Geographical distribution of the giant liver fluke (*Fascioloides magna*) in the Czech Republic and potential risk of its further spread

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ABSTRACT: The giant liver fluke, *Fascioloides magna*, is of interest to wild-life managers, veterinarians and researchers, due to its unusual body size (3–10 cm), high pathogenic potential and because it is continuously spreading to new areas, especially in Europe. Annually, the number of cases of animal infections (mainly cervids and bovids) caused by this fluke is monitored in many European countries, including the Czech Republic (with some foci of prevalence over 90%). During the years 2009 and 2010, 1622 survey forms focused on monitoring of fascioloidosis were distributed in the community of "Czech Inspectors of Hunted Game" (CIHG), and 21.3% of forms containing positive or negative response about *F. magna* occurrence were returned. The administrative units monitored by particular CIHG, who answered the forms, were geographically equally distributed and therefore we believe that also the recorded distribution of *F. magna* in wild-life animals reflects the real situation in the Czech Republic. A significant number of cases of *F. magna* infection were repeatedly reported from areas in the south-west part of the Czech Republic. Moreover, our report contains also some unique records of several new *F. magna* foci in the western (close to the German border), northern (close to the Polish border) and central parts of the Czech Republic, supporting the assumption that the parasite is spreading further throughout Europe. In five game administrative units *F. magna* infection was directly confirmed by examination of dissected deer livers or by microscopic examination of coprological samples, followed by isolation of DNA from adults and eggs and further molecular analyses. *Fascioloides magna* intermediate host snails (*Galba truncatula* and *Radix* spp.) were collected during 2009 and 2010 from different localities of the Czech Republic, kept in aquaria, examined for shedding of *F. magna* cercariae, dissected and parasite/snail DNA was isolated. After PCR with specific primers for parasite/snail internal transcribed region number two (ITS-2) the obtained sequences confirmed identification of the following species: *F. magna*, *G. truncatula*, *R. peregra*, *R. lagotis*, *R. labiata* and *R. auricularia*. Although it has been demonstrated that the number of areas with positive cases of fascioloidosis is still growing, the risk of pathogenic impact of *F. magna* on populations of free-living animals and farming cervids/bovids is generally underestimated.

Keywords: diagnostics; emerging diseases; *Fascioloides magna*; *Galba*; geographical distribution; *Radix*; trematodes.

List of abbreviations

AN = accession number, **BLAST** = Basic Local Alignment Search Tool, **CIHG** = Czech Inspectors of Hunted Game, **FE** = forest game enclosures, **GF** = game farms, **HD** = hunting districts, **ITS-2** = internal transcribed region number two, **NCBI** = National Center for Biotechnology Information, **NGF** = non-productive game farms, **PCR** = polymerase chain reaction

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Fascioloides magna (giant liver fluke) is one of the most pathogenic and in veterinary biology, most important helminths parasitizing a number of mammalian species (primarily ruminants). It causes severe health problems, often leading to death (Foreyt and Todd 1972; Pybus 2001).

The habitat of *F. magna* formerly included several areas of North America (Swales 1935; Pursglove et al. 1977; Lankester and Luttich, 1988; Smits, 1991; Mulvey et al. 1994; Pybus 2001; Lotfy et al. 2008; Kralova-Hromadova et al. 2011). However, in the second half of the 19th century the parasite spread to Europe due to the repeated import of game-park animals from the overseas (Balbo et al. 1987; Pybus 2001; Spakulova et al. 2003; Ursprung et al. 2006; Kralova-Hromadova et al. 2011)[1]. *Fascioloides magna* has successfully adapted to European conditions and is periodically recorded in populations of free-living and domestic ruminants of several European countries, including the Czech Republic (Ullrich 1930; Zahor 1965; Erhardova-Kotrla 1971; Novobilsky et al. 2007a; Kralova-Hromadova et al. 2011), Slovak Republic (Rajsky et al. 2002; Kralova-Hromadova et al. 2011), Austria (Pfeiffer 1983; Ursprung et al. 2006), Germany (Novobilsky et al. 2007a), Hungary (Majoros and Sztojkov 1994), Croatia (Marinculic et al. 2002; Janicky et al. 2005; Rajkovic-Janje et al. 2008) and Italy (Balbo et al. 1987; Kralova-Hromadova et al. 2011). Fascioloidosis has sporadically been recorded also in Spain (Almarza 1935), Poland (Slusarski 1955), and coincidentally in South Africa, Australia and Cuba (Boomker and Dale-Kuys 1977; Arundel and Hamir 1982; Lorenzo et al. 1989). In the last three cases, the infected animals (brahman heifer, ox and wapiti) were imported from North America.

Fascioloides magna adults are mostly localized in the liver tissue of specific definitive hosts (in Europe most frequently in *Cervus elaphus*), where they survive in pseudocysts for a long time, and produce eggs which are released via ducts of pseudocysts into the bile duct system and then gut; they leave the host body with faeces. The eggs are continuously disseminated in high quantities, contaminating the outer environment of hosts (Erhardova-Kotrla 1971; Pybus 2001; Spakulova 2003). After several weeks (4–6) of embryonation under proper conditions (determined mainly by humidity, temperature and oxygen level) miracidia start to hatch from eggs in water and are able to infect snails.

Several species of lymnaeid snails from Central Europe were shown to be susceptible to *F. magna*; natural infections of *Galba truncatula* (O.F. Müller, 1774) (Erhardova-Kotrla 1971; Hirtova et al. 2003; Vignoles et al. 2006) and eventually *Radix* sp. Montfort, 1810 (Faltynkova et al. 2006)[2], and experimental infections of *Omphiscola glabra* (O.F. Müller, 1774) (Rondelaud et al. 2006; Dreyfuss et al. 2007), *Stagnicola palustris* (O.F. Muller, 1774) (Chroustova 1979) and *Lymnaea stagnalis* (Linnaeus, 1758) (Slusarski 1955) were reported. Due to the increasing trade in aquarium plants and global warming, there is a potential for transmission of *F. magna*-susceptible snail species from glasshouses to the outdoors. For example, along the banks of the Lot River in south-western France, Pointier et al. (2007) discovered *Pseudosuccinea columella* (Say, 1817), a lymnaeid snail which has previously been found in Europe only in glasshouses of botanical gardens and which was demonstrated to be a susceptible intermediate host of *F. magna* (Krull 1933).

Considering the cumulative contamination of the environment by metacercariae (the stage after cercariae spread by infected snails in the environment over the course of approximately half a year, i.e., May–October), together with the continuous dissemination of eggs, we can hypothesize that the circulation of this trematode in Europe is intensive and that the potential risk of *F. magna* infection for both wild and domestic animals (cervids and also bovids) is high (Zahor 1965; Zahor et al. 1968). Although domestic ruminants (bovids) are not specific definitive hosts, they are susceptible to *F. magna* infection (Erhardova-Kotrla and Blazek 1970; Chroustova et al. 1980); a higher pathogenic effect for bovids than that for cervids has been described (Foreyt and Todd 1976a).

In the Czech Republic there is a long tradition of intensive forest management and hunting. The game administration is divided into four main administrative branches, including hunting districts

[1] The first report of *F. magna* in Europe is dated to 1875 when Bassi recorded adult flukes in *Cervus elaphus canadensis* (wapiti) from the La Mandria Regional Park, near Turin in Italy.

[2] The taxonomic validity of some species of the genus *Radix* is currently under discussion, because their identification was partly based on unstable morphological characteristics (e.g. conchological).

(HD; about 5750 administrative units), forest game enclosures (FE; about 200 administrative units), productive game farms (GF; about 250 administrative units) and non-productive game farms (NGF, "hobby" farms; about 100 administrative units; Ministry of Agriculture of the Czech Republic – report 2009, 2011; Deer Farmers´Association of the Czech Republic – annual report 2011). Game is represented mainly by free-living animals of HD and FE: *Capreolus capreolus* (roe deer; about 320 000 heads), *Cervus elaphus* (red deer; 30 000 heads), *Dama dama* (fallow deer; 26 000 heads), but there is also a significant number of farmed animals, mostly red deer (about 6000 heads) and fallow deer (about 3000 heads) (Ministry of Agriculture of the Czech Republic – report 2011)[3]. Numerous populations of game from all four mentioned administrative branches are highly affected by *F. magna* (e.g. Novobilsky et al. 2007a).

The present work concerns the actual geographical distribution of *F. magna* in the Czech Republic based on a questionnaire survey combined with direct examination and molecular identification of *F. magna* samples. It confirms the process of *F. magna*'s continuous adaptation and spread in Europe. We believe that the epizootological situation with fascioloidosis in the Czech Republic and central Europe should be met with relevant control measures by local and European authorities. The methods included in this paper might represent a recommendation for administrators of game and farm animals, and veterinarians on how to better estimate the potential risk of *F. magna* infection, with consideration also of the spectrum and geographical distribution of intermediate hosts (*G. truncatula* and *Radix* spp.).

MATERIAL AND METHODS

Fascioloidosis and snail intermediate host survey

1622 "Czech Inspectors of Hunted Game" (CIHG) were twice (2009 and 2010) approached via email and asked for cooperation in a *F. magna* monitoring survey; they were requested to fill in the brief survey form (questions included: Name, geographical location and area of administrated unit. Did you recently, in the last three years, record *Fascioloides magna* infection in the livers of a hunted wild-life animal? Please, specify the species of animal.), and to provide detailed information in the case of positive records of fascioloidosis. In this way, basic data on the historical (in some cases) and current occurrence of *F. magna* in game in particular administrative units was collected.

The information about the geographical distribution of snail intermediate hosts (*G. truncatula* and *Radix* spp.) in the Czech Republic is based on the co-author's (L. Beran) database containing more than 51 000 records of freshwater snails. The majority of records were obtained by field monitoring over the last 15 years, and the rest come from the collections of Czech museums, published papers and unpublished records of other malacologists. At present, the taxonomic validity of some species within the genus *Radix* is under discussion and a re-classification based on a combination of morphological, ecological and molecular criteria seems to be inevitable. Therefore, we will generally use the term *Radix* spp. for all *Radix* snails included in this work, but for those which were molecularly characterized the species names sensu Bargues et al. (2001) will be used.

Collection of parasites and snails

The parasite (*F. magna*) and snail intermediate host (*G. truncatula* and *Radix* spp.) surveys (see above) were accompanied by direct collection of material and its examination.

The livers of hunted animals and coprological samples originating from selected localities where fascioloidosis was reported by CIHG (e.g. National Park Sumava, Military Area Boletice, Kokorinsko Protected Landscape Area, district Kutna Hora and Brdy) were examined for *F. magna* adults and eggs.

About 1100 *G. truncatula* snails and approximately 1800 *Radix* spp. snails were collected during 2009 and 2010 at different localities in the Czech Republic, kept in aquaria (25 °C tap water, fed with lettuce), examined for the shedding of *F. magna* cercariae, and

[3] According to European Union legislation (regulation issued by the European Parliament and Council, ES No. 853/2004), all animals hunted in Czech Republic must be inspected by trained hunters or veterinarians – "Inspectors of Hunted Game" holding a special licence. Inspectors are obliged to produce a report for each animal, which should also contain the information regarding any parasitoses.

subsequently dissected for isolation of sporocysts/rediae/cercariae (Faltynkova et al. 2006).

The *F. magna* adults were washed in sterile H_2O and the eggs were isolated and concentrated from faeces using a sedimentation method (Thienpont et al. 1980). *F. magna* developmental stages – sporocysts/rediae/cercariae – and samples of snails were microscopically examined, or fixed in 70% ethanol for further analyses.

Taxonomic identification of parasites and snails

Fascioloides magna adults, eggs and developmental stages (sporocysts/rediae/cercariae) from naturally infected snails collected and maintained in the laboratory were identified microscopically (using a stereomicroscope and microscope) according to morphological characteristics and sequence of the internal transcribed region number two (ITS-2) (described below).

Genomic DNA of *F. magna* adults, eggs (1–3 samples from each locality), sporocysts/rediae/cercariae (three samples) and *G. truncatula*/*Radix* spp. snails (fresh or fixed in 70–96% ethanol, one to five snails from each locality) was isolated using QIAamp DNA Mini Kit or QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's recommendations, and stored at –20 °C. The concentration of DNA was measured using a NanoDrop 1000 (Thermo Scientific).

Each 25 µl PCR reaction for the detection of *F. magna* contained the following: 12.5 µl 2× concentrated PPP Master Mix (Top-Bio; which includes 10× PCR buffer, 100 IU/ml of Taq-Purple DNA polymerase and 0.4mM dNTP), 2.5 µl 25mM $MgCl_2$, 6 µl PCR H_2O, 1 µl 10µM forward 5'-ACCAGTTATCGTTGTGTTG-3' and reverse 5'-CCGTCTTTAAACAACAG-3' primers specific for the *F. magna* (ITS-2) 152 bp region (designed according to Kralova-Hromadova et al. 2008 and Bazsalovicsova et al. 2010) and 20 ng of DNA template. Amplification proceeded in a My Cycler (Bio-Rad) using the protocol: 94 °C, 5 min; 30 times 94 °C, 30 s; 55 °C, 30 s; 72 °C, 60 s and a final 10 min extension at 72 °C.

Each 25 µl PCR reaction for the detection of *G. truncatula*/*Radix* spp. contained the following: 0.3 µl (5 IU/µl) Sahara DNA Polymerase (Bioline), 2.5 µl 10× Sahara Reaction Buffer, 1 µl 50mM $MgCl_2$, 2 µl 2.5mM dNTP, 16.5 µl PCR H_2O, 1 µl 10µM forward 5'-TGTGTCGATGAAGAACGCAG-3' and 5'-TTCTATGCTTAAATTCAGGGG-3' primers specific for the lymnaeid snail ITS-2 region (572 bp for *G. truncatula* and 549 bp for *Radix* spp.; designed according to Almeyda-Artigas et al. 2000) and 50 ng of DNA template. Amplification proceeded in a My Cycler (Bio-Rad) using the protocol: 94 °C, 10 min; 30 times 94 °C, 30 s; 50 °C, 30 s; 72 °C, 60 s and final 10 min extension at 72 °C. Ten µl of the PCR products were analyzed using 1% agarose electrophoresis and purified using a MinElute® PCR purification Kit or MinElute® Gel Extraction Kit (Qiagen). The purified PCR products were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems). The obtained sequences were adjusted using DNAStar-Lasergene Core Suite software tool and identified with the aid of the Basic Local Alignment Search Tool (BLAST) and the nucleotide database (NCBI).

RESULTS

In total, 345 (21.3%) of the approached 1622 CIHG filled in and returned forms, including 316 (91.6%) negative and 29 (8.4%) positive answers regarding the regular occurrence of *F. magna*. The administrative units (HD, FE, GF, NGF) monitored by CIHG who answered the questionnaire, were geographically relatively evenly distributed within the Czech Republic. The majority of cases of fascioloidosis were distributed in areas in the south-west of the Czech Republic. New foci in the western (close to the German border), northern (close to the Polish border) and central part, and one possible unique report in the north-eastern part of the Czech Republic were also recorded. Based on documentation provided by CIHG it was concluded that *C. elaphus* and *D. dama* are the predominant animals parasitized by *F. magna*. In five game administrative units (Sumava National Park, Boletice Military Area, Kokorinsko Protected Landscape Area, Kutna Hora and Brdy districts) *F. magna* infection was directly confirmed by examination of dissected deer livers or microscopic examination of coprological samples, followed by molecular analyses of adults and also, in some cases, eggs. The amplified ITS-2 regions of suspected *F. magna* were sequenced and checked using BLAST and NCBI; 98–100% identity with *F. magna* ITS-2 reference sequence (Lotfy et al. 2008; GenBank accession number, a.n., EF612487; Table 1) was found.

Table 1. BLAST analysis of *F. magna*, *G. truncatula*, and *Radix* spp. nucleotide sequences of the ITS-2 regions (samples of collected parasites and snails)

	Species	Number of localities	ITS-2 region (bp)	Sequence identity (%)	GenBank (a.n.)	Author
Parasite	*Fascioloides magna*	5	152	98–100	EF612487	Lotfy et al. 2008
Snail*	*Galba truncatula*	10	517	100	HQ283262	Correa et al. 2010
	Radix lagotis	2	378	99	AJ319638	Bargues et al. 2001
	Radix labiata	18	370	99	AJ319636	Bargues et al. 2001
	Radix peregra	2	395	99	AJ319633	Bargues et al. 2001
	Radix auricularia	21	401	99	AJ319628	Bargues et al. 2001

*the list of snail species corresponds to our findings in particular localities

The geographical distribution of *G. truncatula* and *Radix* spp. covers at least 90% of the Czech Republic and their occurrence was monitored at altitudes of 130–1020 m. The geographical distribution of snails overlaps in 100% of cases with the reported distribution of *F. magna*. In four areas with *F. magna* occurrence (three in South Bohemia and one in North Bohemia), only species of the genus *Radix* (and not *Galba*) were recorded. In total, 26.7% of *G. truncatula* snails out of 1100 collected specimens from 10 localities during 2009–2010 were infected with *F. magna*. The infected snails were found only in one locality (Brdy district) where the prevalence was 98%. No snail out of the approximately 1800 collected and examined *Radix* spp. from 36 localities was positive for *F. magna* infection.

All the amplicons of the ITS-2 region obtained using PCR on the DNA of snails and parasites – *G. truncatula* (collected and naturally infected snails from 10 localities), *Radix* spp. (snails collected from 36 localities) and *F. magna* larval stages from the above intermediate hosts (sporocysts/rediae/cercariae) – were also sequenced and checked using BLAST and NCBI: the snails were identified as *G. truncatula* (100% sequence identity with ITS-2 reference sequence, GenBank a.n. HQ283262, was found, Correa et al. 2010) and *Radix* spp.–*R. lagotis* (99%, GenBank a.n. AJ319638), *R. labiata* (99%, GenBank a.n. AJ319636), *R. peregra* (99%, GenBank a.n. AJ319633) and *R. auricularia* (99%, GenBank a.n. AJ319628) (all *Radix* spp. sensu Bargues et al. 2001); sporocysts/rediae/cercariae were determined as *F. magna* (98–100%, GenBank a.n. EF612487, Lotfy et al. 2008; Table 1).

DISCUSSION

According to the results of our study we believe that the set of 1622 CIHG who were approached twice (2009 and 2010) and who cover about 6300 game administrative units (HD, FE, GF, NGF) and the 345 collaborating CIHG who answered the form (21.3%), represent a relevant sampling group documenting the situation of fascioloidosis occurrence in the Czech Republic (central Europe). In addition, the game administrative units with responding CIHG were geographically evenly distributed. Our results revealed that a significant number of *F. magna* infections were repeatedly located in areas of the south-west of the Czech Republic (Erhardova-Kotrla, 1971, Novobilsky et al. 2007a). This "wet region" with a fish farming tradition offers optimal terrestrial/hydrological conditions for intermediate hosts (freshwater snails), definitive hosts (deer) and transmission of *F. magna*. Some of the localities in this region were formerly monitored by e.g. Erhardova-Kotrla (1971) and Novobilsky et al. (2007a) as permanent enzoonotic areas where the prevalence of infection in the red deer population often reached 90%. Historically, the maximum parasite burden reported was 144 worms/deer liver (Erhardova-Kotrla, 1971). The sudden death of free or game ranging red and fallow deer were documented, a significant mortality in free ranging roe deer was recorded, and *F. magna* was also found in the livers of slaughtered cattle originating from these localities (Zahor 1965; Zahor et al. 1968, Erhardova-Kotrla 1971). In our study we recorded some new areas of *F. magna* occurrence

in western (close to the German border, presumed by Novobilsky et al. 2007a), northern (close to the Polish border) and central part, and one unique finding in the north-eastern part of the Czech Republic (close to the Karvina city), which is the first indication of *F. magna* infection in that area. It would, however, be necessary to re-confirm the latter report directly by finding the adult worms, eggs or larvae, and for their taxonomical identification to be validated by a specialist (veterinarians and parasitologists) using relevant microscopical and molecular techniques. The reason(s) for the large geographical distance between particular enzoonotic areas (e.g., south-west Slovakia and south-west Czech Republic) remain unexplained. The results of this study and those obtained formerly by Czech and Slovak scientists (e.g. Zahor 1965; Erhardova-Kotrla 1971; Novobilsky et al. 2007a; Kralova-Hromadova et al. 2011) imply that the number of areas with fascioloidosis is continuously growing, i.e., *F. magna* is probably still in the exponential phase of its adaptation process to the new conditions, at least in central Europe, including the Czech Republic. The risk of *F. magna* transmission from free-living (cervids) to domestic animals (bovids) is therefore high, also in the shared pastures of the Czech-German borderland (Novobilsky et al. 2007b, Kralova-Hromadova et al. 2011). Wet pastures shared by infected and non-infected intermediate host snails and definitive hosts were recognized as the most suitable biotopes for the transmission of *F. magna* (Olsen 1949; Kingscote 1950; Zahor et al. 1968; Foreyt and Todd 1972; Chroustova et al. 1980). With regard to domestic/farming animals, fascioloidosis was recorded as the most fatal disease for goats (Foreyt and Leathers 1980, Novobilsky et al. 2007b) and sheep (Erhardova-Kotrla and Blazek 1970; Foreyt and Todd 1976a).

Based on our observations it is evident that the animals most threatened by fascioloidosis are those kept in enclosures with a source of naturally flowing water (e.g. creek) – the animals are hemmed in by a fence, while the intermediate snail hosts (infected/non-infected) and parasite eggs may continuously be supplied by water flow or occasional local floods. This view is supported by our direct monitoring at one GF (Central Bohemia, Brdy district) where repeated treatment of infected definitive hosts, *D. dama*, by Rafendazol premix (Biopharm; effective compounds rafoxanid/mebendazol) was unsuccessful, and the examination of dissected livers showed that the animals were probably cured and re-infected several times. In the dissected livers of these animals, scarred tissue, calcified pseudocysts, pseudocysts with degraded dead worms, and live worms were observed. Similarly unsuccessful treatment with Rafendazol premix was documented also by other authors (e.g., Foreyt and Todd 1976b, Pybus 2001, Rajsky et al. 2002). Unfortunately, in these cases no efficient and reliable intravital diagnostic method suitable for the preventive survey of definitive hosts is available. Currently, the diagnosis of fascioloidosis is primarily based on postmortal dissection of the host liver (in search of adult flukes), and unreliable coprological examination (in search of eggs). Other diagnostic methods, especially intravital methods such as ELISA/Immunoblot or PCR, could represent an appropriate alternative, and their optimization is in progress (Novobilsky et al. 2007b; Dixit et al. 2008; Oberhauserova et al. 2010).

The absence of fascioloidosis control measures for definitive hosts (no reliable intravital diagnosis, no efficient treatment) and relatively favourable conditions in the Czech Republic for the widely distributed intermediate hosts – *G. truncatula*/*Radix* spp. significantly contribute to the dissemination of this fluke to new localities. Ten species of lymnaeid snails are regarded as natural intermediate hosts of *F. magna* in North America (Dunkel et al. 1996). In Europe only *G. truncatula* was confirmed as an important natural intermediate host of *F. magna* (Erhardova 1961), although some members of the genus *Radix* are also suspected to be susceptible under natural conditions (Faltynkova et al. 2006); therefore, their role in parasite transmission should not be neglected. Our preliminary results with snails experimentally infected with *F. magna* miracidia confirm the susceptibility of several species of the genus *Radix*, and suggest that susceptibility to infection can differ among *Radix* species (not shown). Further investigation of *Radix* spp. is therefore desirable in order to predict the risk of *F. magna* transmission at localities inhabited by this snail genus.

Fascioloides magna was introduced from North America to Europe more than one hundred years ago, and started to successfully spread via local species of snails and cervids (e.g., *G. truncatula*, *Radix* spp. and *C. elaphus*, *D. dama*, *C. capreolus*, respectively). It has been demonstrated that the number of Czech localities with cases of fascioloidosis is continuously growing. Up to now, in the Czech Republic and Europe, only marginal economic

losses caused by *F. magna* infections are regularly monitored, i.e., impaired function of cervid livers and lower quality of trophy. Unfortunately, the risk of transmission of *F. magna* from game to domestic animals, and the role of (new) intermediate hosts – freshwater snails – are not receiving attention. This work underlines the necessity of filling this gap. We show here that questionnaires followed by direct examination and molecular confirmation of parasites and their host snails could be an effective tool for monitoring fascioloidosis in the Czech Republic and other European countries. An understanding of host-parasite interactions and mechanisms of disease transmission may enable the introduction of new strategies for *F. magna* control among cervids and domestic animals.

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