

Research Paper

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# Equines as reservoirs of human fascioliasis: transmission capacity, epidemiology and pathogenicity in *Fasciola hepatica*-infected mules

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## Abstract

Fascioliasis is a zoonotic disease caused by liver flukes transmitted by freshwater lymnaeid snails. Donkey and horse reservoir roles have been highlighted in human endemic areas. Liver fluke infection in mules has received very limited research. Their role in disease transmission, epidemiological importance and *Fasciola hepatica* pathogenicity are studied for the first time. Prevalence was 39.5% in 81 mules from Aconcagua, and 24.4% in 127 from Uspallata, in high-altitude areas of Mendoza province, Argentina. A mean amount of 101,242 eggs/mule/day is estimated. Lymnaeids from Uspallata proved to belong to ribosomal DNA internal transcribed spacer (ITS) markers ITS-1 and ITS-2 combined haplotype 3C of *Galba truncatula*. These lymnaeids were experimentally susceptible to infection by egg miracidia from mules. Infectivity, number of cercariae/snail and shedding period fit the enhanced *F. hepatica*/*G. truncatula* transmission pattern at very high altitude. This indicates that the mule is able to maintain the *F. hepatica* cycle independently. Individual burdens of 20 and 97 flukes were found. Mule infection susceptibility is intermediate between donkey and horse, although closer to the latter. Anatomic-pathology and histopathology indicate that massive infection may cause mule death. Haematological value decreases of red blood cells, haemoglobin, leucocytes and lymphocytes indicate anaemia and strong immunosuppression. Strongly increased biochemical marker values indicate liver function alterations. The mule probably played a role in the past exchanges with Chile and Bolivia through Mendoza province. Evidence suggests that mules could contribute to the spread of both *F. hepatica* and *G. truncatula* to human fascioliasis-endemic areas in these countries.

## Introduction

The knowledge on fascioliasis has pronouncedly changed in recent years (Mas-Coma *et al.*, 2014a).

From the veterinary point of view, epidemiological research focuses on the influences of climate change and global change, similarly as with other trematodiasis (Boissier *et al.*, 2015). Indeed, fascioliasis is a vector-borne disease of rural areas caused by the liver fluke species *Fasciola hepatica* of almost worldwide distribution and *Fasciola gigantica* restricted to parts of Africa and Asia. Their vectors are amphibious freshwater lymnaeid snails, which are markedly influenced by changing climatic factors (Mas-Coma *et al.*, 2009a; Afshan *et al.*, 2014). Among lymnaeid vector species, *Galba truncatula* is the most important transmitter of the disease to humans almost in all endemic areas where *F. hepatica* is the causal agent, as in Europe and throughout all human hyperendemic areas in Andean countries (Mas-Coma *et al.*, 2001, 2009b; Bargues *et al.*, 2007a, 2012, 2020; Artigas *et al.*, 2011). In areas where this lymnaeid species is not present, human infection sustained by other species of the *Galba*/*Fossaria* group shows relatively low transmission levels (Zumaquero-Rios *et al.*, 2013; Bargues *et al.*, 2016, 2017).

Regarding global change, aspects related to liver fluke spread include anthropogenic modifications of the environment, such as irrigation systems (Esteban *et al.*, 2002; Afshan *et al.*, 2014) and animal transport inside countries, as, for example, transhumance (Mas-Coma

*et al.*, 2009b; Chougar *et al.*, 2020), and livestock import/export between countries (Villavicencio *et al.*, 2019) or even different continents (Mas-Coma *et al.*, 2009b).

From the public health point of view, human fascioliasis was considered a pathogenic disease mainly in the first short acute phase of 3–4 months. Consequently, it was considered of secondary public health importance until 1990 (Chen & Mott, 1990). Complex clinical pictures with very severe neurological, meningeal, psychiatric and ophthalmological disorders (Mas-Coma *et al.*, 2014b) may be caused at distance by numerous migrating juveniles during the acute phase but also by a few adult flukes in the liver during the chronic phase (Gonzalez-Miguel *et al.*, 2019). Such situations may leave handicapped subjects behind (Mas-Coma *et al.*, 2014b). Patients not completely recovered have been reported many years after having been appropriately treated, despite their infections initially considered to be not problematic (Rondelaud *et al.*, 2006).

The marked diversity of human infection sources adds to the complexity and public health importance of this zoonotic disease, which is characterized by a low specificity at animal host level. In domestic animal reservoirs, similarly as in lymnaeids, anthropophily stands out as a crucial factor because it assures a transmission closer to the human environment and, therefore, increases human infection probabilities (Mas-Coma *et al.*, 2018).

Among the domestic animal species involved as reservoirs in the human endemic areas, species differ slightly from one area to another and mainly from one continent to another. In the Americas, cattle and sheep are the most usual, followed by pigs. In Africa and Asia, besides goats, the buffalo is an additional reservoir, mainly in areas where *F. gigantica* is present. There are other herbivorous mammal species that have been reported as secondary reservoirs at local level. Equines have been given attention only sporadically, despite their closeness to humans and their crucial role in human development in the past and still in rural areas worldwide in the present. However, the risk of climate and global changes to fascioliasis of equines has been recently highlighted (Williams & Hodgkinson, 2017).

Donkeys appear to be the equines having received more focus. Indeed, recent palaeoparasitological studies have demonstrated that old donkeys, known as onagers, were already infected by *F. hepatica* during the Sassanid period, 224–651 AD, in the former Fertile Crescent area, in present Iran (Askari *et al.*, 2018). At present, donkeys appear to be usually infected by fasciolids in countries of the Fertile Crescent–Ancient Egypt region, or neighbouring that region, such as in Iran, Iraq, Turkey, Egypt and Ethiopia, with prevalence from low to very high (see review in Askari *et al.*, 2018). However, they have been reported to show liver fluke infection in many other countries, by *F. hepatica* in countries such as Morocco (Everaert *et al.*, 1974; Pandey, 1983), Mexico (Collins, 1961), Bolivia (Mas-Coma *et al.*, 1997; Valero *et al.*, 2001a) and Argentina (Mera y Sierra *et al.*, 2009), as well as by *F. gigantica* in Chad (Guilhon & Graber, 1963; Graber, 1969). The reservoir role of donkeys in human endemic areas has been highlighted in Bolivia (Mas-Coma *et al.*, 1997; Valero *et al.*, 2001a) and Egypt (Haridy *et al.*, 2002). The high pathogenicity and mortality induced by *Fasciola* in donkeys is an important factor to be considered (Askari *et al.*, 2018).

Horses are known to be infected by *Fasciola* since two centuries ago (Galli-Valerio, 1893). A review of the countries in which natural infection of horses has been reported was made in 1988 in a report from South Africa (Alves *et al.*, 1988). Additional findings in horses include countries as different as Italy (Galli-Valerio,

1893), Ireland (Quigley *et al.*, 2017), the UK (Howell *et al.*, 2020), Egypt (Haridy *et al.*, 2002), Chile (Alcaino & Aguilar, 1985; Alcaino *et al.*, 1988, 1990, 1992), Uruguay (Sanchis Polto *et al.*, 2014; Bargues *et al.*, 2017), Argentina (Mera y Sierra *et al.*, 2009) and Australia (Bucknell *et al.*, 1995). Infected racehorses have also been the focus of research, as in Europe (Krawiecki, 1986) and Chile (Alcaino *et al.*, 1983, 2005; Montes *et al.*, 1984; Muñoz *et al.*, 2008). Also in Chile, studies highlighted the infection of horses in human fascioliasis-endemic areas (Apt *et al.*, 1993). Compared to donkeys, horses appear to be more resistant to liver fluke infection (Alves *et al.*, 1988; Soulé *et al.*, 1989; Dorchies, 2010). Even ponies have been reported to be experimentally susceptible to liver fluke infection (Grelck *et al.*, 1977) and also infected in their natural environment (Owen, 1977).

Infection of mules by the liver fluke has, however, been the focus of only very limited research. *Fasciola* infection was diagnosed by serology in five mules in Turkey (Acici *et al.*, 2013) and by coprology in another five mules in Catamarca, Argentina (Malandrini *et al.*, 2009). Their role in the transmission of the disease, epidemiological importance as reservoirs regarding human infection and pathogenicity caused by *F. hepatica* in them, are still aspects in need of investigation. The present study analyses these three aspects in mules for the first time.

This study was performed in high-altitude areas of the Andean region in Mendoza province, Argentina. Fascioliasis is endemic in this country, posing an important public health problem much more widespread than traditionally supposed, particularly in mountainous regions (Malandrini *et al.*, 2002; Mera y Sierra *et al.*, 2011, Bargues *et al.*, 2016), and an animal fascioliasis impact in most of its territories (Olaechea, 2007; Mera y Sierra *et al.*, 2009, Cuervo *et al.*, 2013, 2015). These mules are currently used in diverse farming activities and mainly for the transport of people and goods from their stations, following different itineraries close to the Chile border, according to the weather conditions of the different seasons.

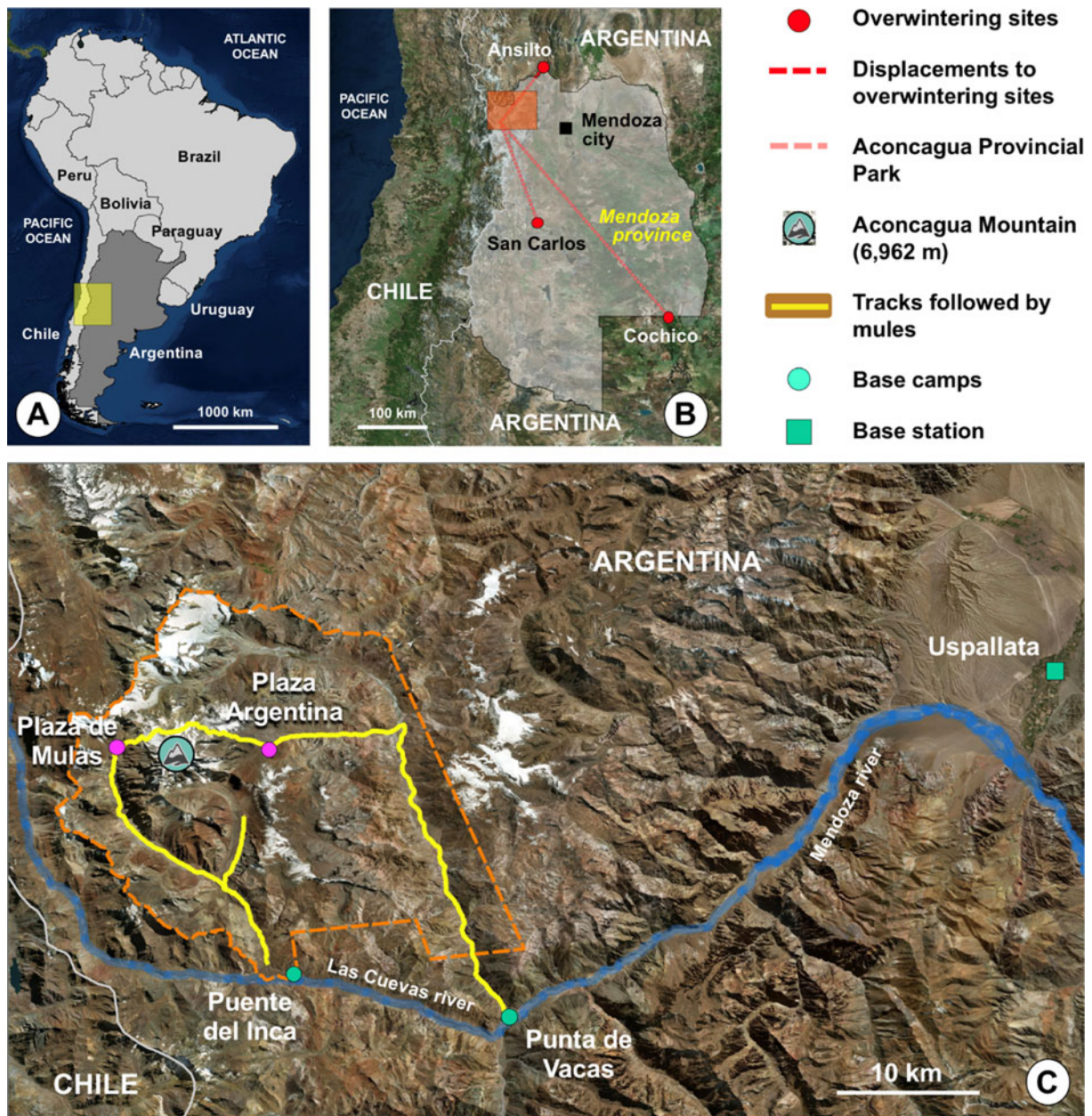
## Materials and methods

### Mule groups analysed

Mules studied were sampled in Mendoza province. These mules are moved to different Andean localities of Midwestern Argentina throughout the year, depending on the work that has to be done and the weather conditions in relation to the different seasons. Two groups of mules were studied.

A group of mules was stationed at Puente del Inca at 2790 m above sea level (asl) during the summer period (December to March), where they transport goods and persons through the tracks of the Aconcagua Provincial Park up to Plaza de Mulas at 4379 m asl (fig. 1). The site called Punta de Vacas is used as the base for these expeditions. Mules continuously arrive to and leave from Puente del Inca during the period between December and the beginning of March, in which all is closed due to the start of the great snowfalls. After activities in the Aconcagua Provincial Park, this group overwinters in Ansilta, located in the province of San Juan, or in San Carlos (Mendoza province). The second group of mules is permanently stationed in Uspallata (fig. 1).

In addition to the aforementioned usual paths, these mules sometimes follow another less transited route to Plaza Argentina, which involves a more dangerous climb to the Aconcagua peak through the Glaciar de los Polacos.



**Fig. 1.** Maps showing the geographical area where the working mules are used. (A) Argentina and neighbouring countries in South America showing the area of working mules (yellow rectangle); (B) the Argentinian province of Mendoza showing mule displacements to overwintering sites; (C) Aconcagua Provincial Park in the Mendoza province close to the Chile border, showing tracks followed by the mules, the base camps of Puente del Inca and Punta de Vacas, and the base station of Uspallata.

Interestingly, several of these mules are sometimes sent to the locality of Cochico, at only 468 m asl, in the province of La Pampa. It should be highlighted that Cochico is very far away from the fascioliasis-endemic areas of Mendoza.

Even though both male and female mules are used, females are preferred due to their greater docility, and included 75% of the animals. The breeds of donkeys and mares used in their production varied; thus, they are not genetically uniform and the donkeys and mares used are frequently crossbreeds. The army uses male Catalan donkeys and the mares are either of Argentine Creole breed or Thoroughbred crossbreeds. The mules analysed included 4–19-year-old specimens, with a mean value of  $11 \pm 3.8$  years old.

### *Mule coprology and fasciolid egg morphometry*

Faecal samples from 208 mules were obtained at the beginning of the mule routes in Puente del Inca and taken directly from the rectum. A total of 81 mules studied were from Aconcagua and another 127 mules were from Uspallata. The samples were stored at 4°C in plastic bags until they arrived at the laboratory. They were then analysed using the Lumbreras sedimentation technique (Lumbreras *et al.*, 1962), by initially weighing 10 g of the sample to quantify the eggs per gram (epg). The Wisconsin sugar flotation technique was performed to investigate and quantify the presence of other parasites (Cox & Todd, 1962) in 36 mules that were positive for *F. hepatica* in the coprological analysis.

Larvae culture (Roberts & O'Sullivan, 1950) was performed to obtain third-stage larvae in animals with higher strongyle egg output.

To estimate the amount of fasciolid eggs that each mule could eliminate, the amount of faeces produced by an adult equine (USDA-NRCS, 2000) was multiplied by the mean of egg produced.

*Fasciola hepatica* eggs were obtained from mule faeces. A total of 179 eggs were measured, of which 110 eggs were from mules of Aconcagua and 69 eggs were from mules of Uspallata. Egg measurements were carried out under a calibrated microscope and obtained according to standardized methods (Valero *et al.*, 2005), including egg length (EL), egg width (EW), egg shape (EL/EW ratio) and egg size (EL multiplied by EW). For each measure, minimum and maximum values, mean and standard deviation were determined.

For statistical analyses, the proportion of positive animals from both sites was compared by means of the Fisher's test. The comparison of egg measurement data was done by means of the Mann-Whitney *U*-test, and considered significant when  $P < 0.05$ .

### DNA marker sequencing of lymnaeid snails

#### Markers used

Lymnaeids used for the experimental infection to assess transmission capacity of mule fasciolids were from a snail population in Uspallata. They were molecularly characterized to allow for their systematic classification. DNA sequencing of the complete nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) markers ITS-2 and ITS-1 was thus performed. The usefulness of these markers has been already proved for the classification of lymnaeid vector species and the comparative assessment of their intraspecific variability in many countries of Latin America (Bargues *et al.*, 2011a, 2012), including Argentina (Bargues *et al.*, 2006, 2007a, b, 2016; Mera y Sierra *et al.*, 2009) and neighbouring countries such as Bolivia (Bargues *et al.*, 2020), Chile (Artigas *et al.*, 2011) and Uruguay (Bargues *et al.*, 2017). It should be considered that, in the Americas, *F. hepatica* is transmitted by lymnaeid species belonging to the *Galba/Fossaria* group, which are undifferentiable morphologically (Bargues *et al.*, 2007b, 2011a, b).

#### DNA extraction

The head-foot tissue of each snail, preserved in 70% ethanol at room temperature after collection, was used for DNA extraction with phenol-chloroform and DNA was precipitated with ethanol. Ten specimens randomly selected from the Uspallata population were suspended in 400  $\mu$ l of lysis buffer (10 mM Tris Hydrochloride, pH 8.0, 100 mM ethylenediamine tetra-acetic acid (EDTA), 100 mM sodium chloride, 1% sodium dodecyl sulphate) containing 500  $\mu$ g/ml Proteinase K (Promega, Madison, Wisconsin, USA) and digested for 2 h at 55°C with alternate shaking each 15 min. The procedure steps were performed according to methods outlined previously (Bargues *et al.*, 1997, 2007b). The pellet was dried and resuspended in 30  $\mu$ l sterile TE buffer (pH 8.0) and this suspension was finally stored at -20°C until use.

#### Sequencing of rDNA markers

Each one of the two markers were polymerase chain reaction (PCR) amplified independently for each specimen and each PCR product was sequenced for a *bona fide* haplotype characterization. The complete rDNA spacers ITS-2 and ITS-1 were

amplified using primers previously described (Bargues *et al.*, 2003, 2007b). Amplification procedures and thermal cyclers conditions for each one of the DNA markers were carried out in a Mastercycle ep Gradient (Eppendorf, Hamburg, Germany), as previously described (Bargues *et al.*, 2007b).

PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, California, USA) according to the manufacturer's protocol, and resuspended in 50  $\mu$ l of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm on an Eppendorf BioPhotometer (Hamburg, Germany).

The sequencing of each molecular marker was performed on both strands by the dideoxy chain-termination method. It was carried out with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) using PCR primers.

#### Sequence analyses

Sequences were edited and assembled using Sequencher v5.4.6. (Gene Codes Co., Ann Arbor, Michigan, USA) and aligned using CLUSTALW2 (Larkin *et al.*, 2007) in MEGA 6.0.6 (Tamura *et al.*, 2013), using default settings. Minor corrections for a better fit of nucleotide or indel correspondences were made. Homologies were performed using the BLASTN programme from the National Center for Biotechnology information website (<http://www.ncbi.nlm.nih.gov/BLAST>). Comparative sequence analyses and haplotype identification of lymnaeids were made using available ribosomal sequence data downloaded from GenBank.

#### DNA haplotype nomenclature

The haplotype (H) terminology used for the sequences obtained follows the standard nomenclature proposed for lymnaeid snails previously described (Bargues & Mas-Coma, 2005; Mas-Coma *et al.*, 2009b). It shall be noted that haplotype codes are only definitive in the case of complete sequences. When dealing with fragments or incomplete sequences, haplotype codes are provisional.

#### Experimental infection of lymnaeids

Fasciolid eggs were washed in water and preserved in darkness at 4–6°C until used. For embryonation, they were kept in darkness in Petri dishes at 24°C for 14 days. Afterwards, they were exposed to light to stimulate hatching of the miracidia, which was observed under the stereomicroscope.

Specimens of a snail population from Uspallata (32° 35'25.25"S, 69°21'04.95"W; 1881 m asl) and systematically classified by the aforementioned molecular methods were used. The lymnaeids were maintained in Petri dishes in climatic chambers with a temperature of 16–18°C, according to previous experiments having demonstrated that higher temperatures were associated with a high mortality. A 12 h/12 h light/darkness photoperiod was applied (Mas-Coma *et al.*, 2001). They were fed *ad libitum* with fresh lettuce and commercial fish flakes (Schulet S.A., Provincia de Buenos Aires, Argentina) following indications of previous studies (Rondelaud *et al.*, 2002; Belfaiza *et al.*, 2004). The water was changed weekly, always using water that was brought from the original location.

Snails that reached at least 4 mm in length were put in individual Petri dishes (one snail per dish) with natural freshwater from the original site. The miracidia were collected by a micropipette. Snails were placed individually in Petri dishes at 22°C with natural

**Table 1.** Measurements of fasciolid eggs found in mule faeces.

Egg measurements	Total studied (63 infected mules) 179 eggs measured	Aconcagua (32 infected mules) 110 eggs measured	Uspallata (31 infected mules) 69 eggs measured	Site comparison ( <i>P</i> -value)
Length (in $\mu\text{m}$ )	136.0 $\pm$ 10.5 (82.6–155.0)	134.0 $\pm$ 11.3 (82.6–152.2)	139.4 $\pm$ 8.1 (122.5–155.0)	0.0023
Width (in $\mu\text{m}$ )	78.2 $\pm$ 6.7 (49.5–106.0)	77.8 $\pm$ 7.8 (49.5–106.0)	78.8 $\pm$ 4.3 (67.5–90.0)	0.0712
Size (in $\mu\text{m}^2$ )	10665.2 $\pm$ 1393.0 (4082.6–16134.6)	10465.2 $\pm$ 1597.8 (4082.6–16134.6)	10984.0 $\pm$ 905.2 (9243.8–12825.0)	0.0036
Shape	1.8 $\pm$ 0.2 (1.3–2.2)	1.7 $\pm$ 0.2 (1.3–2.2)	1.8 $\pm$ 0.1 (1.4–2.2)	0.1759

Mean  $\pm$  standard deviation, with range of extreme values in parenthesis; *P*-value from Mann–Whitney *U*-test.

water and exposed to one miracidium. Meanwhile, they were observed under a stereomicroscope until confirmation that the miracidium had penetrated the snail. The snails were then returned to the climatic chamber. From day 30 onwards, the snails were observed under the stereomicroscope for the presence of metacercariae in the Petri dishes and quantification of their metacercarial production.

### Haematological parameters and biochemical markers

From 13 mules that were positive for *F. hepatica* in the coprological analysis, blood samples were collected from the jugular vein. Due to the lack of adequate installations in the remote regions where the samples were taken, difficulties were encountered in the handling of some of the animals. Thus, collection of blood samples or the necessary blood volume was not always possible. The sample for haemogram (tube with EDTA) was prioritized and biochemical parameters were determined according to the amount of sample (tube without anticoagulant) available. Haemograms were performed in 13 mules and different biochemical parameters determined in 12 mules. The whole blood sample was stored at 4°C right after collection for subsequent haematological analysis. The non-anticoagulant tube sample was allowed to clot (for serum) and then the sample was centrifuged at 1000g for 5 min to separate the blood components. The serum samples were then stored in 1.5 ml microcentrifuge tubes at 4°C until they were analysed at the laboratory.

The following haematological parameters were determined in an Abacus Junior Vet® (Diatron, Budapest, Hungary) automated haematology analyser: red blood cell count (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), platelets (Plt) and total leucocyte count (WBC). A blood smear was fixed with methanol and stained with Giemsa to perform manual relative and differential cell counts to obtain absolute counts for neutrophils, eosinophils, lymphocytes, monocytes and basophils.

Biochemical markers were determined using an InCCA® (Diconex S.A., Buenos Aires, Argentina) auto analyser. The following markers were obtained according to their value for fascioliasis diagnosis (Valero *et al.*, 2016):

- (1) liver damage: aspartate aminotransferase (AST), gamma glutamyl transferase (or  $\gamma$ -glutamyl transpeptidase (GGT)), total bilirubin, alkaline phosphatase (ALP);
- (2) protein metabolism: creatinine, urea;
- (3) plasma proteins: albumin, globulin (Glb), albumin/globulin ratio (A/G), total protein (T-Pro);
- (4) hepatic lipid metabolism: cholesterol; and
- (5) muscular enzymes: creatine phosphokinase (CPK).

For the interpretation of haematological parameters and biochemical markers, there are very few published reports furnishing data useful as reference values for mules. Data available are from reports in which values were noted only when different from in horses (Greene *et al.*, 2006; McLean & Wang, 2013; McLean *et al.*, 2016). When no mule reference values were available, the results were compared to the reference values of horses.

### Post-mortem liver examination and histopathology

Additionally, in two mules that died of natural cause, the necropsy was performed. Their livers were extracted, sent refrigerated to the laboratory, inspected and dissected for the classification of macroscopic lesions and the presence of flukes (the entire organ was dissected in 1-cm slices). Samples of liver tissue were taken, fixed in 10% formalin and anatomo-pathologically studied by means of histological sections stained with haematoxylin–eosin.

## Results

### Prevalence, intensities and egg measurements

Of the 208 mules analysed, 63 (30.3%) were positive for *F. hepatica* eggs. The egg quantification showed a mean value of 4.5 egg  $\pm$  8.0, with a range of 0.1–35. Of the 81 mules from Aconcagua, 32 (39.5%) were positive for *F. hepatica*, with a mean egg value of 3.6  $\pm$  2.5, and a range of 0.5–8. Of the 127 mules from Uspallata, 31 (24.4%) were positive for *F. hepatica*, with a mean egg value of 4.7  $\pm$  8.7, and a range of <1–35.

The difference of prevalence in both sites proved to be statistically significant (*P* = 0.0298) (Fisher's test). However, the difference between the egg of the two sites was not statistically significant (*P* = 0.5839) (Student's *t*-test).

An adult equine produces approximately 22.7 kg of faeces per day. The resulting estimate is that, on average, a mule may be releasing 2270–794,500 (mean 101,242) fasciolid eggs to the environment on a daily basis.

The presence of other parasites could be assessed in 36 faecal samples of *F. hepatica*-positive mules. A total of 32 (86.5%) were positive for strongyle eggs, with a mean value of 30.5 egg  $\pm$  37.9 and a range of 1–120. Due to the low egg counts, successful larvae culture could be performed in only two animals, in which larvae of *Strongylus vulgaris* and cyathostomins (small strongyles) were identified. No eggs of paramphistomids were found.

A total of 179 eggs from 63 infected mules of the two sites were measured (table 1). There were significant differences between both sites in egg length and size, but not in width and shape.

**Table 2.** Cercarial shedding by *Galba truncatula* specimens from Uspallata experimentally infected with one miracidium from *Fasciola hepatica* eggs collected in mule faeces.

No. of infected lymnaeid	Cercarial shedding days post infection (dpi)			Total
	30–50 dpi	50–70 dpi	70–90 dpi	
1	4	0	0	4
2	20	0	0	20
3	4	0	0	4
4	3	0	0	3
5	0	128	0	128
6	0	93	144	237
7	0	22	5	27
8	0	0	11	11
Total	31	243	160	434

### Molecular classification and experimental infection of lymnaeid snails

The ITS-1 sequences from the ten lymnaeid specimens from the Uspallata population analysed presented the same length of 504 base pairs (bp) and a scarcely biased guanine-cytosine (GC) content of 57.5%. The nucleotide sequence was in all cases the same. An initial BLASTN search showed marked sequence similitude with *G. truncatula*. Consequently, the following ITS-1 sequences present in GenBank were used for comparisons: *G. truncatula* haplotype A (accession number AJ243018), haplotype B (AJ296270) and haplotype C (AJ272052). In the comparison alignment, the ITS-1 sequence of the Uspallata specimens proved to be identical with that of *G. truncatula* haplotype C from Bolivia and Chile.

The ITS-2 sequences from the same lymnaeid specimens presented a length of 401 bp and a scarcely biased GC content of 58.6%. A BLASTN analysis indicated that the sequences belonged to the species *G. truncatula*. Consequently, the following ITS-2 sequences present in GenBank were used for comparisons: *G. truncatula* haplotype 1 (AJ296271), haplotype 2 (AJ243017) and haplotype 3 (AJ272051). No one nucleotide difference appeared between the Uspallata sequence and that of haplotype 3 from the Northern Bolivian Altiplano and Chile when analysing the corresponding alignment.

Of 22 monomiracidially infected snails, eight (36.4%) became infected and shed cercariae. The total cercarial shedding capacity per snail ranged from four to 237 cercariae/snail, with a mean of 54.3 cercariae/snail and a standard deviation of 84.8. Detailed results are presented in table 2.

### Haematological parameters and biochemical markers in liver-fluke-infected mules

The values of the haematological parameters determined in 13 *Fasciola*-infected mules are presented in table 3. Altered values regarding reference values were shown by PCV, RBC, haemoglobin, leukocytes and lymphocytes in a high percentage of the mules. No parameter proved to be modified in every mule studied.

The values of the biochemical markers determined in the serum of 12 *Fasciola*-infected mules are presented in table 4. When compared to the reference ranges, pronouncedly increased values were found in mainly GGT and AST in all the mules analysed, secondarily ALP and CPK in half of them, and also creatinine in several specimens.

### Liver examination and histopathology

The livers of two infected mules were analysed after autopsy. A total of 20 and 97 fasciolid flukes were found. The mule infected with the larger amount of 97 flukes showed an evident out-of-condition and emaciated aspect (fig. 2). In both livers, the flukes obtained showed the typical *F. hepatica* form with cephalic cone, pronounced shoulders and non-parallel lateral body walls.

The liver of the mule infected by the lower burden of 20 flukes showed no lesions or pathological changes upon macroscopic exam of the Glisson capsule or the underlying parenchyma. Resistance was perceived upon the parenchyma incisions. There was mild thickening of the bile ducts, as well as an increased bile viscosity. Thickening of the bile ducts and increased bile viscosity were observed.

The liver of the mule infected by 96 flukes in the biliary ducts showed blunt lobe borders, Glisson capsule with moderate opacity, parenchyma with multifocal subcapsular haemorrhages and firm and stringy organ consistency. A protuberance was found in the right lobe. At the dissection of this protuberance, an opaque brownish fluid was observed, and an additional immature fluke of *F. hepatica* immersed in the fluid was found. There was mild thickening of the bile ducts, as well as an increased bile viscosity. When performing the incisions of the parenchyma, a resistance was perceived, probably due to fibrosis.

Within the anatomic-pathological study of the liver carrying 20 adult flukes, the examination of the histological sections led to a diagnosis of mild lymphocytic hepatitis. The hepatic parenchyma showed modifications of the trabecular pattern with polyhedral hepatocytes of irregular distribution. Several of these hepatocytes showed granular cytoplasm, thickening of the nuclear membrane and increased size indicating reactive hepatocytes. The portal triads presented a scarce lymphocytic inflammatory infiltrate and oedema of the epithelium of the biliary duct. Several sections appeared covered by a thick fibro-collagenous capsule. Arterial vessels of medium size showed marked dilatation and vascular congestion.

The anatomic-pathological study of the liver with 97 flukes led to a diagnosis of chronic hepatitis, with areas of acute inflammation and pronounced interstitial fibrosis. The interlobular connective tissue was markedly thickened and forming thick tracts that delimited smaller-sized lobules in several sectors. Hepatocytes showed an acidophilic cytoplasm and intracellular cholestasis was frequent. Portal triads presented a slight lymphocytic inflammatory infiltrate, marked fibrosis and hyperplasia of the bile ducts, some of which were dilated and with bile emboli. The sinusoids were permeable and occupied by a moderate amount of erythrocytes. Thickening of the space of Disse was frequent. There were sectors of hyperplastic fibrous tissue, with a marked mixed inflammatory infiltrate consisting of numerous neutrophils with a scarce amount of lymphocytes and eosinophils with areas that penetrated adjacent plates. There were haemorrhagic areas with irregular and poorly defined limits with aggregation of neutrophils in which

**Table 3.** Haematological parameters in 13 mules infected with *Fasciola hepatica*.

Haematological parameters (units)	RR	Infected mules studied mean $\pm$ SD (range)	infected mules < reference intervals	Infected mules > reference intervals
<b>PCV (%)</b>	32–43 <sup>a</sup>	39.1 $\pm$ 8.6 (30–56)	3 (23.1%)	5 (38.5%)
<b>RBC (<math>10^6 \times \text{mm}^3</math>)</b>	7.6–10.2 <sup>a</sup>	7.4 $\pm$ 1.1 (6.3–10.2)	8 (61.5%)	1 (7.7%)
VGM (fl)	43.4–52.2 <sup>a</sup> 46.4–50.0	52.2 $\pm$ 5.5 (45–62)	0	6 (46.2%)
<b>Haemoglobin (gdl)</b>	11.1–13.1 <sup>a</sup>	13.2 $\pm$ 2.2 (10.3–17.8)	7 (53.9%)	2 (15.4%)
Platelets (no.)	100.000–600.000 <sup>b</sup>	187.2 $\pm$ 116.2 (24–314)	2 (15.4%)	0
<b>Leucocytes (<math>10^3 \times \text{mm}^3</math>)</b>	7.6–10.2 <sup>a</sup> 5.0–6.7 <sup>c</sup>	6.7 $\pm$ 1.8 (3.9–9.7)	10 (76.9%)	0
Segmented neutrophils ( $10^3 \times \text{mm}^3$ )	3.0–11.5 <sup>b</sup>	4.7 $\pm$ 1.6 (1.9–7.3)	2 (15.4%)	1 (7.7%)
Band neutrophils ( $10^3 \times \text{mm}^3$ )	0–0.1 <sup>a</sup>	0.09 $\pm$ 0.1 (0–0.3)	0	4 (30.8%)
<b>Lymphocytes (<math>10^3 \times \text{mm}^3</math>)</b>	2.4–3.4 <sup>c</sup>	1.2 $\pm$ 0.5 (0.5–1.8)	7 (53.9%)	0
Monocytes ( $10^3 \times \text{mm}^3$ )	0.16–0.42 <sup>c</sup>	0.2 $\pm$ 0.2 (0–0.6)	0	1 (7.7%)
Eosinophils ( $10^3 \times \text{mm}^3$ )	0–0.8 <sup>b</sup>	0.4 $\pm$ 0.6 (0–2.4)	0	1 (7.7%)
Basophils ( $10^3 \times \text{mm}^3$ )	0–0.2 <sup>b</sup>	0.006 $\pm$ 0.021 (0–74)	0	0

RR, reference ranges; SD, standard deviation; VGM, mean globular volume.

<sup>a</sup>RR according to McLean *et al.* (2016).

<sup>b</sup>RR according to reference ranges for horses. Parameters out of the reference range in at least half of the studied mules are highlighted in bold.

<sup>c</sup>RR according to McLean & Wang (2013).

**Table 4.** Biochemical markers in mules infected with *Fasciola hepatica*.

Biochemical marker (units)	RR	Number of mules studied	Infected mules studied mean $\pm$ SD (range)	infected mules < reference intervals	Infected mules > reference intervals
<b>AST (IU/L)</b>	152–204 <sup>a</sup>	12	433.7 $\pm$ 212.8 (10–748)	1 (8.3%) <sup>a</sup>	12 (91.7%) <sup>a</sup>
<b>GGT (IU/L)</b>	15–29.5 <sup>a</sup>	12	40.1 $\pm$ 18.5 (27–95)	0 <sup>a</sup>	13 (100%) <sup>a</sup>
Total bilirubin (mg/dl)	0.2–1.3 <sup>b</sup>	12	0.6 $\pm$ 0.4 (0.1–1.4)	0	1 (8.3%)
Direct bilirubin (mg/dl)	0.1–0.8 <sup>b</sup>	9	0.3 $\pm$ 0.2 (0.1–0.8)	0	0
Indirect bilirubin (mg/dl)	0.1–0.7 <sup>b</sup>	9	0.4 $\pm$ 0.2 (0.2–0.9)	0	1 (8.3%)
<b>ALP (IU/L)</b>	<290 <sup>b</sup>	12	351.3 $\pm$ 116.2 (188–571)	0	7 (53.8%)
<b>Creatinine (mg/dl)</b>	0.9–1.2 <sup>a</sup>	12	1.2 $\pm$ 0.3 (0.7–1.7)	2 (16.7%) <sup>a</sup>	4 (33.3%) <sup>a</sup>
Urea (mg/dl)	15–50 <sup>b</sup>	12	34.8 $\pm$ 11.6 (12–48)	0	0
Albumin (g/dl)	2.5–3.8 <sup>b</sup>	12	3.0 $\pm$ 0.3 (2.5–3.2)	1 (8.3%)	0
Globulin (g/dl)	2.3–4.1 <sup>b</sup>	12	3.5 $\pm$ 0.4 (2.9–4.1)	0	0
A/G ratio (-)	0.5–1.5	12	0.9 $\pm$ 0.2 (0.6–1.1)	0	0
Total proteins (g/dl)	5–7.9 <sup>b</sup>	12	6.5 $\pm$ 0.4 (5.9–7.1)	0	0
Cholesterol (mg/dl)	75–150 <sup>b</sup>	11	94.4 $\pm$ 24.4 (59–151)	3 (27.8%)	0
<b>CPK (IU/L)</b>	184–285 <sup>a</sup>	7	458.7 $\pm$ 177.4 (239–707)	0	6 (85.7%)

RR, reference ranges; SD, standard deviation; AST, aspartate aminotransferase; IU/L, international units per litre.

<sup>a</sup>RR according to McLean *et al.* (2016).

<sup>b</sup>RR according to reference ranges for horses. Markers out of the reference range in at least half of the studied mules are highlighted in bold.

karyorrhexis was frequent. There was presence of pseudocysts lined partially by a fine fibrinous leucocitary exudate with a lymphoplasmacytic inflammatory infiltrate and subadjacent fibrosis. The wall was thick and formed by fibrocollagenous tissue, with some bile ducts and wide brown pigment-laden macrophage aggregates.

## Discussion

### Liver fluke infection in mules

This is the first fascioliasis study in which the mule is the target host. The mule shows a liver fluke infection susceptibility intermediate between donkey and horse, although closer to the latter.



**Fig. 2.** Out of condition and pronouncedly emaciated mule, which proved to be massively infected by 98 fasciolid adult flukes in the post-mortem liver dissection.

Anatomo-pathological differences between the mules with low and high burden were reflected macro- and microscopically. The low-burden animal showed no macroscopic abnormalities and only mild histological liver alterations, similar to reports in horses (Nansen *et al.*, 1975). The high-burden animal presented severe chronic and acute lesions. Reinfection may occur and, thus, the mules suffer seasonal exacerbations of the disease. A decrease in red blood cells and haemoglobin indicates anaemia – an effect well known in other liver-fluke-infected livestock species (Valero *et al.*, 2008, 2017) and also long since highlighted in horses (Krajl *et al.*, 1960).

The decrease in leucocyte number but mainly the pronounced lymphocyte decrease indicates a strong immunosuppression. This *Fasciola* infection consequence has been emphasized in other animals in the chronic phase (Girones *et al.*, 2007) and reinfections (Valero *et al.*, 2017). Indeed, reinfection probabilities are likely to be high in mules repeatedly using the same routes, base camps and stations.

The strongly increased values of GGT, AST and ALP indicate pronounced liver function alterations, as reported in other animal species (Valero *et al.*, 2016), including horses (Montes *et al.*, 1984; Soule *et al.*, 1989; Howell *et al.*, 2020). Furthermore, an increase in creatinine suggests alterations in protein metabolism, and the increase of the CPK enzyme may underlie potential effects as endocrine disorders, heart damage and skeletal muscle disease.

Such disorders, together with the anaemia and other typical chronicity effects such as lithiasis and bacteriobilia (Valero *et al.*, 2003, 2006), may be of severe consequences for working mules daily brought under strong efforts for weight transport in rugged mountain climbing routes and very high-altitude hypoxia. High-altitude hypoxia conditions were noted as the potential origin of a reduced egg production by the flukes (Valero *et al.*, 2001b) when considering that oxygen is required for egg production in *F. hepatica* (McGonigle & Dalton, 1995).

Haematological, biochemical and pathological findings indicate that *F. hepatica* may severely affect mule health. Particularly striking was the death of the high-burden mule (fig. 2). Evidence suggests reinfection, which could lead to a cumulative fluke effect, causing its death. Moreover, *F. hepatica*-infected animals are at a higher risk of suffering the effects of other insults to the liver, which would ultimately impact on their performance and welfare (Quigley *et al.*, 2017).

Altered biochemical markers may be useful for *Fasciola* infection diagnosis. An indirect enzyme-linked immunosorbent assay (ELISA) test may also be used for the diagnosis in mules, similarly as in other hosts (Mas-Coma *et al.*, 2014a), and also donkeys (Haridy *et al.*, 2007; Awad *et al.*, 2009; Acici *et al.*, 2013; Quigley *et al.*, 2017).

Ultimately, the easiest method of diagnosis is the consideration that fasciolids produce eggs in this host. It should be taken into account that fasciolids present different egg sizes, depending on the host species, even inside the same endemic area (Valero *et al.*, 2005; Mas-Coma *et al.*, 2014a). Thus, in the Bolivian Altiplano, where only *F. hepatica* is present, fasciolid eggs shed by donkeys proved to be shorter and wider than eggs shed by sheep and cattle – donkey: 96.4–140.8/63.3–84.7  $\mu\text{m}$  (mean: 125.4  $\pm$  8.3/75.0  $\pm$  3.7  $\mu\text{m}$ ); sheep: 114.8–151.2/65.5–81.4  $\mu\text{m}$  (mean: 130.8  $\pm$  7.1/72.6  $\pm$  3.9  $\mu\text{m}$ ); cattle: 105.3–155.9/61.7–82.5  $\mu\text{m}$  (mean: 132.0  $\pm$  10.5/71.1  $\pm$  4.4  $\mu\text{m}$ ) (Valero *et al.*, 2001a). The EL/EW of 82.6–155.0/49.5 106.0  $\mu\text{m}$  (mean 136.0/78.2  $\mu\text{m}$ ) found in the Argentinian mules fits well with the aforementioned egg size in donkeys of Bolivia (Valero *et al.*, 2001a) and also the egg measurements of 105–147/72–90  $\mu\text{m}$  (mean: 126/81  $\mu\text{m}$ ) from donkeys in Egypt (Badawy *et al.*, 2014). Interestingly, egg size in mules also fits with the size of fasciolid eggs found in the archaeological remains of Persian onagers, old donkeys which were of a similar body size as mules (Askari *et al.*, 2018). However, limits of egg-finding coproscopy in fascioliasis diagnosis have been emphasized in horses (Soule *et al.*, 1989), in which a copro-antigen ELISA has been seen to be useless (Palmer *et al.*, 2014).

Only very low burdens of strongyles were found, as previously described in horses of the same region (Neira *et al.*, 2019). The extremely cold winter temperatures and very low precipitation do not favour their larval development. Concerning trematodes, *F. hepatica* is the sole species described so far in the province.

For the treatment of fascioliasis in mules, drugs successfully used in horses may be applied. Triclabendazole continues to be the drug of choice because it is the only one efficient against both migrating fluke juveniles and fluke adults in the bile ducts, for both animals and humans (Gandhi *et al.*, 2019). Triclabendazole is widely used for equines nowadays (Dorchies, 2010). Other drugs used in the past were closantel (Alcaino & Aguilar, 1985), oxclozanide (Owen, 1977), rafoxanide (Moisant *et al.*, 1972) and piperazine phloroglucinate (Guilhon & Graber, 1963).

### **Epidemiological role of mules**

The high prevalence rates of 24.4% and 39.5% found in the two mule groups and the estimated amounts of 2270–794,500 eggs/mule/day (mean 101,242 eggs/mule/day) fit well in the ranges reported from the Northern Bolivian Altiplano: 9000–808,000 eggs/day in donkeys; 800–721,800 eggs/day in sheep; 3750–840,000 eggs/day in cattle; and 2000–195,000 eggs/day in pigs (Mas-Coma *et al.*, 1997). In this Bolivian hyperendemic area, donkeys are considered one of the four important reservoir species. Interestingly, horses from the UK do not appear to be 'high' egg excretors (Howell *et al.*, 2020).

The very high transmission capacity of *F. hepatica* through *G. truncatula* in this very high-altitude hyperendemic area of Bolivia allows us to highlight the findings in the mules. Indeed, the routes followed by the working mules are also at very high altitude. It should be considered that the limit between high altitude and



very high altitude is usually put at 3200 m asl. The life cycle of *F. hepatica* with *G. truncatula* as vector species has been proven to be enhanced under the very high-altitude conditions, with a longer cercarial shedding period and a higher metacercarial production per snail individual than in the lowlands (Mas-Coma *et al.*, 2001; Bargues *et al.*, 2017). Additionally, it should be taken into account that *G. truncatula* does not only play this transmitter role under Altiplanic flatland conditions, but also in other high-altitude Andean areas, such as Cajamarca (Bargues *et al.*, 2012), where the highest human prevalence rates in a valley endemiotope have been reported (Gonzalez *et al.*, 2011).

Therefore, four results obtained in the present study deserve special epidemiological importance:

- (1) the field finding of *Galba/Fossaria* lymnaeids in the mule overwintering station of Uspallata;
- (2) the molecular conclusion that the lymnaeids found in Uspallata belong to the species *G. truncatula*;
- (3) the complete DNA sequences of rDNA ITS-1 and ITS-2 proving that the Uspallata *G. truncatula* population belongs to the same rDNA ITS combined haplotype 3C, which ensures the high disease transmission at the very high altitude in the Bolivian Altiplano; and
- (4) the experimental demonstration that miracidia obtained from fasciolid eggs found in mule faecal samples are infective to the Uspallata *G. truncatula* combined haplotype 3C.

The infectivity of miracidia from mule origin, the number of cercariae/snail and the cercarial shedding period of longer than 70 days fit the enhanced transmission pattern in *F. hepatica*/*G. truncatula* at very high altitude (Mas-Coma *et al.*, 2001). This pattern differs from that known in the lowlands (Bargues *et al.*, 2017). These features, together with the prevalence rates and intensities detected, indicate that the mule is able to maintain the *F. hepatica* cycle independently.

Throughout human history, mule size, power and endurance have led to their increased use. The added value of mules includes their usefulness as pack animals in facilitating trade and communication over short and mainly long distances along difficult rugged mountainous routes and arid or semi-arid flatlands, as well as their capacity to replace oxen in ploughing (Mitchell, 2018). Indeed, besides mainly Old World camelids and donkeys, mules participated as pack animals (Mitchell, 2018) and were also included in the tribute paid to the Chinese overlords (Kim, 2011) along the long routes of the Silk Road. This many centuries-long trade event has contributed to the spread of both *F. hepatica* and *F. gigantica* throughout Asia (Mas-Coma *et al.*, 2009b).

The question is posed as to whether mules may also play a role in the transmission of *F. gigantica*. Indeed, there are very important mule populations in countries where this fasciolid is present, such as the People's Republic of China, which has over three million mules (ChartsBin, 2015). However, the pronounced higher physiopathogenicity of *F. gigantica* should be considered (Valero *et al.*, 2016), and indicates that appropriate studies are needed to assess such a potential role.

In the New World, horses and donkeys were introduced by the Spanish 'conquistadores' during the early colonial period around 500 years ago. In South America, like the donkeys that fathered them, mules were raised at lower altitudes because of the lack of suitable pastures in very high-altitude areas and the challenges that an oxygen-deficient environment posed to equine

reproduction. By the 1770s, as many as 500,000 mules and donkeys were estimated to be present in Peru and Bolivia, with two million across Latin America as a whole (Mitchell, 2018). By the early 1800s, donkeys and mules had largely supplanted llamas and alpacas everywhere below 3000 m asl (Cade, 1992). On the one hand, the American camelids could not carry more than 30 kg, whereas a mule could carry three to four times this weight. On the other hand, llamas and alpacas could not be ridden and were harder to drive (Mitchell, 2018). Huge numbers of mules were imported from as far as northern Argentina, totalling up to 60,000 per year, to work in the Bolivian mines in around 1610 (Flynn & Giraldez, 2002).

In Argentina, there are currently 44,000 mules (SENASA, 2016). In its Andean regions, mules have been used since colonial times (Martinez, 1961). The army has traditionally employed them for transport in mountainous regions and still has a mule production centre in Mendoza province (Junin & Queirolo, 2018). Mules are also used by mountain-climbing expeditions to carry equipment and food to base camps.

The Aconcagua Provincial Park and Uspallata are located in Mendoza province. Mendoza city, founded in 1561, traded northward with Alto Peru (what is now Bolivia and southern Peru) and westward with Chile during the colonial period. Until commerce through the port of Buenos Aires was liberated in 1778, goods imported from Spain to Mendoza usually came either by land from Lima, or crossing the Andes from Valparaiso (Encina, 1952). All goods travelling northward had to pass through Cordoba (Giberti, 1954). All commerce from the Virreynato del Río de la Plata, created in 1776, to Chile was done through Mendoza (Lacoste, 2008). All land transport of goods and persons was done with pack animals, mainly mules or ox-driven carts, depending on the type of terrain – mules in the mountains and ox carts almost exclusively in the plains.

Mules were important because of their carrying capacity of up to 150 kg (Lacoste, 2008) and transport capacity of up to 60 km/day (Farro, 2008). Mules were bred in the lowlands in Buenos Aires and Santa Fe provinces, fattened in Cordoba and Salta and sold in Peru (Lacoste, 2008). From Salta, more than 30,000 mules were exported to Peru around 1778 and 1779 (Serrera, 1992). The crossing of the Andes was done exclusively with mules, with up to 25,000 making the journey between Mendoza and Chile, as was also the case for most of the commerce between Argentina and Bolivia.

In summary, Mendoza province was a crucial trade crossroad for more than two centuries, and the mule played an important role as a pack animal in exchanges with neighbouring countries. Thus, mules could contribute to the spread of both *F. hepatica* and *G. truncatula* combined haplotype 3C to areas where human fascioliasis-endemic areas have been described in Chile (Apt *et al.*, 1993; Artigas *et al.*, 2011) and Bolivia (Mas-Coma *et al.*, 1999, 2001; Bargues *et al.*, 2020). All this may also help in historically understanding why Cordoba is by far the province in which more human fascioliasis cases have been reported (Mera y Sierra *et al.*, 2011).

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## References

- Acici M, Bolukbas CS, Gurler AT, Umur S and Buyuktanir O (2013) Seroprevalence of fasciolosis in equines of the Black Sea Region, Turkey. *Journal of Equine Veterinary Science* **33**, 62–66.
- Afshan K, Fortes-Lima CA, Artigas P, Valero MA, Qayyum M and Mas-Coma S (2014) Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. *Geospatial Health* **8**, 317–334.
- Alcaino H and Aguilar A (1985) Actividad antihelmíntica del Closantel y de la combinación febantel + triclorfon en caballos fina sangre de carrera. *Archivos de Medicina Veterinaria* **17**, 103–109.
- Alcaino H, Gorman T, Guevara G and Fernandez JD (1983) Distomatosis y parásitos del intestino grueso de equino de la zona centro sur de Chile. *Archivos de Medicina Veterinaria* **15**, 27–35.
- Alcaino H, Vega F, Klein P, Gorman T and Apt W (1988) Fascioliasis en caballos y conejos silvestres (*Oryctolagus cuniculus*) en la provincia de Curicó, Chile. *Parasitología al Día* **12**, 136–140.
- Alcaino H, Gorman T, Gonzalez V, Vega F and Apt W (1990) Fascioliasis en caballos, cerdos y conejos silvestres en la provincia de Talca, VII Región, Chile. *Parasitología al Día* **14**, 9–13.
- Alcaino H, Apt W, Vega F, Gorman T and Apt P (1992) Fascioliasis animal en la VII Región de Chile: áreas de distribución e infección en caballos y conejos silvestres. *Parasitología al Día* **16**, 11–16.
- Alcaino H, Parra L and Gorman T (2005) Fascioliasis en equinos fina sangre de carrera de los hipódromos de la zona central de Chile 2002-2003. *Parasitología Latinoamericana* **60**, 61–64.
- Alves RM, van Rensburg LJ and van Wyk JA (1988) *Fasciola* in horses in the Republic of South Africa: a single natural case of *Fasciola hepatica* and the failure to infest ten horses either with *F. hepatica* or *Fasciola gigantica*. *Onderstepoort Journal of Veterinary Research* **55**, 157–163.
- Apt W, Aguilera X, Vega F, et al. (1993) Prevalencia de fascioliasis en humanos, caballos, cerdos y conejos silvestres en tres provincias de Chile. *Boletín de la Oficina Sanitaria Panamericana* **115**, 405–414.
- Artigas P, Bargues MD, Mera y Sierra R, Agramunt VH and Mas-Coma S (2011) Characterisation of fascioliasis lymnaeid intermediate hosts from Chile by DNA sequencing, with emphasis on *Lymnaea viator* and *Galba truncatula*. *Acta Tropica* **120**, 245–257.
- Askari Z, Mas-Coma S, Bouwman AS, Boenke N, Stöllner T, Aali A, Rezaian M and Mowlavi G (2018) *Fasciola hepatica* eggs in paleofaeces of the Persian onager *Equus hemionus onager*, a donkey from Chehrabad archaeological site, dating back to the Sassanid Empire (224-651 AD), in ancient Iran. *Infection, Genetics and Evolution* **62**, 233–243.
- Awad WS, Ibrahim AK and Salib FA (2009) Using indirect ELISA to assess different antigens for the serodiagnosis of *Fasciola gigantica* infection in cattle, sheep and donkeys. *Research in Veterinary Science* **86**, 466–471.
- Badawy A, Abouzaid N and Merwad A (2014) Occurrence of zoonotic fascioliasis in donkeys in Egypt with emphasis on PCR-RFLP of 28S rRNA gene. *Revue de Médecine Vétérinaire* **165**, 167–171.
- Bargues MD and Mas-Coma S (2005) Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology* **79**, 257–267.
- Bargues MD, Mangold AJ, Muñoz-Antoli C, Pointier JP and Mas-Coma S (1997) SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *Journal of Parasitology* **83**, 1086–1092.
- Bargues MD, Horak P, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C and Mas-Coma S (2003) Insights into the relationships of Palaearctic and Nearctic lymnaeids (Mollusca: Gastropoda) by rDNA ITS-2 sequencing and phylogeny of stagnostic intermediate host species of *Fasciola hepatica*. *Parasite* **10**, 243–255.
- Bargues MD, Mera y Sierra R, Gomez HG, Artigas P and Mas-Coma S (2006) Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Animal Biodiversity and Conservation* **29**, 191–194.
- Bargues MD, Mera y Sierra R, Gomez HG, Artigas P and Mas-Coma S (2007a) Caracterización molecular de *Galba truncatula*, vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública. *Enfermedades Emergentes, Barcelona* **9**, 77–82.
- Bargues MD, Artigas P, Mera y Sierra RL, Pointier JP and Mas-Coma S (2007b) Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Annals of Tropical Medicine and Parasitology* **101**, 621–641.
- Bargues MD, Gonzalez C, Artigas P and Mas-Coma S (2011a) A new baseline for fascioliasis in Venezuela: lymnaeid vectors ascertained by DNA sequencing and analysis of their relationships with human and animal infection. *Parasites & Vectors* **4**, 200.
- Bargues MD, Artigas P, Khoubbane M, Flores R, Glöer P, Rojas-García R, Ashrafi K, Falkner G and Mas-Coma S (2011b) *Lymnaea schirazensis*, an overlooked snail distorting fascioliasis data: genotype, phenotype, ecology, worldwide spread, susceptibility, applicability. *PLoS ONE* **6**, e24567.
- Bargues MD, Artigas P, Khoubbane M, Ortiz P, Naquira C and Mas-Coma S (2012) Molecular characterisation of *Galba truncatula*, *Lymnaea neotropica* and *L. schirazensis* from Cajamarca, Peru and their potential role in transmission of human and animal fascioliasis. *Parasites & Vectors* **5**, 174.
- Bargues MD, Malandrini JB, Artigas P, Soria CC, Velazquez JN, Carnevale S, Mateo L, Khoubbane M and Mas-Coma S (2016) Human fascioliasis endemic areas in Argentina: multigene characterisation of the lymnaeid vectors and climatic environmental assessment of the transmission pattern. *Parasites & Vectors* **9**, 306.
- Bargues MD, Gayo V, Sanchis J, Artigas P, Khoubbane M, Birriel S and Mas-Coma S (2017) DNA multigene characterization of *Fasciola hepatica* and *Lymnaea neotropica* and its fascioliasis transmission capacity in Uruguay, with historical correlation, human report review and infection risk analysis. *PLoS Neglected Tropical Diseases* **11**, e0005352.
- Bargues MD, Artigas P, Angles R, Osca D, Duran P, Buchon P, Gonzales-Pomar RK, Pinto-Mendieta J and Mas-Coma S (2020)

- Genetic uniformity, geographical spread and anthropogenic habitat modifications of lymnaeid vectors found in a One Health initiative in the highest human fascioliasis hyperendemic of the Bolivian Altiplano. *Parasites & Vectors* **13**, 171.
- Belfaiza M, Abrous M, Rondelaud D, Moncef M and Dreyfuss G** (2004) The use of Tetraphyll as food for snails increases the intensity of cercarial shedding in *Galba truncatula* infected with *Fasciola hepatica*. *Parasitology Research* **94**, 86–90.
- Boissier J, Moné H, Mittra G, Bargues MD, Molyneux D and Mas-Coma S** (2015) Schistosomiasis reaches Europe. *Lancet Infectious Diseases* **15**, 757–758.
- Bucknell DG, Gasser RB and Beveridge I** (1995) The prevalence and epidemiology of gastrointestinal parasites in horses of Victoria, Australia. *International Journal for Parasitology* **25**, 711–724.
- Cade DW** (1992) Landscape, system, and identity in the post-Conquest Andes. *Annals of the Association of American Geographers* **82**, 460–477.
- ChartsBin** (2015) Mules stock by country, ChartsBin.com. Available at <http://chartsbin.com/view/35644>, (accessed 2 August 2020).
- Chen MG and Mott KE** (1990) Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Tropical Diseases Bulletin* **87**, R1–R38.
- Chougar L, Mas-Coma S, Artigas P, Harhoura K, Aissi M, Agramunt VH and Bargues MD** (2020) Genetically “pure” *Fasciola gigantica* discovered in Algeria: DNA multimarker characterization, trans-Saharan introduction from a Sahel origin and spreading risk into northwestern Maghreb countries. *Transboundary and Emerging Diseases*. [published online ahead of print, 2020 Apr 18]. DOI:10.1111/tbed.13572.
- Collins DR** (1961) Fascioliasis in Mexican burro. *Journal of the American Veterinary Medicine Association* **139**, 1321–1323.
- Cox DD and Todd AC** (1962) Survey of gastrointestinal parasitism in Wisconsin dairy cattle. *Journal of the American Veterinary Medical Association* **141**, 706–709.
- Cuervo P, Sidoti L, Fantozzi MC, Neira G, Gerbeno L and Mera y Sierra R** (2013) *Fasciola hepatica* infection and association with gastrointestinal parasites in Creole goats from western Argentina. *Revista Brasileira de Parasitologia Veterinaria* **22**, 53–57.
- Cuervo P, Cataldo SD, Fantozzi MC, et al.** (2015) Liver fluke (*Fasciola hepatica*) naturally infecting introduced European brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk. *Acta Parasitologica* **60**, 536–543.
- Dorchies P** (2010) La fasciolose des équidés, une infestation parasitaire inhabituelle. *Buletin des Groupements Techniques Vétérinaires* **57**, 71–78.
- Encina FA** (1952) *Historia de Chile desde la prehistoria hasta 1.891*. 3rd edn. 694 pp. Santiago de Chile, Editorial Nascimento.
- Esteban JG, Gonzalez C, Bargues MD, Angles R, Sanchez C, Naquira C and Mas-Coma S** (2002) High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Tropical Medicine and International Health* **7**, 339–348.
- Everaert GPJ, Jawhari M and Gaufreteau A** (1974) De la presence de grandes douves *Fasciola gigantica* sur des foies d’asins du Maroc. *Revue de Medecine Veterinaire* **125**, 541–544.
- Farro ME** (2008) Redes y medios de transporte en el desarrollo de expediciones científicas en Argentina (1850-1910). *História, Ciências, Saúde – Manguinhos* **15**, 679–696.
- Flynn DO and Giraldez A** (2002) Cycles of silver: global economic unity through the mid-eighteenth century. *Journal of World History* **13**, 391–427.
- Galli-Valerio B** (1893) Lésions déterminées par la *Fasciola hepatica* chez le cheval. *Archivio per le Scienze Mediche* **9**, 173.
- Gandhi P, Schmitt EK, Chen CW, Samantray S, Venishetty VK and Hughes D** (2019) Triclabendazole in the treatment of human fascioliasis: a review. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **113**, 797–804.
- Giberti H** (1954) *Historia económica de la ganadería Argentina*. 217 pp. Buenos Aires, Editorial Raigal.
- Girones N, Valero MA, Garcia-Bodelon MA, Chico-Calero MI, Punzon C, Fresno M and Mas-Coma S** (2007) Immune suppression in advanced chronic fascioliasis: an experimental study in a rat model. *Journal of Infectious Diseases* **195**, 1504–1512.
- Gonzalez-Miguel J, Valero MA, Reguera-Gomez M, Mas-Bargues C, Bargues MD, Simon-Martin F and Mas-Coma S** (2019) Numerous *Fasciola* plasminogen-binding proteins may underlie blood-brain barrier leakage and explain neurological disorder complexity and heterogeneity in the acute and chronic phases of human fascioliasis. *Parasitology* **146**, 284–298.
- Gonzalez LC, Esteban JG, Bargues MD, Valero MA, Ortiz P, Naquira C and Mas-Coma S** (2011) Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province, Peru. *Acta Tropica* **120**, 119–129.
- Graber M** (1969) Helminthes parasites de certains anirnaux domestiques et sauvages du Tchad. *Bulletin of Epizootic Diseases of Africa* **17**, 403–428.
- Greene HM, Hurson MJ and Wickler SJ** (2006) Haematological and respiratory gas changes in horses and mules exercised at altitude (3800 m). *Equine Veterinary Journal, Equine Exercise Physical Supplement* **36**, 551–556.
- Grelck H, Horchner F and Wohrl H** (1977) Experimental infection of ponies with *Fasciola hepatica*. *Berliner und Muenchner Tieraerztliche Wochenschrift* **90**, 371–373.
- Guilhon J and Graber M** (1963) Action du phloroglucinate de piperazine sur quelques Helminthes des Equides. *Revue d’Elevage et de Medecine Veterinaire des Pays Tropicaux* **16**, 305–308.
- Haridy FM, Morsy TA, Gawish NI, Antonios TN and Abdel Gawad AG** (2002) The potential reservoir role of donkeys and horses in zoonotic fascioliasis in Gharbia Governorate, Egypt. *Journal of the Egyptian Society of Parasitology* **32**, 561–570.
- Haridy FM, Morsy GH, Abdou NE and Morsy TA** (2007) Zoonotic fascioliasis in donkeys: ELISA (Fges) and postmortem examination in the Zoo, Giza, Egypt. *Journal of the Egyptian Society of Parasitology* **37**, 1101–1110.
- Howell AK, Malalana F, Beesley NJ, et al.** (2020) *Fasciola hepatica* in UK horses. *Equine Veterinary Journal* **52**, 194–199.
- Junin B and Queirolo M** (2018) Los valores ocultos de Campo Los Andes. Available at <http://www.unidiversidad.com.ar/los-valores-ocultos-de-campo-los-andes> (accessed 22 February 2018).
- Kim BJ** (2011) Trade and tribute along the Silk Road before the third century A.D. *Journal of Central Eurasian Studies* **2**, 1–24.
- Krajl M, Srebocvan V, Marzan B, Turner V and Wikerhauser T** (1960) Fasciolose aiguë chez les chevaux avec un aperçu particulier sur le diagnostic différentiel de l’anémie infectieuse du cheval. *Veterinarski Arhiv Zagreb* **30**, 192–199.
- Krawiecki JM** (1986) A propos d’un cas clinique de fasciolose hépatique chez un cheval de sport. *Pratique Vétérinaire Equine* **18**, 173–176.
- Lacoste P** (2008) El arriero y el transporte terrestre en el cono sur (Mendoza 1780-1800). *Revista de Indias* **68**, 35–68.
- Larkin M, Blackshields G, Brown N, et al.** (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.
- Lumbreras H, Cantella R and Burga R** (1962) Acerca de un procedimiento de sedimentación rápida para investigar huevos de *Fasciola hepatica* en las heces, su evaluación y uso en el campo. *Revista Médica Peruana* **31**, 167–174.
- Malandrini, JB, Soria CC, Carnevale S, Velazquez J and Pizarro MC** (2009) Estudio de la fascioliasis desde una perspectiva multidisciplinaria. In *Colegio Medico Veterinario de la Provincia de Córdoba, XXVIII Jornadas de Actualización en Ciencias Veterinarias*, Villa Giardino, Córdoba, 25–27 September 2009.
- Malandrini JB, Soria CC, Foresi I and Correa S** (2002) Fasciolosis en Catamarca. Primeros resultados. pp. 287–293 in Universidad Nacional de Catamarca (Ed) *La Investigación Científica en la Facultad de Ciencias de la Salud, en los Umbrales del Siglo XXI*. Catamarca, Editorial Universitaria.
- Martinez PS** (1961) *Historia económica de Mendoza durante el Virreinato 1776-1810*. 478 pp. Madrid, Universidad Nacional de Cuyo – Instituto Gonzalo Fernández de Oviedo.
- Mas-Coma S, Rodriguez A, Bargues MD, Valero MA, Coello JR and Angles R** (1997) Secondary reservoir role of domestic animals other than sheep and cattle in fascioliasis transmission in the Northern Bolivian Altiplano. *Research and Reviews in Parasitology* **57**, 39–46.
- Mas-Coma S, Angles R, Esteban JG, Bargues MD, Buchon P, Franken M and Strauss W** (1999) The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Tropical Medicine and International Health* **4**, 454–467.

- Mas-Coma S, Funatsu IR and Bargues MD (2001) *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology* **123**, S115–S127.
- Mas-Coma S, Valero MA and Bargues MD (2009a) Climate change effects on trematodiasis, with emphasis on zoonotic fascioliasis and schistosomiasis. *Veterinary Parasitology* **163**, 264–280.
- Mas-Coma S, Valero MA and Bargues MD (2009b) *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology* **69**, 41–146.
- Mas-Coma S, Bargues MD and Valero MA (2014a) Diagnosis of human fascioliasis by stool and blood techniques: Update for the present global scenario. *Parasitology* **141**, 1918–1946.
- Mas-Coma S, Agramunt VH and Valero MA (2014b) Neurological and ocular fascioliasis in humans. *Advances in Parasitology* **84**, 27–149.
- Mas-Coma S, Bargues MD and Valero MA (2018) Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. *Parasitology* **145**, 1665–1699.
- McGonigle S and Dalton JP (1995) Isolation of *Fasciola hepatica* haemoglobin. *Parasitology* **111**, 209–215.
- McLean AK and Wang W (2013) Pilot study comparing hematologic and serum biochemical parameters in healthy horses (*Equus caballus*) and mules. *Journal of Equine Veterinary Science* **33**, 352–354.
- McLean AK, Wang W, Navas Gonzalez FJ and Rodriguez JB (2016) Reference intervals for hematological and blood biochemistry reference values in healthy mules and hinnies. *Comparative Clinical Pathology* **25**, 871–878.
- Mera y Sierra RL, Artigas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S and Bargues MD (2009) Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Veterinary Parasitology* **166**, 73–79.
- Mera y Sierra R, Agramunt VH, Cuervo P and Mas-Coma S (2011) Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasites & Vectors* **4**, 104.
- Mitchell P (2018) *The donkey in human history. An archaeological perspective*. 306 pp. Oxford, Oxford University Press.
- Moisant C, Jolivet J and Pitre J (1972) La fasciolose des équidés. Observations en Normandie. Essais de traitement avec le rafoxanide. *Recueil de Médecine Vétérinaire de l'Ecole d'Alfort* **148**, 444–450.
- Montes GA, Zurita L and Uhart R (1984) Medición de aspartato amino transferasa, gamma glutamil transpeptidasa, proteína total y albúmina sérica en fascioliasis equina. *Zentralblatt fuer Veterinaermedizin* **34B**, 338–342.
- Muñoz L, Rubilar L, Zamora D, Sepulveda O, Reh Hof C and Ortiz R (2008) Fasciolosis en equinos fina sangre de carrera del Club Hípico Concepción, Chile. *Parasitología Latinoamericana* **63**, 88–91.
- Nansen P, Andersen S and Hesselholt M (1975) Experimental infection of the horse with *Fasciola hepatica*. *Experimental Parasitology* **37**, 15–19.
- Neira G, Godoy D, Logarzo L and Mera y Sierra R (2019) Helmintos digestivos en caballos de la provincia de Mendoza, Argentina. *Revista Jornadas de Investigación* **11**, 44.
- Olaechea FV (2007) Tremátodos y Céstodos: *Fasciola hepatica*. pp. 159–168 in Suárez VH, Olaechea FV, Rossanigo CE and Romero JR (Eds) *Enfermedades parasitarias de los ovinos y otros ruminantes menores en el cono sur de América*. Buenos Aires, Instituto Nacional de Tecnología Agropecuaria – INTA.
- Owen JM (1977) Liver fluke infection in horses and ponies. *Equine Veterinary Journal* **9**, 29–31.
- Palmer DG, Lyon J, Palmer MA and Forshaw D (2014) Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses. *Australian Veterinary Journal* **92**, 357–361.
- Pandey VS (1983) Observations on *Fasciola hepatica* in donkeys from Morocco. *Annals of Tropical Medicine and Parasitology* **77**, 159–162.
- Quigley A, Sekiya M, Egan S, Wolfe A, Negrodo C and Mulcahy G (2017) Prevalence of liver fluke infection in Irish horses and assessment of a serological test for diagnosis of equine fasciolosis. *Equine Veterinary Journal* **49**, 183–188.
- Roberts FHS and O'Sullivan PJ (1950) Methods for egg counts and larval cultures for strongyles infesting the gastro-intestinal tract of cattle. *Australian Journal of Agricultural Research* **1**, 99–102.
- Rondelaud D, Abrous M and Dreyfuss G (2002) The influence of different food sources on cercarial production in *Lymnaea truncatula* experimentally infected with Digenea. *Veterinary Research* **33**, 95–100.
- Rondelaud D, Dreyfuss G and Vignoles P (2006) Clinical and biological abnormalities in patients after fasciolosis treatment. *Médecine et Maladies Infectieuses* **36**, 466–468.
- Sanchis Polto J, Madeira de Carvalho LM, Bonilla R, Duque de Araujo AM, Arroyo F, Suarez J, Solari MA, Romero JA and Sanchez Andrade R (2014) Horse handling conditions and emergence of Neglected Infections: Fasciolosis. pp. 127–145 in Paz-Silva A, Arias Vázquez M and Sánchez-Andrade Fernandez R (Eds) *Horses: breeding, health disorders and effects on performance and behavior*. New York, Nova Publishers.
- SENASA (2016) Distribución de Existencias Equinas por Categoría - Marzo 2016. Available at <https://datos.agroindustria.gov.ar/dataset/existencias-equinas> (accessed 31 January 2019).
- Serrera RM (1992) *Tráfico terrestre y red vial en las Indias Españolas*. 336 pp. Barcelona, Editorial Lunverg.
- Soulé C, Boulard C, Levieux D, Barnouin J and Plateau E (1989) Fasciolose équine expérimentale: évolution des paramètres sérologiques, enzymatiques et parasitaires. *Annales des Recherches Vétérinaires* **20**, 295–307.
- Tamura K, Stecher G, Peterson D, Filipksi A and Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.
- USDA-NRCS (2000) *Agricultural waste management field handbook*. Washington, DC, United States Department of Agriculture.
- Valero MA, Darce NA, Panova M and Mas-Coma S (2001a) Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Veterinary Parasitology* **102**, 85–100.
- Valero MA, Panova M and Mas-Coma S (2001b) Developmental differences in the uterus of *Fasciola hepatica* between livestock liver fluke populations from Bolivian highland and European lowlands. *Parasitology Research* **87**, 337–342.
- Valero MA, Santana M, Morales M, Hernandez JL and Mas-Coma S (2003) Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *Journal of Infectious Diseases* **188**, 787–793.
- Valero MA, Panova M and Mas-Coma S (2005) Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. *Journal of Helminthology* **79**, 217–225.
- Valero MA, Navarro M, Garcia-Bodelon MA, Marcilla A, Morales M, Garcia JE, Hernandez JL and Mas-Coma S (2006) High risk of bacterobilia in advanced experimental chronic fasciolosis. *Acta Tropica* **100**, 17–23.
- Valero MA, Girones N, Garcia-Bodelon MA, Periago MV, Chico-Calero I, Khoubbane M, Fresno M and Mas-Coma S (2008) Anaemia in advanced chronic fasciolosis. *Acta Tropica* **108**, 35–43.
- Valero MA, Perez-Crespo I, Chillón-Marinás C, Khoubbane M, Quesada C, Reguera-Gomez M, Mas-Coma S, Fresno M and Girones N (2017) *Fasciola hepatica* reinfection potentiates a mixed Th1/Th2/Th17/Treg response and correlates with the clinical phenotypes of anemia. *PLoS ONE* **12**, e0173456.
- Valero MA, Bargues MD, Khoubbane M, *et al.* (2016) Higher physiopathogenicity by *Fasciola gigantica* than by the genetically close *F. hepatica*: experimental long-term follow-up of biochemical markers. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **110**, 55–66.
- Villavicencio AF, Bargues MD, Artigas A, Guaman R, Ulloa SM, Romero J, Osca D and Mas-Coma S (2019) Lymnaeid snail vectors of fascioliasis, including the first finding of *Lymnaea neotropica* in Ecuador, assessed by ribosomal DNA sequencing in the southern zone close to the Peru border. *Acta Parasitologica* **64**, 839–849.
- Williams DJL and Hodgkinson JE (2017) Fasciolosis in horses: a neglected, re-emerging disease. *Equine Veterinary Education* **29**, 202–204.
- Zumaquero-Rios JL, Sarracent-Perez J, Rojas-García R, Rojas-Rivero I, Martínez-Tovilla Y, Valero MA and Mas-Coma S (2013) Fascioliasis and intestinal parasitoses affecting schoolchildren in Atlixco, Puebla State, Mexico: epidemiology and treatment with nitazoxanide. *PLoS Neglected Tropical Diseases* **7**, e2553.