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Authors: Bildfell, Robert J., Whipps, Christopher M., Gillin, Colin M., and Kent, Michael L.

Source: Journal of Wildlife Diseases, 43(4) : 762-769

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-43.4.762

Journal of Wildlife Diseases, 43(4), 2007, pp. 762–769

DNA-based Identification of a Hepatic Trematode in an Elk Calf

Robert J. Bildfell,[1,4] Christopher M. Whipps,[2] Colin M. Gillin,[3] and Michael L. Kent[1] [1]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Magruder Hall, Corvallis, Oregon 97331, USA; [2]Department of Microbiology, College of Science, Nash Hall, Corvallis, Oregon 97331, USA; [3]Oregon Department of Fish and Wildlife, E. E. Wilson Wildlife Refuge, Adair, Oregon 97330, USA; [4]Corresponding author (email: rob.bildfell@oregonstate.edu)

ABSTRACT: Liver fluke infection was identified as a probable cause of clinical disease in an approximately 6-mo-old elk (*Cervus elaphus*) in coastal Oregon. Clinical pathology and necropsy findings are described. The alcohol-fixed flukes that were submitted for identification were similar in size to *Fasciola hepatica*, but their shape resembled *Fascioloides magna* in that they lacked a distinctive anterior cone. A few structures consistent with the eggs of *F. magna* were observed in liver lesions, suggesting that at least some of the worms were sexually maturing. Due to difficulties in morphologic identification associated with improper fixation technique, DNA analysis was used to compare small subunit (SSU) and internal transcribed spacer 2 ribosomal RNA gene sequences of the recovered parasites with those of *F. hepatica* and *F. magna*, confirming these small, but sexually mature flukes were *F. magna*. This is the first publication of the SSU gene sequence for *F. magna*. Phylogenetic analysis showed that it is related to, but is an outlier, to the genus *Fasciola*. Due to the high mortality rate associated with this disease outbreak, the overall significance of trematodiasis in the herd is unclear.

Key words: *Cervus elaphus*, elk, *Fascioloides magna*, fascioloidiasis, hepatitis, ribosomal DNA.

Increased mortalities were noted in a herd of approximately 70 Roosevelt elk (*Cervus elaphus* subsp. *roosevelti*) in Clatsop County of northwestern Oregon. Twelve mortalities were recorded during a 1-mo period that commenced in early December. Adults and juveniles were affected, and no gender bias was noted. Animals in the herd were in good body condition, with abundant forage available. Clinical signs observed in some of the affected animals included frothing at the mouth and generalized weakness. In early January, an approximately 6–8-mo-old male calf was observed to be hunched-up, weak, and reluctant to move. This animal was euthanized via gunshot and transported to the Oregon State Veterinary Diagnostic Laboratory (OSU VDL) for necropsy examination. A serum sample was also recovered from blood collected at the time of euthanasia. This serum was initially frozen but subsequently submitted for a complete biochemical profile.

Necropsy revealed the 86 Kg calf to be in good body condition, with small numbers of sucking and chewing lice present. There was gunshot trauma to the chest wall, lungs, and thoracic aorta. Generalized tissue pallor was attributed to hemothorax associated with laceration of the aorta. Approximately 40–50 nematodes were found in the major bronchi and trachea. Unilateral pulmonary atelectasis and congestion were present and interpreted as postmortem change.

The abdominal cavity contained approximately 500 ml of blood-tinged watery fluid. The retroperitoneal tissues were mottled by black-and-white/grey patches of variable size and shape, with no corresponding change in texture. The liver was pale with rounded edges. Irregularly located serpentine tracts, fissures, and depressions interrupted the capsular surface. Some of these tracts were demarcated by red-black pigmentation, whereas others had the opaque character typical of fibrosis. On cut surface, more tracts, cavitations, and foci of black discoloration were present (Fig. 1) as well as oval trematodes approximately 1.5–2 cm long (Fig. 2). Examination of the liver via "bread-loafing" into 2-cm-wide slices yielded five of these parasites. Further efforts at quantification, such as dicing of the parenchyma and soaking in warm water, were not performed. No abscesses

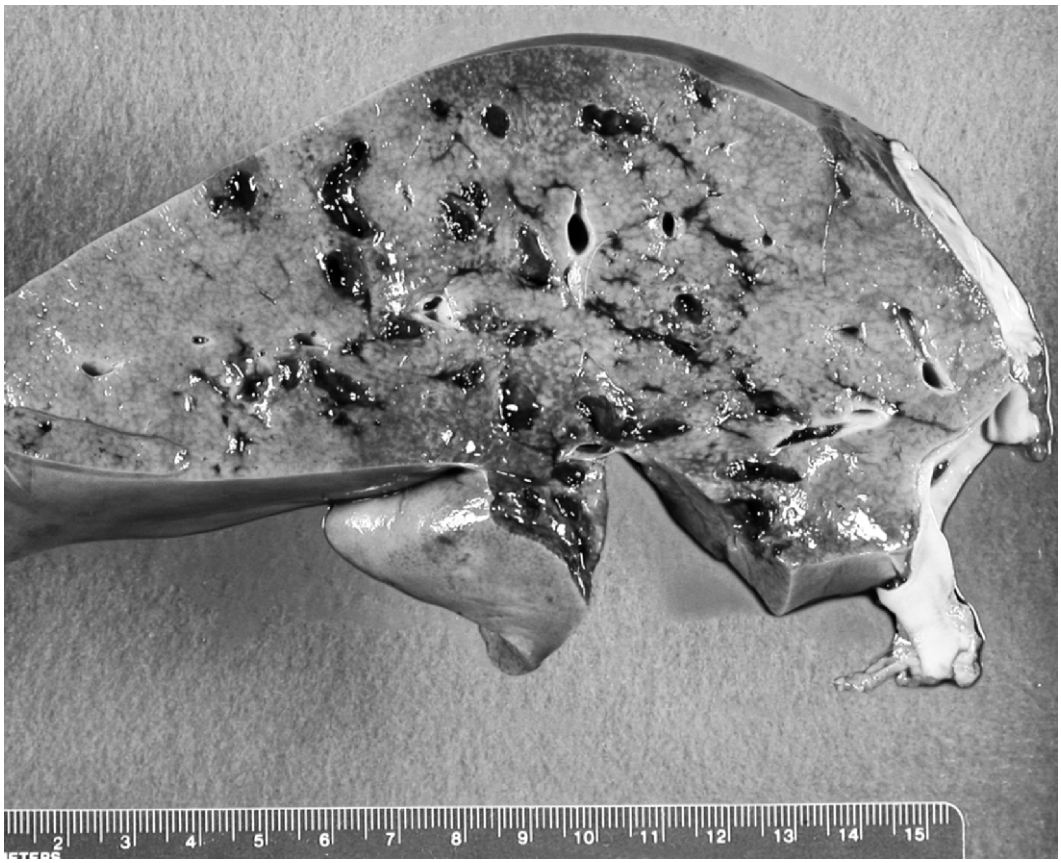

FIGURE 1. Cut surface of liver of elk calf with trematodiasis. Note loss of hepatic tissue along irregularly shaped hemorrhagic tracts.

or encapsulated parasite-filled cysts were identified, but irregularly shaped foci of firm white fibrous tissue were present, often associated with a large blood vessel. The entire liver was affected by this process, with the hemorrhagic tracts on cut surface suggesting a loss of 15–20% of the total hepatic parenchyma. No parasites were found in the bile duct. Perihepatic lymph nodes were enlarged but not discolored. No other significant gross findings were noted.

The ectoparasites were collected into 70% alcohol and submitted to the USDA National Veterinary Services Laboratory, Ames, Iowa, USA, for identification. Flukes were also collected into 70% alcohol for in-house identification at the OSU VDL. Lungworms were placed in saline and submitted to the National Parasite Collection and Animal Parasitic Disease Laboratory, Beltsville, Maryland, USA. A sample of the atelectatic lung was submitted for aerobic bacterial culture. Samples of lung, liver, diaphragm, and perihepatic lymph node were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Serum biochemistry values were compared with those published by Flach (2003) for moose (*Alces alces*) and elk, and these ranges are given in parentheses: total protein, 7.6 g/dl (7.6–9.0); albumin, 2.0 g/dl (3.28–6.38); creatine kinase (CK), 5830 U/l (48–159), γ-glutamyltransferase (GGT), 234 U/l (7–12); and aspartate aminotransferase (AST), 677 U/l (39–

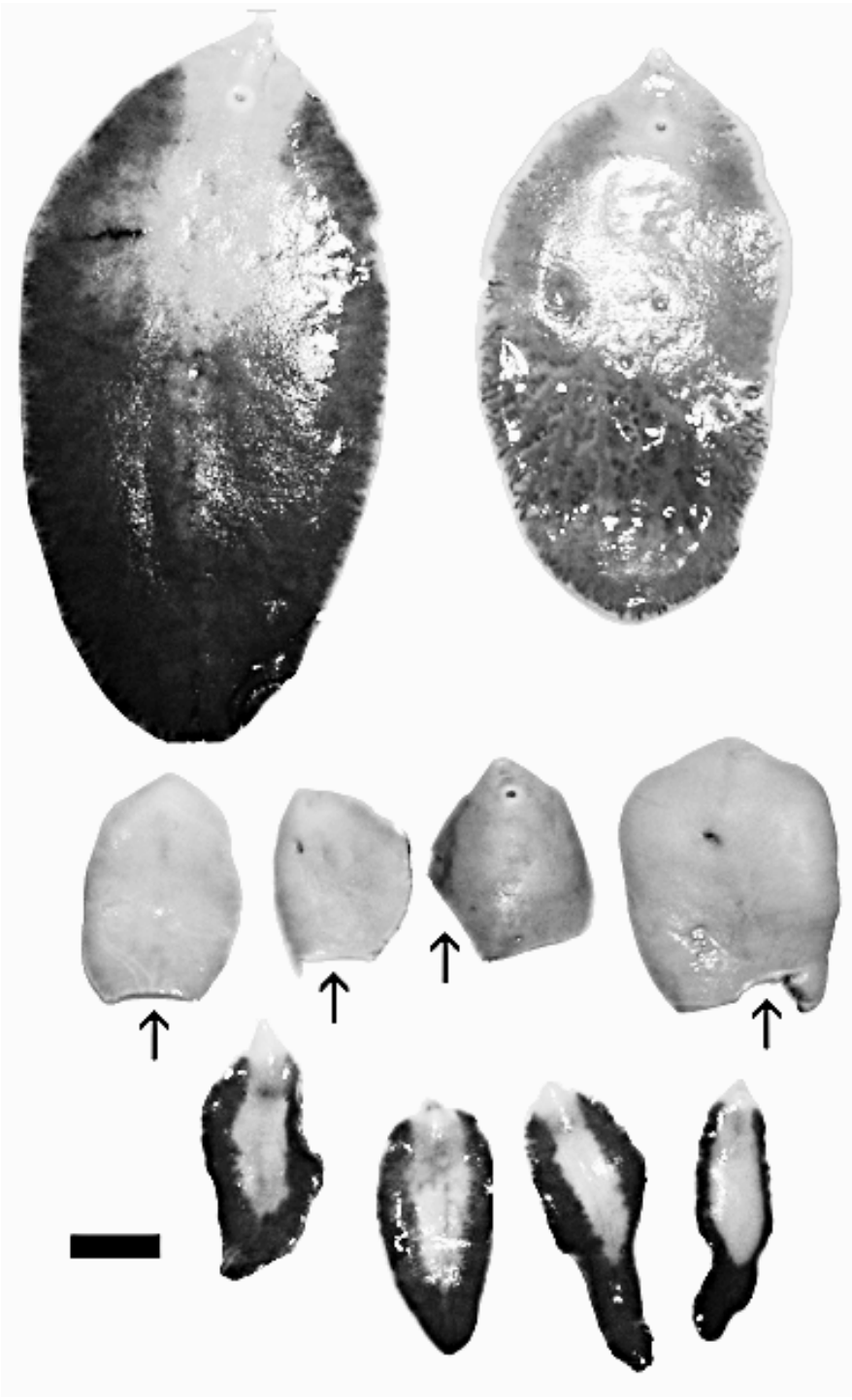

FIGURE 2. Fasciolid flukes. Top row: typical *F. magna* from a fallow deer. Middle row: unusual *F. magna* from elk (present study). Bottom row: *F. hepatica* from sheep. Distortion in the shape of the trematodes from elk were suspected to be a result of lack of relaxation of specimens before fixation. Arrows indicate sites of tissue collection for DNA analysis. Bar=5 mm.

108). Total bilirubin was 0.3 mg/dl and thought to be at the high end of normal, although a reference range was not available for this parameter. Electrolyte abnormalities were also present, but they were discounted as an artifact of hemolysis. The GGT and AST values are consistent with severe hepatic damage, although a portion of the AST may also reflect the muscular damage indicated by the increased CK. Hypergammaglobulinemia, hypoalbuminemia, and increased AST were reported in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Fasciola hepatica* (Presidente et al., 1975), and the latter two findings are also commonly noted in ovine fascioliasis (Radostits et al., 2000).

The ectoparasites were identified as the chewing louse *Damalinia* (*Cervicola*) sp. and the sucking louse *Solenoptes ferrisi*. Both have been previously identified on cervids necropsied at the OSU VDL. The lungworms were identified as *Dictyocaulus eckerti*, a species commonly recovered from cervids and previously often classified as *D. viviparus* (Hoberg, pers. comm.). Due to fixation in alcohol, the trematodes could not be definitively identified on the basis of morphology. No bacterial pathogens were cultured from lung.

Histopathologic examination of the liver revealed a chronic active inflammatory process with foci of periportal fibrosis as well as tortuous tracts of acute hepatic necrosis and hemorrhage. Features such as thrombosis, phlebitis, intracellular black pigment, and infiltrates of eosinophils, macrophages, multinucleate giant cells, and other leukocytes were also common. Some of the cavitated areas were rimmed by immature fibrous tissue with entrapped fluke eggs. The eggs measured about 70 μm long with a golden, refractile surface. Changes in the perihepatic lymph node included lymphoid follicular hyperplasia of the cortex and small amounts of black cytoplasmic pigment in the medullary histiocytes. Sections of diaphragm revealed foci of muscular necrosis, atrophy and fibrosis, and eosinophils and clusters of macrophages laden with coarse black pigment. Regenerative myocytes were visible, as well as a few sarcocysts. Other than trauma-associated hemorrhage, the pulmonary changes were minimal, consisting of atelectasis, a few lymphoplasmacytic periarteriolar cuffs, and mild lymphoplasmacytic bronchiolitis.

Fascioloides magna and *F. hepatica* have both been found in elk (Shimalov and Shimalov, 2000; Pybus, 2001), although the literature suggests that *F. magna* is the more common species and that it is a more significant cause of disease in elk. Various published reports and web pages on *F. magna* provide a range in length from 30 to 100 mm and note that this fluke is not found in bile ducts (Foreyt and Todd, 1976; Schell, 1985; Foreyt, 1992, 2001; Marinculić et al., 2002; Bowman, 2003), whereas *F. hepatica* is about 15 to 40 mm long (Foreyt, 2001; Bowman, 2003; Roberts and Janovy, 2005), and adults occur in bile ducts of domestic animals and cervids (Pybus, 2001). Although infection with *F. magna* was suspected due to the parenchymal location and general morphology of the trematodes, the small size of these flukes (1.5 cm in length) combined with the presence of eggs in tissue sections prompted us to consider *F. hepatica* or a novel fluke infection in this animal. Morphometric identification of trematodes is best accomplished by clearing and mounting specimens that have been relaxed overnight in saline before initial fixation in warmed formalin; thus, measurements of these improperly fixed elk flukes could not be reliably compared with those of the literature. Although suboptimal for morphometrics, these alcohol-fixed specimens were ideal for molecular analyses.

Highly sensitive and specific, DNA sequence-based approaches are exceptionally informative for parasite identification

(Prichard and Tate, 2001). Parasites that possess limited morphological features for identification or that are nearly identical by morphology can be distinguished by their DNA sequences (Adlard et al., 1993; Casiraghi et al., 2006). In addition, DNA analysis is applicable to any life stage of the parasite, and the sequences generated are useful for taxonomy and systematics (Gasser, 2006). Ribosomal DNA sequences are noted as particularly useful for discrimination of digenean trematode species (Nolan and Cribb, 2005). Indeed, in an early study of fasciolids, Adlard et al. (1993) documented differences in partial sequences of the internal transcribed spacer 2 (ITS-2) of the ribosomal DNA between of *F. hepatica*, *F. gigantica*, and *F. magna*, but little intraspecific variation. Thus, our approach was to sequence the ITS-2 of the unidentified specimen, identify the species; and then examine additional gene regions (small subunit [SSU], ITS-1, and 5.8S) to determine whether any further genetic variation occurs in this morphologically unique specimen.

Samples of the fixed parasites (Fig. 2) were processed to recover DNA (described below). GenBank archives did not include any rDNA sequence for *F. magna*, and the ITS-2 sequence of Adlard et al. (1993) was only a partial sequence. Therefore, we processed an alcohol-fixed archived specimen of this species that had been collected from a captive adult fallow deer (*Dama dama*) in 2003. The DNeasy tissue kit (QIAGEN, Valencia, California, USA) was used to extract DNA. Overlapping regions of the SSU, ITS-1, 5.8S, and ITS-2 genes were amplified by polymerase chain reaction and sequenced using standard methods (Jirků et al., 2006) and the following primers (start position relative to GenBank EF051080 in parentheses). In the SSU, modified universal primers 18E (1), 18I (368), and 18J (1294) of Hillis and Dixon (1991) as described by Jirků et al. (2006) were used, along with primer 18R (1928) of Whipps et al. (2003), and Elk1F (694) 5′-CCTGGTTACTACCGGGTC. In the 5.8S and large subunit, we used reverse primers NC13R and NC2, respectively, of Chilton and Gasser (1999). Nucleic acid sequence alignments were created in ClustalX (Thompson et al., 1997) using default settings. The resultant sequence alignment was then edited by eye to remove ambiguous regions or suspected base calling errors where positions of homology were questionable, yielding alignments 1,871 (SSU) and 555 (ITS) characters in length. Maximum parsimony analyses were conducted in PAUP*4.01 (Swofford, 1998) using a heuristic search algorithm with 10 random additions of sequences and tree bisection-reconnection (TBR) branch swapping. Bootstrap values were calculated with 1,000 replicates using a heuristic search algorithm with simple sequence addition and TBR branch swapping.

The 2,920-nucleotide rDNA sequence from the unknown parasite and *F. magna* were identical, confirming their conspecificity. Furthermore, the ITS-2 was identical to a sequence that has since been deposited from *F. magna* in Austria (GenBank DQ683545). Consistent with *F. magna*, little variation is also expected between regional specimens of *F. hepatica* and *F. gigantica* (Adlard et al., 1993; Huang et al., 2004; Nolan and Cribb, 2005). Phylogenetically, *F. magna* was sister to the two *Fasciola* species by both SSU and ITS-2 sequence analysis (Fig. 3). Internal transcribed spacer-2 sequences tended to cluster by species, with the exception of a *F. gigantica* sequence (GenBank AB010975) that was more like *F. hepatica*. Sequenced by Itagaki and Tsutsumi (1998), no explanation was provided whether this was a potential misidentification. Our DNA results are consistent with the traditional taxonomy of the family Fasciolidiae, and they support the distinctness of the genera *Fasciolopsis*, *Fascioloides*, and *Fasciola*. There was no evidence (i.e., dissimilar or multiple sequence peaks) to suggest that the smaller

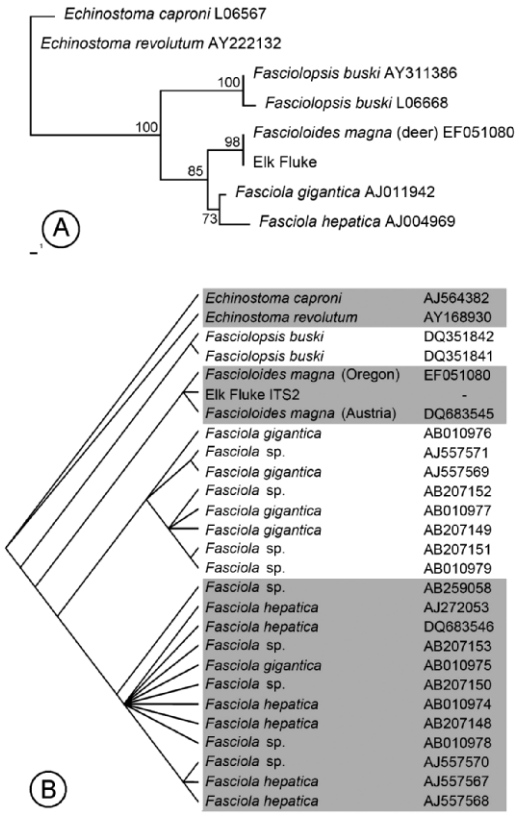

FIGURE 3. Phylogenetic trees resulting from parsimony analysis of small subunit (A) and internal transcribed spacer 2 (B) ribosomal DNA sequences from fasciolid trematodes. *Echinostoma* species were used as outgroup taxa. Bootstrap confidence values generated from 1,000 replicates are shown at nodes.

morphologic variant of *F. magna* studied here was a distinct species, or a hybrid species as suggested for some morphologic variants of *Fasciola* spp. (Itagaki and Tsutsumi, 1998; Huang et al., 2004; Itagaki et al., 2005). This case confirms that DNA analysis is a relatively easy and precise tool for identification of parasites in which specimens are not preserved appropriately for precise morphologic examinations. Additional specimens and analysis of other genes, that is, mitochondrial DNA, are needed to determine the degree of variation that exists within the species.

The life cycle of *F. magna* has been reviewed previously (Pybus, 2001). The hepatic changes in this case were typical of migrating immature flukes (Foreyt, 1996; Pybus, 2001), and the blood-filled cavities within the parenchyma likely represented the early stages of the parasitic cysts typical of chronic infections. Experimental infections of elk calves by Foreyt (1996) indicate the prepatent period in this host is 6–7 mo. The small size of the flukes and age of the host suggest that the former might be immature. However, a few eggs consistent with *F. magna* were found in liver sections. Given that no *F. hepatica* were found, we conclude that these small *F. magna* were in early stages of egg production (i.e., sexually maturing), and this is supported by the observation of an egg in one of the collected flukes. Immature *F. magna* may migrate for months to years (Pybus, 2001) with egg production usually occurring within the capsules that surround adult forms. The parasitic hematin pigment seen in various tissues is a form of iron porphyrin regurgitated by the migrating parasites. The damage to retroperitoneal tissues and release of CK from damaged myocytes are attributed to the migration of metacercariae/immature flukes as they leave the intestinal tract and seek the liver. Myositis, muscular degeneration and necrosis, fibrosis, and granuloma formation were all documented in the musculature of the flank and diaphragm of white-tailed deer experimentally infected *F. magna* (Presidente et al., 1980).

The herd moved off this winter pasture shortly after this elk calf was euthanized, and it did not return to the area for several months. Biologists reported that a total of 27 mortalities had occurred during the winter period, but no further necropsies were performed. The severity of hepatic and muscular damage in this calf could have produced the clinical signs observed, but the contribution of trematodiasis to such a high herd mortality rate remains uncertain. Significant hepatic pathology due to *F. magna* infection does occur (Bassi, 1875; Pybus, 1990; Foreyt, 1996;

Hood et al., 1997); yet, clinical disease is generally thought to be uncommon, because elk are a definitive host for this parasite (Pybus, 2001). With such a high mortality rate, the involvement of some other disease process seems likely, and additional investigation is required should the herd suffer further losses.

This work was partially funded by the Oregon Department of Fish and Wildlife. Our thanks to field biologists H. Biederbeck and T. Laws. We gratefully acknowledge Drs. E. Hoberg and J. Mertins for parasite identifications, and J. Becker for photography. Thanks also to B. Barr for assistance in DNA sequencing of *F. magna*.

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Received for publication 11 October 2006.