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NOVEL-RESULT

Helminth infection-induced carcinogenesis: spectrometric insights from the liver flukes, *Opisthorchis* and *Fasciola*

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Abstract

Earlier reports revealed oxysterol metabolites of *Opisthorchis* spp. liver fluke origin conjugated with DNA bases, suggesting that the generation of these DNA-adducts may underlie the mutagenicity and carcinogenicity of the infection with these food-borne pathogens. Here, we employed liquid chromatography-mass spectrometry to investigate, compare and contrast spectrograms of soluble extracts from *Fasciola hepatica* adult worms from bile ducts of cattle with those from *O. viverrini* and *O. felineus* from experimentally infected hamsters. *F. hepatica* and *Opisthorchis* spp. shared common compounds including oxysterol-like metabolites, bile acids and DNA-adducts, but the spectrometric profiles of *F. hepatica* included far fewer compounds than *Opisthorchis* species. These findings support the postulate that parasitic oxysterol-like metabolites could be related to carcinogenesis associated to infection and they point to a molecular basis for the differences among major groups of liver flukes concerning infection-induced malignancy.

Keywords: *Fasciola hepatica*; *Opisthorchis viverrini*; Opisthorchis felineus; oxysterols; DNA adducts

Introduction

More than 20% of cancer in the developing world are caused by infections (Brindley et al., 2015). The World Health Organization's International Agency for Research on Cancer (IARC) recognizes the infection with about 12 pathogens as group 1 biological carcinogens, *i.e.*, definitive causes of cancer. These group 1 agents include three helminth parasites, specifically the fish-borne trematodes (FBT) *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke, *Schistosoma haematobium* (IARC, 2012). In addition, we have previously reported findings from hamster infection that support the inclusion of *Opisthorchis felineus*, also an FBT, to this list of biological carcinogens and definitive cause

of cholangiocarcinoma (Gouveia et al., 2017). We hypothesized that these helminths produce and release derivatives of oestrogens and oxysterols that promote oxidation of host DNA and have the ability of parasite metabolites to directly promote DNA lesions adducts and mutations that ultimately lead to cholangiocarcinoma (Brindley et al., 2015; Costa et al., 2014; Gouveia et al., 2015; 2017; Vale et al., 2013). The findings supported the postulate that these infection-associated cancers originate from a biological and/ or chemical insult followed by chronic inflammation, fibrosis, and a change in the tissue micro-environment that leads to a pre-cancerous niche (Brücher & Jamall, 2014; Cavalieri et al., 2017). Paradoxically, infections with other close phylogenetic relatives of these carcinogenic helminths, also food borne trematodes of the Phylum Platyhelminthes, have not been categorized as group 1 biological carcinogens (Chapman et al., 1999; Montero et al., 1999; Kawanishi et al., 2006; Kolodziejczyk et al., 2006; Machicado et al., 2016; Mayer & Fried, 2007; Tsocheva-Gaytandzhieva, 2005).

For instance, *Fasciola hepatica* has a wide geographical range, causes major economic loss in sheep and cattle worldwide, and also is an important zoonosis in humans (Villegas et al., 2012). It has been shown that fascioliasis can induce host DNA damage through action of reactive nitric species (RNS) or oxygen species (ROS) (Mayer & Fried, 2007; Tsocheva et al., 1992); however, the infection has not been associated to carcinogenesis. Seeking new insights in this apparent paradox among closely related food-borne trematodes, here we conducted an analysis of soluble extracts of adult worms of *F. hepatica, O. viverrini and O. felineus* by liquid chromatography coupled with mass spectrometry (LC–MS/MS). Remarkably, the LC–MS/MS chromatograms for each of the liver fluke species exhibited clear differences in regard the presence of oxysterols. These metabolites were minor components of the extract from *F. hepatica*, in contrast to the relatively high abundance and diversity of oxysterols in *O. viverrini and O. felineus.* The presence of abundant oxysterols in the of *Opisthorchis* liver flukes would support the hypothesis that these molecules may act as initiators during the liver fluke infection-induced biliary tract malignancy.

Material

Ethics Statement

Procedures undertaken complied with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Syrian hamsters (*Mesocricetus auratus*) were purchased from the stock of the Puschino Animal Facility (Russia) and bred at the Animal Facility of the ICG SB RAS (RFMEFI61914X0005) (Russia). The hamsters were maintained according to protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics (Permit Number: 25 of 12.12.2014).

Soluble extracts from F. hepatica, O. viverrini *and* O. felineus *adult liver flukes*

Adult worms of *F. hepatica* were obtained from the bile ducts of infected cattle at a local slaughterhouse (Silva et al., 2004). It should be noted that the animals were processed as part of normal work of the slaughterhouse. *O. viverrini* and *O. felineus* were obtained as previously described (Gouveia et al., 2017; Vale et al., 2013). In brief, metacercariae of *Opisthorchis* species were obtained from naturally infected cyprinoid fish in Khon Kaen province, Thailand or from naturally infected fish (*Leuciscus idus*) in the Ob River near the city of Novosibirsk, Siberia Russia, respectively. The fish were digested with pepsin-HCl (Gouveia et al., 2017). Fifty metacercariae were used to infect hamsters (*Mesocricetus auratus*) and three months after infection, the animals were euthanized and adult *O. viverrini* or *O. felineus* flukes recovered from their bile ducts. The worms were washed extensively in phosphate buffered saline (PBS, pH 7.4) supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin G and cultured overnight in serum free RPMI-1640 medium (Lonza, Basel, Switzerland) containing 1% glucose, and protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 2 µM E-64 and 10 µM leupeptin) (Sigma-Aldrich, St. Louis, Missouri) at 37 °C, 5% CO_2.

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Soluble extracts from all samples were prepared by sonication (5 x 5 s burst, output cycle 4, Branson Sonifier 450, Germany) in PBS supplemented with protease inhibitors [500 µM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.3 µM aprotinin, 10 µM E-64, 10 µM bestatin and 10 µM leupeptin] (M221, Amresco, Solon, OH, USA), followed by 30 min centrifugation at 10,000 rpm, 4 °C. The protein concentration of supernatants was determined using a commercial kit. Ascorbic acid was added to 1 mg/ml to these extracts, which were stored in aliquots at −80 °C (Gouveia et al., 2017; Vale et al., 2013).

Sample preparation and LC–MS/MS analysis

Samples were prepared and processed using liquid chromatography diode array detection electron spray ionization mass spectrometry, as previously described (Gouveia et al., 2015; 2017; Vale et al., 2013). Due to the acceptable chromatographic performance of methanol as the solvent in terms of separation and sensitivity, with short gradient times (Wang et al., 2000), this solvent was added up to 20% (v/v). High performance liquid chromatography coupled with mass spectrometer was employed to investigate molecular species from liver flukes, with samples of 25 µl injected into the LC–MS/MS instrument for analysis. The mass analysis was performed within an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photo diode array (PDA) detector. Analysis involved a Macherey-Nagel Nucleosil C18-column (250 mm x 4 mm internal diameter; 5 µm particle diameter, end-capped), proceeding at a flow rate of 0.3 ml/min. The capillary voltage of the electrospray ionization was 28 kW, capillary temperature was 310 °C, flow rates of the sheath gas and auxiliary N2 were set to 40 and 10 (arbitrary unit as provided by the software settings), respectively, and gas temperature was 275 °C (Gouveia et al., 2015; 2017; Vale et al., 2013). The mobile phase consisted of 1% formic acid in water (A)/acetonitrile (B) mixtures. Eluates were monitored for 75 min, run with a mobile phase gradient of 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A, 10–15 min 80% A, 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% to 100% B. Washing for 15 min with acetonitrile was carried out to stabilize the column. Data were collected in negative electrospray ionization negative mode scanning a mass to charge ratio (m/z) range of 50–2,000.

Results

Both species of Opisthorchis *shared identical mass spectra profiles*

We have developed a sensitive LC–MS/MS-based protocol to identify new steroids-derived molecules not only in extracts of helminth parasites (Gouveia et al., 2017; Vale et al., 2013), but also from experimental infected rodents (Gouveia et al., 2017) and naturally-infected humans (Gouveia et al., 2015). Extracts obtained from *F. hepatica* adult worms were analyzed in order to provide insights related to their composition and complexity.

Comparing data obtained for *O. viverrini* with *O. felineus* we observed that both these liver flukes displayed highly similar mass spectra (MS) and shared most peaks detected (indicated in grey in Fig 1) which were attributed to oxysterol-like metabolites, *e.g.* mass/charge (m/z) 356, 307, bile acids in oxidized form, *e.g.* m/z 443, 479, 488 and DNA-adducts, *e.g.* m/z 599, 639, 667 (Gouveia et al., 2017; Vale et al., 2013).

F. hepatica *extracts exhibited striking differences to those of the* Opisthorchis *species*

Notable differences were apparent among the MS profiles of *F. hepatica* and the *Opisthorchis* species. Most of compounds present in both *Opisthorchis* species were absent from *F. hepatica*, specifically m/z 356, 357, 425 and 307. Remarkably, these specific compounds were attributed to be oxysterols with ability to react with host DNA as described (Gouveia et al., 2017). The MS profile of *F. hepatica* was much more

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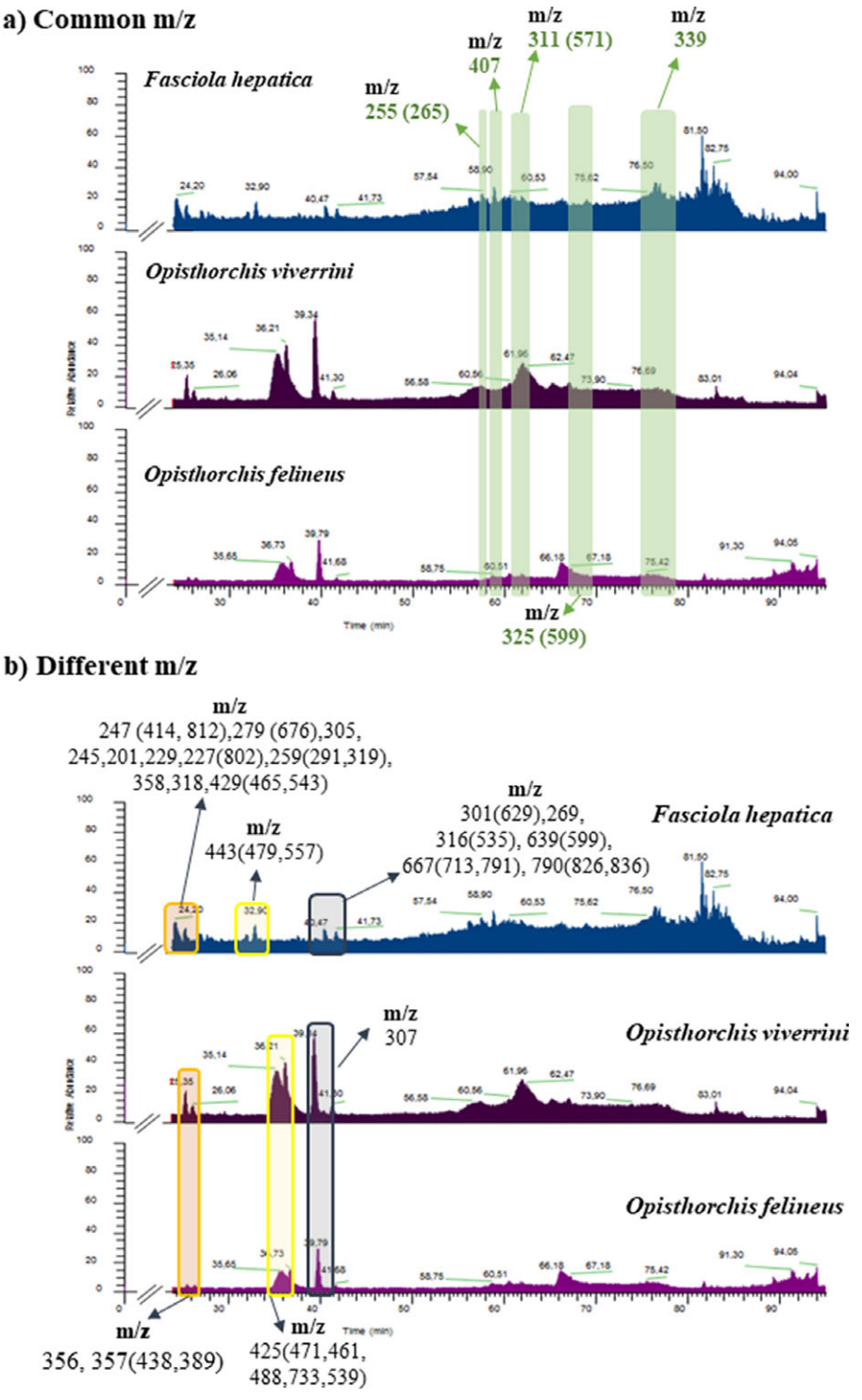

Fig. 1 Comparison of mass spectral profiles obtained for *Fasciola hepatica* and *Opisthorchis* spp. Panel A, common m/z between the three liver flukes; panel B, major differences among the liver flukes.

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complex than those obtained for *Opisthorchis* spp. (Fig. 1). The major differences were observed at retention intervals of approximately 24, 32, and 40 min – as indicated in orange, yellow and blue, respectively, on the chromatographs (Fig. 1). On these retention times, *F. hepatica* showed greater number of compounds in comparison to those observed on *Opisthorchis* species (Fig. 1 and Table 1). Remarkably, most of these compounds were detected only in *F. hepatica* extracts (Table 1).

Unlike *Opisthorchis*, *F. hepatica* displayed a higher number of compounds with elevated m/z (between 600 and 800), mostly between retention interval of 38 to 42 min (Table 1), which might suggest that they are more complex than the majority of those detected on *Opisthorchis* spp.

Table 1. Comparison of mass/charge (m/z) obtained for *Fasciola hepatica* during this study with *Opisthorchis* spp. previously reported for *O. viverrini* (Gouveia et al., 2017; Vale et al., 2013) and *O. felineus* (Gouveia et al., 2017). The structures of common m/z (signed at green) are depicted on Table 2.

Retention time (min)	m/z	*Fh*	*Ov*	*Of*
17.64	337.08	✓	✓	
23.24	320.17	✓		
23.25	353.13	✓		
23.95	293.12	✓		
24.17	245.12	✓		
	414.22	✓		
24.26	279.14	✓		
	676.30	✓		
24.36	305.08	✓		
24.41	245.12	✓		
24.75	229.16	✓		
	201.13	✓		
25.08	227.14	✓		
25.13	259.13	✓		
	291.10	✓		
	319.10	✓		
25.41	358.20	✓		
25.54	318.11	✓		
28.12	429.23	✓		
	465.20	✓		
	543.22	✓		
32.89	443.24	✓		
37.65	301.07	✓		
38.18	629.30	✓		
39.50	316.17	✓		
	535.23	✓		
40.50	677.50	✓		
	713.48	✓		
	724.51	✓		

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Table 1. *Continued*

Retention time (min)	m/z	*Fh*	*Ov*	*Of*
41.70	790.58	✓		
	826.56	✓		
	837.59	✓		
51.02	447.14	✓		
54.86	321.18	✓		
58.70	255.23	✓	✓	✓
58.92	407.28	✓	✓	✓
59.93	571.29	✓	✓	✓
61.06	311.17	✓	✓	✓
64.25	325.19	✓	✓	✓
64.15	599.32	✓	✓	✓
70.16	367.25	✓		
77.95	339.20	✓	✓	✓
76.38	391.29	✓		
81.63	465.31	✓		

Table 2. Structures of m/z common to *Fasciola hepatica* and *Opisthorchis* species.

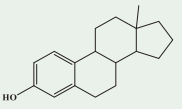
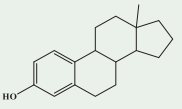
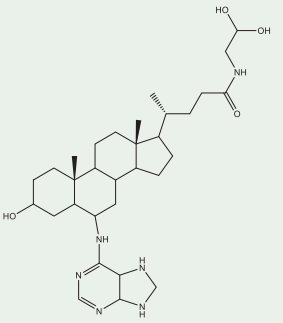
Retention time (min)	m/z	*Fh*	*Ov*	*Of*	Structures
57.54	255.07	✓	✓	✓	
58.92	407.28	✓	✓	✓	
59.93	571.29	✓	✓	✓	

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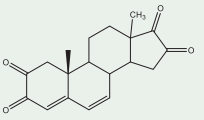
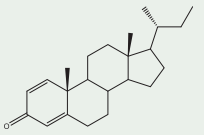
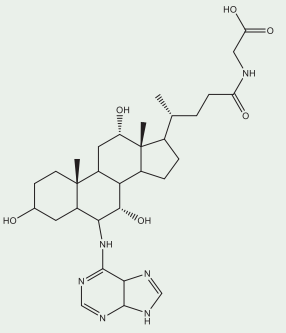
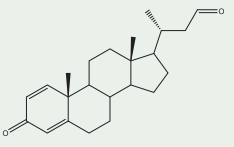
Table 2. *Continued*

Retention time (min)	m/z	*Fh*	*Ov*	*Of*	Structures
61.06	311.17	✓	✓	✓	
64.25	325.19	✓	✓	✓	
64.15	599.32	✓	✓	✓	
77.95	339.20	✓	✓	✓	

Nonetheless, *F. hepatica* and *Opisthorchis* spp. shared several common compounds at retention interval of 58–64 min (signed by green in Fig. 1 and Table 1). These compounds have been ascribed previously to oxysterol-like metabolite (*e.g.* m/z 325), bile acids (*e.g.* m/z 571) and as well as DNA-adducts (m/z 599) (Vale et al., 2013) which is expected since parasites reside on bile ducts. Remarkably, however, oxysterol-like metabolites, bile acids and DNA-adducts in particular were fewer in *F. hepatica* compared to *Opisthorchis* spp. The fact, *F. hepatica* has lesser oxysterol-like metabolites might be one of the reasons that explain why a carcinogenic potential associated with its infection has not been established.

Discussion

Both chronic infection with *Fasciola* spp. and *Opisthorchis* spp. could lead to fibrosis, hyperplasia, and biliary stasis (Gouveia et al., 2017; Machicado et al., 2016; Maksimova et al., 2017; Motorna et al., 2001; Sithithaworn et al., 2012). However, an association between fascioliasis and cancer remains controversial and not definitely established (Machicado et al., 2016). Thus, we decided to investigate extracts of adult

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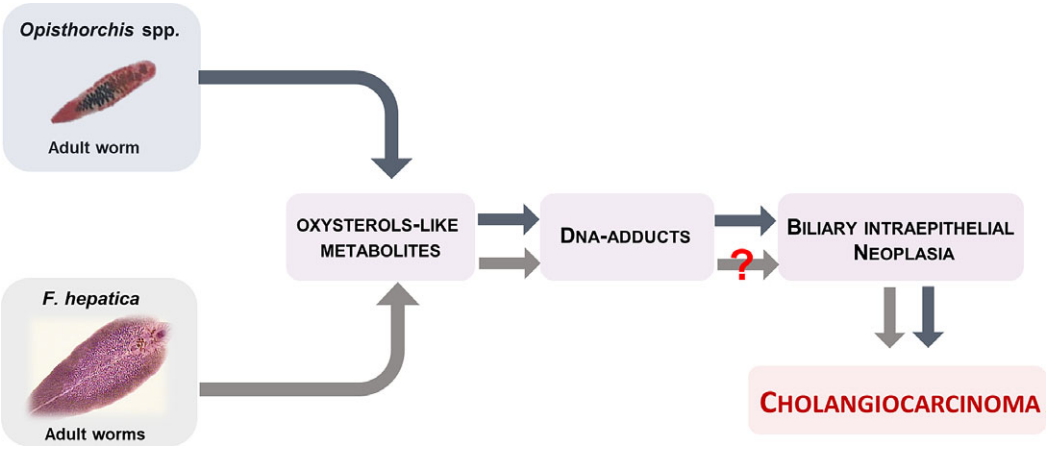

Fig. 2 Adult liver flukes *O. viverrini* and *O. felineus* produces oxysterol-like metabolites that interact with host chromosomal DNA to form DNA-adducts and forms of biliary intraepithelial neoplasia that conducive to cholangiocarcinoma. *F. hepatica* also elaborates oxysterol-like metabolites, but at much lower number, which might be explain, at least in part, why infection with this parasite fails to induce malignancy.

worms of *F. hepatica* and compare with data previously obtained for *Opisthorchis* spp. We aimed to address the following questions: 1) does *F. hepatica* synthesize and excrete metabolites that might promote direct damage on host DNA? The MS profile of *F. hepatica* was found to be far more complex, showing an elevated number of compounds more complex that translate in an elevated m/z rather than *Opisthorchis* spp. This suggested that metabolic process that occur in *F. hepatica* are dissimilar to those in *Opisthorchis* spp. and also could be related to its complex route of migration to the biliary tract might underlie these differences.

Compounds of *F. hepatica* might be related to the different migratory route of the parasite to the biliary tree. Unlike *Opisthorchis* spp., newly excysted juveniles of *F. hepatica* exit the lumen of the small intestine, transverse the intestinal wall and migrate through the abdominal cavity to the Glisson's capsule of the liver (Mas-Coma, 2005; Moazeni & Ahmadi, 2016). This parasite might deploy more complex biochemical processes and secretions, including the secretion of cathepsins (Cancela et al., 2010; Cwiklinski et al., 2015; Young et al., 2010) to accomplish this elaborate organ and tissue migration. The juvenile *F. hepatica* infects the liver by directly penetrating the Glisson's capsule from the abdominal cavity, and thereafter burrows through the hepatic parenchyma to the bile ducts where it eventually matures into the egg-laying adult worm (Moazeni & Ahmadi, 2016). Components detected in the extracts of *F. hepatica* might be related with digestion of host tissues including blood such as hemoglobin, albumin and immunoglobin to support reproductive process including synthesis of eggs (Moazeni & Ahmadi, 2016). On other hand, most of the compounds observed from 23 to 57 minutes were specific of *F. hepatica*, *i.e.* not present in *Opisthorchis.* Juvenile *Opisthorchis* flukes ascend from the duodenum directly into the lumen of biliary tree (Sithithaworn et al., 2012; Pakharukova et al., 2016).

Glycocholic acid in the mammalian small intestine triggers the excystment of the metacercaria and emergence of *F. hepatica* juvenile flukes stimulating the exit of the parasite from the gut lumen and its migration to the abdominal cavity. Intriguingly, the juvenile *F. hepatica* did not survive in bile-containing solutions whereas the adult fluke resides in the bile ducts, bathed in bile (Tielens et al., 1981). Differences in the nature of the juvenile *versus* adult tegument of *F. hepatica* and the selectivity and the permeability of glycocalyx of the tegument may underpin these stage specific differences (Tielens et al., 1981). The complexity of the tegument, a complex metabolically active and highly glycosylated biological matrix (Ravidá et al., 2016) might also underpin complexity of *F. hepatica* MS profile and its components.

Some of these compounds might be precursors of known compounds that were previously attributed to oxysterol-like metabolites, bile acids and DNA-adducts (Gouveia et al., 2017; Vale et al., 2013). This is

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feasible since all three flukes live within the biliary tree. There is evidence that *F. hepatica* induces DNA damage through the action of mutational mediators (Jedina et al., 2011; Kolodziejczyk et al., 2006). The presence of DNA adducts in tissue does not necessarily imply a specific tumorigenic risk for the host tissue. Other factors such as DNA repair and cell proliferation key roles players in determining the overall carcinogenic risk (Povey, 2000). An association between fascioliasis and cancer has only been suggested from *in vitro* studies and, thus far, there have not been satisfactory reports of human cases of bile duct cancer due to chronic infection with *F. hepatica* (Chun et al., 2012; Gentile et al., 1998; Hanahan & Weinberg, 2011; Machicado et al., 2016; Tsocheva-Gaytandzhieva, 2005). Therefore, there is a lack of cogent evidence that relate fascioliasis with cancer (Machicado et al., 2016). By contrast, a number of reports posit opposing effects, *i.e.* tumor growth stimulation and inhibition. Tumor growth stimulation and proliferation of hepatocytes has been observed during acute phase of infection where larval flukes migrate through the parenchyma of the liver and provoke marked inflammation (Montero et al., 1999; Tsocheva et al., 1992). In turn, the chronic inflammation increases oxidative stress that can overwhelm antioxidant system homeostasis to dampen reactive oxygen species and consequent oxidative modification of lipids, nucleic acids and proteins (Kolodziejczyk et al., 2006). Like fascioliasis, opisthorchiasis is characterized by elevated oxidative stress and altered the antioxidant systems (Kawanishi et al., 2006; Kolodziejczyk et al., 2006). We also documented that infection with *O. felineus* induces biliary intraepithelial neoplasia (BilIN). The consonance of findings that the presence of new metabolites and of BilIN-1 and BilIN-2 indicates that *O. felineus* infection induces neoplastic transformation of cholangiocytes and can be expected to promote growth of biliary cancers (Gouveia et al., 2017). Tumor inhibition has been noted during the chronic phase of fascioliasis that may dampen the liver metabolizing activity (Montero et al., 1999). Whereas acute *F. hepatica* infection may increase the metabolizing enzymes in liver and thus increase the activation of exogenous carcinogens (Motorno et al., 2001), chronic infection may reduce hepatic metabolizing activity (Montero et al., 1999). It is noteworthy that chronic infection with *F. hepatica* in a rat model suppressed *N*-nitrosodimethyldiamine-induced carcinogenesis, suggesting a parasite-induced inhibition of carcinogenesis in the liver of rodents experimentally infected with *F. hepatica* (Tsocheva et al., 1992). Could this ability be one of the reasons why the infection with *F. hepatica* does not present carcinogenic potential? Is *F. hepatica* able to neutralize the reactivity of oxysterol-like molecules or other carcinogenic with host DNA? All these questions require further investigation. This study aimed to characterize the differences between *Fasciola* and *Opisthorchis* with a view to identifying the parasite-derived compound that results in the different pathogenic outcomes after infection with these two parasites. Not only is this important to understand mechanisms underlying the pathology, with a view to perhaps developing appropriate therapeutics in the future, but it would also provide information to understand how two flukes have adapted to induce such different outcomes in their hosts. Future studies will evaluate if there is a host-related effect on the production of the metabolites identified in this study.

In conclusion, we discovered that *Fasciola hepatica* displayed more complex mass spectra profile that the *Opisthorchis* species and several specific compounds that might be related to its complex route of migration to the biliary tract. Nonetheless, *F. hepatica* shared few compounds with *Opisthorchis* species, which are related to oxysterols, bile acids and DNA-adducts. The presence of only a few common compounds might explain why fascioliasis has not been causally linked with liver cancer. Indeed, we posit that presence of fewer oxysterol-like metabolites might (partially) explain why definitive carcinogenic potential has not been ascribed to ruminant or human fascioliasis (Fig. 2). More study can be expected to enhance our understanding of the differences in carcinogenicity between these two genera of food borne liver flukes.

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the results. M.J.G. and N.V. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of Interest. On behalf of all authors, the corresponding author states that there is no conflict of interest.

Data availability. The authors confirm that the data supporting the findings of this study are available within the article.

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Peer Reviews

Reviewing editor: Prof. Martin Michaelis
University of Kent, School of Biosciences, Canterbury, United Kingdom of Great Britain and Northern Ireland, CT2 7NJ

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and met required revisions.

doi:10.1017/exp.2020.38.pr1

Review 1: Helminth infection-induced carcinogenesis: spectrometric insights from the liver flukes, Opisthorchis and Fasciola

Reviewer: Dr. Sirikachorn Tangkawatana

Date of review: 30 June 2020

Conflict of interest statement. Reviewer declares none.

Comments to the Author: This works is designed to compare the spectrometric profiles of soluble extracts from F. hepatica, O. viverrini and O. felineus. The authors found 3 common compounds: oxysterol-like metabolites, bile acids and DNA-adducts.

They believe the first compound related to carcinogenesis. The findings are interesting and could explain the different/ common pathogenesis caused by these flukes. However some points should be revised or clarified.

- Fig. 2 : Because the carcinogenesis is multistage-multistep process, other important factors should be added in the diagram.

- Does the host spp. affect the results? F. hepatica adult worms are from natural host but O. viverrini and O. felineus worms are from animal model.

Score Card

Presentation

4.4 /5

Is the article written in clear and proper English? (30%)	4/5
Is the data presented in the most useful manner? (40%)	5/5
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doi:10.1017/exp.2020.38.pr2

Review 2: Helminth infection-induced carcinogenesis: spectrometric insights from the liver flukes, Opisthorchis and Fasciola

Reviewer: Dr. Lydia Leonardo

Date of review: 17 July 2020

Conflict of interest statement. Reviewer declares none.

Comments to the Author: Please discuss the basis for suspected association between fascioliasis and carcinogenesis. Include the initial similarity in life processes and metabolism shown by Fasciola spp and Opisthorchis spp before they diverge with the Opisthorchis becoming carcinogenic and the Fasciola otherwise. Take note of the comments cited in the manuscript.

Score Card

Presentation

4.0 /5

Is the article written in clear and proper English? (30%)	4/5
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Analysis

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Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5