pathogenic effect of nematodes from the subfamily Haemonchiinae is due mostly to the blood-feeding activities of these causative agents, unlike *Ostertagia*. Typical findings such as

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* Corresponding author.
  E-mail addresses: vadlejch@af.czu.cz, jaroslavvadlejch@centrum.cz (J. Vadlejch).

hemorrhagic lesions, ulceration, and mucosal erosion have been described in small domestic ruminants infected with *Haemonchus contortus* (Pérez et al., 2001; Taylor et al., 2015). Similar pathological alterations were observed in red deer, roe deer, and European bison parasitized by *A. sidemi*. Abomasal tissue infected with *A. sidemi* exhibits edema, hyperemia, and infiltration of lymphoid cells and eosinophils (Demiaszkiewicz et al., 2009; Osińska et al., 2010).

Trichostrongylids can also cause damage indirectly via alterations in abomasal secretion; this process may lead to enlarged abomasal glands and reduced mucin formation. The key role in this process is probably the production of excretory-secretory products (ES) that affect parietal cells (Mihi et al., 2013). The surface of abomasal epithelium under physiological conditions is covered by a mucous gel composed predominantly of neutral mucin Muc5AC and acidic mucin Muc6. This layer protects the epithelium from both the acidic environment of the abomasal lumen and autolysis (Lichtenberger, 1999; Simpson, 2000). Adult trichostrongylids in the abomasum substantially decrease the production of neutral mucin by surface mucous cells (SMCs). This decrease was observed immediately after adult *Teladorsagia circumcincta* were transplanted to the abomasa of lambs (Scott et al., 2017) and after the emergence of adult *Ostertagia ostertagi* and *H. contortus* in cattle and sheep (Rinaldi et al., 2011; Simpson et al., 2016).

Such information is available for only a few wild ruminants in comparison to livestock, for which the pathogenicity of abomasal nematodes is well documented (Murray et al., 1970; Laraillet et al., 2012; Pérez et al., 2001). We therefore evaluated the pathologies in wild ruminants induced by abomasal nematodes, with an emphasis on an invasive nematode, *A. sidemi*. This study aimed to: (i) investigate gross lesions in the abomasa of wild ruminants infected by nematodes, and (ii) evaluate both histological abnormalities and histochemical changes of affected abomasal tissues.

2. Materials and methods

2.1. Animals, necropsies, and parasitology

We collected GI tracts of wild ruminants culled from the periodic control of game populations during the hunting seasons of 2017 and 2018. A total of 21 GI tracts from five wild ruminant species (only adults), which commonly occurs in the Czech Republic (CR), were included in this study. The animals originated from eight game reserves defined by the Czech Hunting Act as a fenced area of at least 50 ha and conditions suitable for breeding of certain game species. This study included game reserves in the Liberec Region and Ústí and Labem Region (northern and northwestern parts of the country), Central Bohemian Region, Southern Bohemian Region, and Plzeň Region (western part of the country). The animals had no signs of any disease when they were culled.

All GI tracts were subjected to parasitological post mortem examination using standard techniques (Hansen and Perry, 1994; Wood et al., 1995). The time from culling to collecting tissue samples did not exceed four hours. The abomasum was separated from the rest of the GI tract and opened along the greater curvature, and the abomasal content was gently washed out by saline solution into separate buckets. These organ contents were combined with mucosal washings and then poured through 200- and 150-μm mesh sieves. All recovered nematodes were preserved in 70% ethanol until morphologically identified using identification keys (Lichtenfels and Hoberg, 1992; Dróźdź, 1995; Dróźdź et al., 1998). Quantitative descriptors of the parasites were evaluated as outlined by Bush et al. (1997).

2.2. Gross pathology, histology, and histochemistry

All washed abomasa were subjected to gross examination; gross lesions on the organs were measured and photographically documented. Tissue samples (1 × 1 cm) of both the gross lesions and mucous membranes without macroscopically apparent pathological alterations were collected from the fundic region of all abomasa. The samples were fixed by immersion in 10% buffered formalin for one day and then processed in a certified histopathological laboratory under good laboratory practices with an established system of quality control. The samples were processed by standard histological methods using an automated Leica ASP6025 tissue processor (Leica Microsystems, Germany), and then embedded in paraffin blocks using a Leica EG 1150H paraffin embedding station (Leica Microsystems, Germany). Slices 2–5 μm thick were cut from each sample using a Leica RM2255 microtome (Leica Microsystems, Germany) and mounted on special silanized glass slides (DAKO, Denmark). All tissue sections were stained separately using: (i) hematoxylin-eosin (DiaPath, Italy) for principal tissue visualization, (ii) Masson's trichrome (TRI) (Sigma-Aldrich, Czech Republic) for the histological visualization of collagen I fibers, (iii) Periodic acid-Schiff (PAS) to detect polysaccharides, and (iv) Alcian blue at pH 2.5 (ALC) (Sigma-Aldrich, Czech Republic) for detecting acidic mucin. The prepared sections were evaluated using an Olympus BX51 microscope, and images were acquired using a Promicam 3-3CP Sony Pregius camera running QuickPHOTO MICRO 3.0 software. The sections were independently examined blindly by two veterinary pathologists to confirm the diagnosis, both histopathologically and histochemically. The details of histological and histochemical findings in affected animals are presented in Supplemental Table S1.

2.3. Recovery of arrested larvae

The abomasa were also examined for arrested larvae as outlined by Hansen and Perry (1994). After collecting tissue samples for histologic examination, we placed the organs with the mucous membrane facing down in a bowl containing saline and left them overnight at room temperature. The organs were then discarded, and the saline solution was poured through a 38-μm mesh sieve. The larvae recovered on the sieve were identified to genus based on morphological features (Thomas and Probert, 1990; Dróźdź et al., 2000). All nematode stages were studied using an Olympus BX51 microscope, and the morphometric characters were measured using QuickPHOTO MICRO 3.0 software.

3. Results

3.1. Parasitology

Nematodes were detected in the abomasa of all animals, varying widely in number. The majority (96%) of the nematodes were *A. sidemi*. The prevalence of this parasite was 100%, and abundance varied from six to nearly 2300 individuals (Table 1). Infrapopulations of *A. sidemi* consisted mainly (97%) of fully developed individuals; juvenile or early adults were detected in eight animals (38%), with a maximum intensity of 73 individuals. Several dozen nematodes from the subfamily Ostertagiinae were recovered from the vast majority of the animals (prevalence of 86%); only three individuals contained no Ostertagiinae (Table 1). The Ostertagiinae infrapopulations consisted only of adults and comprised predominantly *Ostertagia* and *Spiculopteragia*. Only a negligible abundance and prevalence (9.5%) of tissue larval stages were detected by the techniques of mucosal larval recovery using the saline solution. Ten *A. sidemi* fourth stage (L4) larvae were recovered from the abomasum of one fallow deer, and 40 early L4 of the subfamily Ostertagiinae were detected in the abomasal tissues of sika deer.

3.2. Gross pathology

All abomasa were of standard size. Grossly visible lesions, thickening, swelling, and extensive areas of hyperemia were observed on all mucous membranes of the abomasa; these findings were particularly restricted to fundic regions (Fig. 1A) The lesions varied in size, shape, and color, from isolated mild petechiae (≥3 mm in diameter) to

Table 1
Summarized details of animals and recovered nematodes.

Host species	Number examined	Nematode abundance							
		Ashworthius sidemi				Subfamily Ostertagiinae			
		Min	Max	Mean	SD	Min	Max	Mean	SD
Red deer	5	6	1084	385	369	0	20	15	14
Roe deer	5	26	2660	944	904	10	99	33	37
Fallow deer	5	62	970	488	411	0	57	24	21
Sika deer	5	3	582	144	247	0	19	11	5

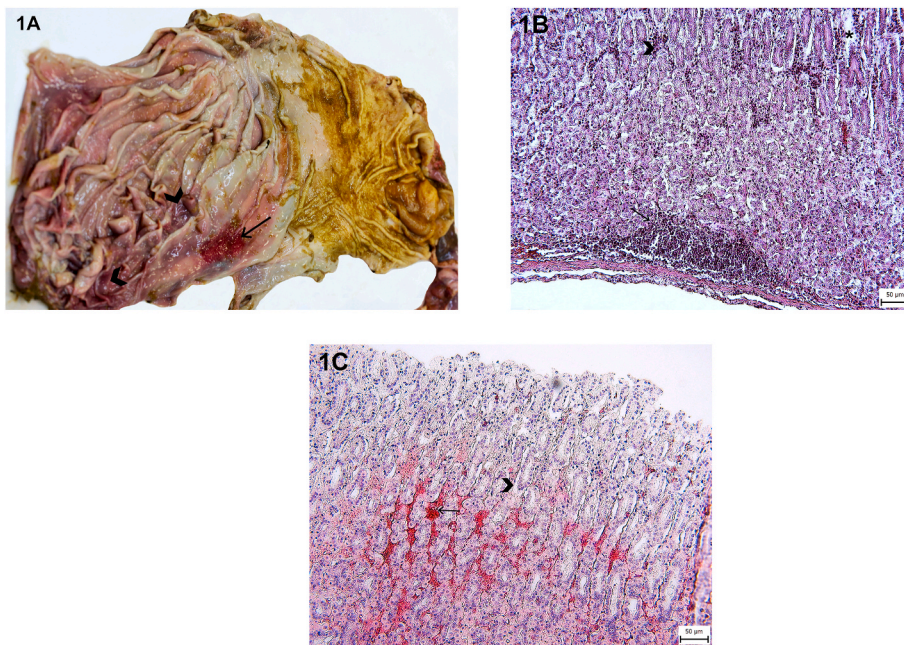Legends: Min/Max, minimum/maximum abundance; SD, standard deviation.

Fig. 1. Abomasal tissue infected predominantly with *A. sidemi*. A. Fallow deer, positive for *Ashworthius sidemi*. Washed abomasal mucosa with apparent extensive areas of hyperemia (arrowheads) and hemorrhagic lesion in the area of the fundus (arrow). B. Red deer, sample from gross lesion. Part of the abomasum with dispersed round-cellular interstitial inflammation at the base of the glands (arrow) and between abomasal pits (arrowheads). Full findings are accompanied by interstitial edema (asterisk). Hematoxylin and eosin (H&E) staining, magnification ×200. C. Fallow deer, sample from gross lesion. Part of the abomasum with hyperemia, consisting of several groups of vital erythrocytes in the interstitial tissue (arrow), including edema (arrowheads). H&E staining, magnification ×200. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

continuous locally extensive hemorrhagic areas a few centimeters in length. Slight pale nodules and typical mucosal corrugation were detected in some cases, primarily in abomasal antra.

3.3. Histology and histochemistry

Histological examination indicated inflammation of the abomasal mucosa in all animals (n = 20). Widespread inflammatory infiltration and accompanying alterations (e.g. edema and necroses) indicating abomasitis were evident in all abomasal sections, regardless of whether they originated from the affected mucosal regions or from regions without gross findings. Infiltrates consisted particularly of lymphocytes and plasmatic cells with a predominantly diffusive distribution. Aggregations of lymphocytes were localized in the lamina propria and interglandular tissue (Fig. 1B). Inflammatory infiltrates pervaded through the entire walls of the abomasa, including the muscular layer, in 11 of 20 animals (55%) with varying intensities of *A. sidemi* infections. The abomasa of one roe deer had necrotic tissue surrounded by collagen fibers in the submucosal layer. Slight local erosions were observed on superficial epithelia affecting the upper abomasal pits in three cases (15.0%). Thickening in the zone of abomasal mucous neck cells (MNCs) was evident in one sample taken from a nodular lesion. All samples collected from gross lesions had hemorrhages in the lamina propria that extended to the upper part of the mucosa in some cases. Histological alterations did not correspond to the intensity of the nematode infections; nematodes were not histologically confirmed in any of the abomasal sections. Pathological changes and their intensities did not differ significantly between host species.

Trichrome staining identified strands of collagenous connective fibers in eight of 20 samples (40%), indicating initial fibrosis. Tissues stained by PAS exhibited low levels or the total loss of polysaccharides. The PAS positivity of the epithelia of the upper abomasal pits (SMC zone) tended to decrease, ranging from an almost complete absence (Fig. 2B) to focal residues of polysaccharides (Fig. 2C). Polysaccharide positivity also tended to decrease in elongated MNC zones. Acidic mucins occurred from the upper pits down to the MNC zone (Fig. 2D). Areas stained by Alcian blue mostly coincided with PAS positivity. The intensity and extent of the pathological changes and the level of staining did not represent the intensity of the nematode infections.

4. Discussion

The allochthonous *A. sidemi* was more prevalent and infections were more intense than other (autochthonous) nematode species in nearly all animals. This finding is in accordance with previous reports of the dynamic spreading of this invasive parasite (Demiaszkiewicz et al., 2017; Kuznetsov et al., 2018). The host animals were culled during winter, but infrapopulations of *A. sidemi* consisted primarily of adults; only low numbers of juveniles and negligible numbers of arrested larvae were detected. This finding supports the hypothesis that arrested development is not a strategy essential for *A. sidemi* to survive winter in the current climatic conditions of central Europe (Vadlejch et al., 2017).

The samples of abomasal tissue exhibited characters of abomasitis; the intensity of inflammation varied from mild to severe. The majority of the histological alterations corresponded with those previously detected in red deer and European bison infected by *A. sidemi* (Demiaszkiewciz
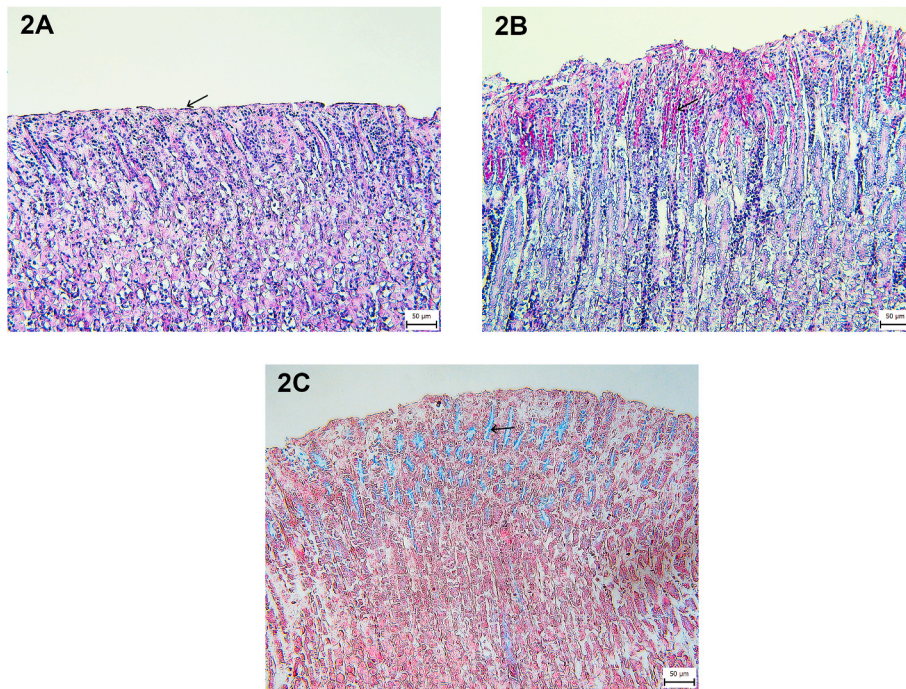
Fig. 2. Histochemical staining of fundic tissue for mucin detection. A. Roe deer, sample from gross lesion. Superficially apparent decreasing PAS positivity at the luminal surface (arrow). PAS, magnification ×100. B. Fallow deer, sample from gross lesion. Residual PAS positivity in the upper pits of abomasum (arrow). PAS, magnification ×100. C. Fallow-deer, sample from gross lesion. Residual mucin located from upper abomasal pits (arrow) content to lower parts, including basal glands. Alcian blue, magnification ×200. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2009; Osińska et al., 2010). The inflammatory infiltrate in the samples was composed of lymphoid cells (lymphocytes and plasmatic cells). The number of lymphoid cells was higher in calves with secondary infections with *O. ostertagi* than in calves with primary infections (Gasbarre, 1994). Also, European bison infected by *A. sidemi* had numerous lymphocytes in the abomasal mucosa and lymph nodes (Osińska et al., 2010). We examined only adult individuals, so the observed condition was most likely due to reinfection by trichostrongylid nematodes rather than to primary infections. In contrast, only a small amount (or the absence) of eosinophils was identified, perhaps due to the negligible numbers of larval stages recovered from the animals. Eosinophils are more involved in defensive mechanisms against larval stages than in killing adult parasites (Meeusen and Balic, 2000). Eosinophil levels likewise gradually decreased in abomasal mucosa 10 days after sheep were experimentally infected by *T. circumcincta* (Scott et al., 2017). The composition of the infiltrate and the developmental stage of the nematodes we recovered indicated a later phase of infection.

Some parts of the examined tissues (40%) exhibited initial or mild fibrosis of the mucosal tissue. Fibrosis may have various causes but usually occurs after chronic inflammation due to the release of cytokines in a Type 2 immune response, which is involved in the regulation of fibrogenesis (Wynn, 2004; Van Dyken and Locksley, 2013). Type 2 responses occur during allergic reactions and also during helminth infections, including abomasal nematodes such as *T. circumcincta* (Díaz and Allen, 2007; Knight et al., 2007). The presence of collagenous fibers in abomasal mucosa can be considered as a mechanism to repair host tissue, but it could lead to pathological changes under the chronic inflammation that we observed or during altered regulation (Gieseck et al., 2018).

The gastric epithelium of mammals is covered by a layer of secreted mucin that comprises saccharides O-linked to a protein core by glycosylation. Mucous cells at the surface of the lumen and in the upper region of gastric pits mostly produce the neutral gel-forming mucin Muc5AC, and the lower zone of MNCs produce the acidic mucin Muc6 (Ota and Katsuyama, 1992; Hoorens et al., 2011). Polysaccharide levels and mucin secretion were considerably lower in the abomasal tissue of all animals in our study. The luminal surfaces appeared virtually clear of mucus in some cases, consistent with recent studies (Rinaldi et al., 2011;

Simpson et al., 2016; Scott et al., 2017) that reported decreases in neutral superficial mucin in livestock experimentally infected by trichostrongylids such as *H. contortus*, *O. ostertagi*, or *T. circumcincta*. Moreover, the expression of Muc5AC was significantly downregulated in the epithelial tissue of sheep infected by *H. contortus* (Rowe et al., 2009). Whether the decrease in mucin was due to an effort by the parasites to make their environments more favorable or to a defensive mechanism of the hosts is not yet entirely clear. Muc5AC is not only a barrier that protects epithelia from physical and chemical damage, but is also an effective line of defense against several pathogens (Lichtenberger, 1999). For example, Muc5AC in mice is essential to expel various intestinal parasites (Sharpe, 2018). The anthelmintic effect of Muc5AC in ruminants was supported by stronger Muc5AC gene expression in resistant than susceptible sheep (Ingham et al., 2008). The residual PAS positivity could therefore indicate an effort to restore normal mucin formation after parasite-induced reduction. This finding is consistent with our assumption of a later phase of infection, in which some low-level infected hosts probably expelled most of their worm burdens. A substantial decrease in superficial mucous gel layers was apparent, even in samples from animals infected by negligible numbers of abomasal nematodes. Muc5AC, however, can allow some pathogens to colonize host gastrointestinal tracts (Van den Brink et al., 2000; Van de Bovenkamp et al., 2003). The exposure of adult *T. circumcincta* and *H. contortus* to acidic environments causes rapid death (Rowe et al., 2009; Hoorens et al., 2011). Alternatively, a layer of neutral Muc5AC can provide protection to nematodes from the low pH of the gastric lumen, and its weakening or loss may be detrimental to the maintenance of infection (Scott et al., 2017).

The consequence of reduced neutral Muc5 is nevertheless the loss of the pH gradient and barrier protecting hosts against their own enzymes that can lead to epithelial autodigestion. Glandular cells exhibited various degrees of residual positivity in the abomasal pits and at the base of glands identified by PAS and Alcian blue (pH 2.5). The overlap of PAS/ALC positivity suggested that the abomasal pits contained predominantly acidic Muc6 mucins from the upper pits down to the MNC zone. This suggestion is consistent with increases in acidic mucin previously found in sheep infected by *H. contortus* and *T. circumcincta* (Simpson, 2016) that represented elevated Muc6 levels as well as in

cattle during *O. ostertagi* infection (Rinaldi et al., 2011). Alterations in mucin formation are probably due to the inhibition of parietal cells, because its onset is contemporary with infection (Scott et al., 2017). This inhibition is common during infections with abomasal nematodes, probably caused by inflammation and nematode ES products (Simpson et al., 1999; Lawton et al., 1996) Decline of negative feedback from a low pH increases gastrin levels that induce the proliferation and subsequent hyperplasia of MNCs. Inhibition of the maturation of mucosal cells into parietal and chief cells occurs at the same time. We found only slight thickening in the MNC zone, despite significant alterations in mucin formation, except the single case of nodular lesions that exhibited hyperplasia in the MNCs. Variation in abomasal pH and serum gastrin level and alterations in mucin oligosaccharide structure in wild ruminants infected with nematodes should be subjects of further study.

The pathological alterations detected during our study surprisingly did not correspond with the intensity of nematode infection. The histological alterations, however, may have been induced by a serious initial nematode infection of the gastric lumen; infection intensity may have decreased over time due to the natural ageing of the nematodes, the immunological expulsion of nematodes, or anthelminthic treatment when pathological alterations persisted in infected tissue. The alterations in mucin secretion and the pathological changes of abomasal tissue were probably due to a synergistic effect of *A. sidemi* and the substantially less numerous nematodes from the subfamily Ostertagiinae.

5. Conclusion

We found that *A. sidemi*, with a lesser or greater contribution of nematodes from the subfamily Ostertagiinae, induced changes in abomasal tissue included chronic abomasitis with lower polysaccharide levels and mucin secretion. These alterations were observed in a wide spectrum of cervid host species, so we assumed that abomasal nematode infections (primarily those caused by *A. sidemi*) may negatively affect the production and welfare of wild ruminants, particularly farmed cervids. Livestock can also be infected by *A. sidemi* due to the low host specificity of this non-native nematode. Free-ranging wild ruminants thus threaten the health of ruminant livestock, particularly in pastures shared by cervids and bovids.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.12.008.

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