Fasciola hepatica and lymnaeid snails occurring at very high altitude in South America

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SUMMARY

Fascioliasis due to the digenean species *Fasciola hepatica* has recently proved to be an important public health problem, with human cases reported in countries of the five continents, including severe symptoms and pathology, with singular epidemiological characteristics, and presenting human endemic areas ranging from hypo- to hyperendemic. One of the singular epidemiological characteristics of human fascioliasis is the link of the hyperendemic areas to very high altitude regions, at least in South America. The Northern Bolivian Altiplano, located at very high altitude (3800–4100 m), presents the highest prevalences and intensities of human fascioliasis known. Sequences of the internal transcribed spacers ITS-1 and ITS-2 of the nuclear ribosomal DNA of Altiplanic *Fasciola hepatica* and the intermediate snail host *Lymnaea truncatula* suggest that both were recently introduced from Europe. Studies were undertaken to understand how the liver fluke and its lymnaeid snail host adapted to the extreme environmental conditions of the high altitude and succeeded in giving rise to high infection rates. In experimental infections of Altiplanic lymnaeids carried out with liver fluke isolates from Altiplanic sheep and cattle, the following aspects were studied: miracidium development inside the egg, infectivity of miracidia, prepatent period, shedding period, chronobiology of cercarial emergence, number of cercariae shed by individual snails, survival of molluscs at the beginning of the shedding process, survival of infected snails after the end of the shedding period and longevity of shedding and non-shedding snails. When comparing the development characteristics of European *F. hepatica* and *L. truncatula*, a longer cercarial shedding period and a higher cercarial production were observed, both aspects related to a greater survival capacity of the infected lymnaeid snails from the Altiplano. These differences would appear to favour transmission and may be interpreted as strategies associated with adaptation to high altitude conditions.

Key words: Human fascioliasis, *Fasciola hepatica*, *Lymnaea truncatula*, rDNA ITS-1 and ITS-2, fluke larval development, snail survival, adaptation strategies, high altitude, Bolivia, Andean countries.

INTRODUCTION

Fascioliasis due to the digenean species *Fasciola hepatica* is a well-known veterinary problem worldwide. However, studies carried out in recent years have shown it to be an important public health problem as well (Chen & Mott, 1990; WHO, 1995; Mas-Coma, Bargues & Esteban, 1999). Human cases have been reported in countries of the five continents (Esteban, Bargues & Mas-Coma, 1998), with severe symptoms and pathology being observed (Chen & Mott, 1990; Mas-Coma & Bargues, 1997; Mas-Coma *et al*. 2000). Human endemic areas range from hypo- to hyperendemic (Mas-Coma, Esteban & Bargues, 1999) with characteristic epidemiology (Mas-Coma, 1998).

F. hepatica is a parasite originally associated with Europe, where it is almost exclusively transmitted by the species *Lymnaea truncatula* (see review by Oviedo, Bargues & Mas-Coma, 1996). Although *L. truncatula* may be found even up to 2600 m altitude in Europe, *F. hepatica* is a parasite typical of lowlands and is never found at high altitudes in Europe. This different altitudinal distribution is mainly related to temperature, as it is known that *F. hepatica* larval development is arrested below 10 °C (Mas-Coma & Bargues, 1997), although a lack of compatibility between the fluke and the populations of lymnaeids from high altitudes has also been suggested (Oviedo, Bargues & Mas-Coma, 1995).

Several lowland regions presenting fascioliasis health problems are known, such as human hypoendemic zones in France, Corsica and Chile, mesoendemic areas in Portugal and repetitive epidemics in Cuba and Iran (see review by Esteban *et al*. 1998), and recently even hyperendemic areas in the Nile Delta, Egypt (Curtale *et al*. 2000). Interestingly, however, in the Andean countries human fascioliasis hyperendemic areas appear to be linked to very high altitude regions of 3000–4100 m (Esteban *et al*. 1998; Mas-Coma *et al*. 1999). Fascioliasis transmission at lower altitudes, between 1000 and 2500 m, is known in different zones of Europe (Manga-Gonzalez, Gonzalez-Lanza & Otero-Merino, 1991; Pareau *et al*. 1994), Africa (Van Someren, 1946; Bergeon & Laurent, 1970; Scott & Goll, 1977; Loker *et al*. 1993) and Asia (Kendall, 1954; Kendall

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& Parfitt, 1959; Morel & Mahato, 1987), as well as in Central (Perez-Reyes, Jimenez-Nava & Varela-Ramirez, 1985) and South America (Morales & Pino, 1981, 1983).

Among the latter, the Northern Bolivian Altiplano, located at the very high altitudes between 3800–4100 m, presents the highest prevalences and intensities of human fascioliasis known, affecting mainly children. Up to 72% and 100% prevalences have been recorded in given localities according to coprological and immunological survey methods, respectively (Hillyer *et al*. 1992; Mas-Coma *et al*. 1995, 1999; Bjorland *et al*. 1995; Esteban *et al*. 1997*a*, *b*, 1999; O'Neill *et al*. 1998), and more than 5000 eggs per g of faeces have been observed (Esteban *et al*. 1997*a*, *b*, 1999).

A multidisciplinary project was undertaken to understand the transmission and epidemiology of fascioliasis in the Northern Bolivian Altiplano. Sheep and cattle have been shown to be the main reservoir host species inhabiting the endemic region with high infection prevalences and intensities (Ueno & Morales, 1973; Ueno *et al*. 1975; Mas-Coma *et al*. 1995, 1997; Hillyer *et al*. 1996; Buchon *et al*. 1997; Grock *et al*. 1998). Studies showed that pigs and donkeys may be considered as reservoir hosts of secondary importance and that horses, llamas and alpacas (Mas-Coma *et al*. 1997), as well as rabbits, wild hares, domestic guinea pigs and wild rodents (Fuentes *et al*. 1997) do not significantly participate in the transmission of the disease.

The endemic zone proved to be stable, isolated and apparently unable to extend from its present outline, boundaries being marked by geographic, climatic and soil-water chemical characteristics. Altiplanic lymnaeids were found to inhabit mainly permanent water bodies, which enables parasite transmission during the whole year (Mas-Coma *et al*. 1999). The transmission foci appear patchily distributed and linked to the presence of appropriate water bodies according to an irregular geographic distribution within the endemic area. The likelihood of transmission can be assessed by means of forecast indices based on meteorological characteristics and appropriately modified for high altitude (Fuentes *et al*. 1999) and even more accurately by the normalized difference vegetation index obtained from remote sensing data (Fuentes, Malone & Mas-Coma, 2001).

In the Northern Bolivian Altiplano a confluence of several factors takes place which mitigates the negative effects of the very high altitude, mainly the cold temperatures and the high evapotranspiration rates. Among such positive factors there are (a) the nearness to the equator with increasing temperature, (b) the neighbourhood of Lake Titicaca moderating temperatures and increasing humidity, (c) the existence of numerous fresh water bodies deriving from the thaw of the perpetual snow amounts of the Eastern Andean Chain which combined with the existence of shallow phreatic layers assure the presence of permanent water collections for the survival of lymnaeids, (d) the absence of shadow because of the lack of trees and shrubs permitting a marked daily increase of the temperature of the water bodies, and (e) the scarcity of land leads human inhabitants to a marked dependence on livestock including various potential definitive host species (Mas-Coma *et al*. 1999).

Nevertheless, a detailed study demonstrated that the very high altitude climatic characteristics of this endemic area markedly differ from those of fascioliasis endemic lowland areas, such as those of the original European geographical distribution of *F. hepatica*. In the Northern Altiplano the following points are worth mentioning regarding the life cycle of *F. hepatica* and the transmission of the disease. The temperature has no marked seasonal character; the mean environmental temperature is very low throughout the year, about 10 °C or a little lower, which is not appropriate for the fluke development. There are large variations in temperature within a daily 24 hour period, both free-living and intra-molluscan larval stages of the parasite needing to adapt to such severe changing daily conditions. The rainfall distribution is seasonal, with a long dry season coinciding with the lowest minimum temperatures and a long wet season in which rainfall is concentrated, thus differing significantly from the European endemic areas in which fascioliasis is traditionally biseasonal, appearing in spring and autumn. Evapotranspiration is very high, temporary water bodies being of very short duration, mainly in the arid period, explaining why Altiplanic lymnaeids are almost always restricted to permanent water collections and transmission can occur throughout the year, contrary to what is typical of fascioliasis in Europe, where *L. truncatula* is markedly amphibious and develops mainly in temporary water collections. Lastly, the solar radiation is high not only because of altitude, but also because of the absence of shade as the consequence of lack of trees and shrubs (Fuentes *et al*. 1999).

Three important questions immediately arise: (a) how has the liver fluke adapted to maintain its life cycle at the extreme conditions associated with this very high altitude zone? (b) What strategies have been developed by the parasite to reach very high transmission rates in such an *a priori*-inhospitable environment? (c) Has this adaptation been reflected in both parasite and intermediate snail hosts at genotype and phenotype levels? To analyse these questions different studies have been performed. The aim of this paper is to present the results obtained in studies on: (1) the genetic characterization of both Altiplanic liver fluke and lymnaeid snails, and (2) the larval stage development of the liver fluke from the Northern Bolivian Altiplano.

For the first objective, the sequences of the first

and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA were obtained and analysed. These spacers were selected taking into account their usefulness for species, subspecies and population differentiation in parasites and vectors in general (Mas-Coma, 1999). For the second objective, experimental infections of Altiplanic lymnaeids kept in the laboratory under standardised rearing conditions with liver fluke isolates from Altiplanic sheep and cattle were carried out to establish the transmission characteristics.

MATERIALS AND METHODS

rDNA ITS sequencing

Liver fluke materials. Adult worms found in naturally infected hosts from the Northern Bolivian Altiplano and Spain were used for ITS-1 and ITS-2 sequencing (Table 1). For sequence comparison purposes, the following rDNA ITS-2 were used: (1) *F. hepatica* from: Australia, Hungary, Mexico and New Zealand (Adlard *et al*. 1993); unknown origin (GenBank Accession No. LO7844) (Michot *et al*. 1993); Australia (Hashimoto *et al*. 1997); Uruguay (Acc. No. AB010974) (Itagaki & Tsutsumi, 1998); (2) *F. gigantica* from Indonesia and Malaysia (Adlard *et al*. 1993); Malaysia (Hashimoto *et al*. 1997); Zambia isolate I (Acc. No. AB010975), Zambia isolate II (Acc. No. AB010976) and Indonesia (Acc. No. AB010977) (Itagaki & Tsutsumi, 1998); (3) *Fasciola* sp. from: Japan (Adlard *et al*. 1993); Japan (Hashimoto *et al*. 1997); Japan isolate I (Acc. No. AB010978) and Japan isolate II (Acc. No. AB010979) (Itagaki & Tsutsumi, 1998).

Lymnaeid snail materials. Snail specimens from the Northern Bolivian Altiplano and different geographic localities of Europe and Morocco were used for ITS-1 and ITS-2 sequencing (Table 1). The absence of infection by helminth parasites was always verified prior to the selection of the lymnaeids for molecular techniques. Taking helminth microhabitats into account, the region of the foot was chosen as the only snail part to be used for DNA extraction.

Molecular techniques. *F. hepatica* adults washed extensively in PBS (37 °C) were subsequently fixed in 70% ethanol. Snail feet were also fixed in 70% ethanol and stored at 4 °C for several weeks before DNA extraction according to the phenol-chloroform method (Sambrook, Fritsch & Maniatis, 1989) following the protocol of Bargues & Mas-Coma (1997).

The fragments corresponding to the ITS-1 and ITS-2 of each trematode and lymnaeid were amplified by the Polymerase Chain Reaction (PCR) using 4–6 μl of genomic DNA for each 50 μl PCR reaction, according to methods outlined previously (Almeyda-Artigas, Bargues & Mas-Coma, 2000; Marcilla *et al*. 2001). The PCR amplification was performed using primers designed in conserved positions of 18S, 5·8S and 28S rRNA genes of several eukaryote Metazoa species. For the ITS-1 and ITS-2 regions, the primers used were as described by Almeyda-Artigas *et al*. (2000). Only one additional primer, LT1 (forward) 5′-TCGTCTGTGTGAGGGTCG was designed for amplification and sequencing purposes.

Amplifications were generated in a GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT, USA), by 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, preceded by 30 sec at 94 °C and followed by 7 min at 72 °C. Ten microliters of the reaction mixture were examined by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol and resuspended in 50 μl of 10 mM TE buffer (pH 7·6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

Sequencing was performed on both strands by the dideoxy chain-termination method, and with the Taq dye-terminator chemistry kit for ABI PRISM 377 (Perkin Elmer, Foster City, CA), using PCR primers. For sequence alignment the CLUSTAL-W version 1·8 (Thompson. Higgins & Gibson, 1994) was used.

Experimental life cycle studies

Liver fluke materials. Living eggs of *F. hepatica* were obtained from sheep and cattle gall-bladders after bile filtration. Gall-bladders from naturally infected sheep and cattle hosts of the Northern Bolivian Altiplano endemic area were obtained in the slaughterhouses of Batallas and El Alto, respectively. Eggs were washed three times with natural water and transported to the laboratory of Valencia, where they were stored in fresh water and complete darkness at 4 °C until required.

Lymnaeid snail materials. Lymnaeid snails used were collected in the Huacullani zone, where fascioliasis is highly prevalent in humans (Esteban *et al*. 1997*a*, 1999: varying between 31·2 and 38·2% in children, depending on year periods; with intensities of up to 5064 eggs per g of faeces), sheep (Grock *et al*. 1998: 70·0%) and cattle (Buchon *et al*. 1997: 45·2%). Lymnaeids were transported under isothermal conditions to the laboratory of Valencia, where they were adapted to experimentally controlled conditions

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Table 1. Populations of liver fluke and lymnaeid populations chosen for sequencing of rDNA ITS-1 and ITS-2. *Lymnaea* sp. morph I (= *L. viatrix sensu* Ueno *et al*. 1975) and morph II (= *L. cubensis sensu* Ueno *et al*. 1975) according to Oviedo, Bargues & Mas-Coma (1995)

			ITS-1		ITS-2	
Populations	Geographic location	Country	Acc. No.	bp length	Acc. No.	bp length
Liver fluke	Batallas, Northern Altiplano	Bolivia	AJ243016	433	AJ272053	364
Fasciola hepatica	Castellon	Spain	AJ243016	433	AJ272053	364
Lymnaea sp. morph I	Tambillo, Northern Altiplano	Bolivia	AJ272052	504	AJ272051	401
Lymnaea sp. morph II	Batallas, Northern Altiplano	Bolivia	AJ272052	504	AJ272051	401
Lymnaea truncatula	Javalambre, Castellon	Spain	AJ243018	504	AJ296271	401
Lymnaea truncatula	Beira	Portugal	AJ243018	504	AJ296271	401
Lymnaea truncatula	Monaccia, Corsica island	France	AJ243018	504	AJ296271	401
Lymnaea truncatula	Albufera, Sueca, Valencia	Spain	AJ243018	504	AJ243017	401
Lymnaea truncatula	Benicasim, Castellon	Spain	AJ243018	504	AJ243017	401
Lymnaea truncatula	Minho	Portugal	AJ243018	504	AJ243017	401
Lymnaea truncatula	Le Taulard, Laussane	Switzerland	AJ243018	504	AJ243017	401
Lymnaea truncatula	Site Oudesa	Morocco	AJ296270	504	AJ243017	401

in 2000 ml fresh water in standard breeding containers at 20 °C, 90% relative humidity and 12 h/12 h light/darkness photoperiod in precision climatic chambers (Heraeus-Vötsch HPS-1500 and HPS-500). The water was changed weekly and lettuce added *ad libitum*.

Experimental procedures. A continuous temperature of 20 °C was selected and used throughout because of two reasons: (1) lymnaeids from the Bolivian Northern Altiplano proved to adapt to 20 °C better than to higher temperatures; (2) a 20 °C temperature has been largely used by other authors in experiments with the European *F. hepatica*/*L. truncatula* model, thus allowing comparisons of results.

Liver fluke eggs in fresh water were maintained under complete darkness at 20 °C to start the embryogenic process. Embryogenesis was followed at intervals of 4 days by counting eggs presenting an incipient morula, eggs including an advanced morula, eggs with outlined miracidium, and fully embryonated eggs containing a developed miracidium.

Developed miracidia were forced to hatch by placing fully embryonated eggs under light and used for the experimental infection of snails. Only 4–5 mm-long first generation snails born in the laboratory were used for experiments. A total of 32 and 25 lymnaeids were infected monomiracidially by the Altiplanic sheep and cattle isolates, respectively, by exposing each snail to 1 miracidium for 4 hours in a small Petri dish containing 2 ml of fresh water. Snails were afterwards returned to the same standard conditions in the climatic chamber (2000 ml containers, 20 °C, 90% r.h., 12 h/12 h light/darkness, dry lettuce *ad libitum*) until day 30 post-infection, in which they were again isolated in Petri dishes to allow daily monitoring of cercarial shedding by individual snails. Lettuce was provided *ad libitum* to each snail in a Petri dish during both shedding and post-shedding periods until death of the snail.

The chronobiology of the cercarial shedding was followed by counting metacercariae in each Petri dish. To test the viability and infectivity of metacercariae, they were stored in natural water in total darkness until required. The storage temperature selected was 4 °C according to the mean winter temperature obtained in five meteorological stations of the Bolivian Altiplano endemic region in the 1949–1990 year period (Mas-Coma *et al*. 1999). This temperature has also been used for storing metacercariae by other authors, thus enabling comparative analysis with lowland liver fluke isolates. Male Wistar rats (Iffa Credo, Barcelona, Spain) aged 4–5 weeks were used for infection with metacercariae inoculated orally by means of a gastric gavage. Rats were housed in Micro-Isolator boxes (Iffa Credo, Barcelona, Spain) and maintained in a pathogen-free room, electrically heated with a 12 h/12 h light/darkness cycle (conditions in compliance with the European Agreement of Strasbourg, 18 March 1986). Food and water were provided *ad libitum*. Infection prevalence and intensity (number of worms successfully developed in each rat) were determined by dissection (Valero & Mas-Coma, 2000).

RESULTS

rDNA ITS-1 and ITS-2 sequences

New nucleotide sequence data reported in this paper on both fasciolids and lymnaeids are available in the EMBL, GenDank and DDBJ databases under the accession numbers listed in Table 1.

Liver fluke. The complete sequence of the ITS-1 showed no nucleotide difference between the Northern Bolivian Altiplano and Spain. It was 433 bp long,

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Table 2. Nucleotide differences found in the comparison of the ITS-2 sequences of *Fasciola* populations [[a] = after Itagaki & Tsutsumi (1998); [b] = after Hashimoto *et al.* (1997); [c] = after Aadlard *et al.* (1993); pb seq. = number of pb sequenced; bp numbers in parentheses indicate partial sequences; * = position not sequenced]

			Positions															
Species	Origin	pb seq.	1	51	53	78	97	101	115	210	221	234	273	279	287	327	330	337
F. hepatica	Bolivia	364	G	T	G	T	G	T	C	T	T	T	C	C	C	T	T	G
F. hepatica	Spain	364	G	T	G	T	G	T	C	T	T	T	C	C	C	T	T	G
F. hepatica	Uruguay[a]	364	C	T	G	T	G	T	C	T	T	T	C	C	T	T	T	G
F. hepatica	Australia[b]	362	C	T	G	T	G	T	C	T	T	T	C	C	C	T	T	G
F. hepatica	Australia[c]	(292)	*	T	—	T	—	T	C	T	T	T	C	C	C	T	T	G
F. hepatica	Hungary[c]	(264)	*	*	*	T	—	T	C	T	T	T	C	C	C	T	T	G
F. hepatica	Mexico[c]	(292)	*	T	—	T	—	T	C	T	T	T	C	C	T	T	T	G
F. hepatica	New Zealand[c]	(292)	*	T	—	T	—	T	C	T	T	T	C	C	C	T	T	G
F. gigantica	Zambia I[a]	362	C	T	G	T	G	T	C	T	T	A	C	C	C	T	—	G
F. gigantica	Zambia II[a]	362	C	T	G	T	G	T	C	T	T	C	T	T	C	T	—	A
F. gigantica	Indonesia[a]	362	C	T	G	T	G	T	C	C	T	C	T	T	C	T	—	A
F. gigantica	Indonesia[c]	(214)	*	*	*	*	*	*	*	C	T	C	T	T	C	—	T	A
F. gigantica	Malaysia[c]	(252)	*	*	*	*	—	—	C	C	T	C	T	T	C	—	T	A
F. gigantica	Malaysia[b]	362	C	T	G	T	G	T	C	C	T	C	T	T	C	T	—	A
Fasciola sp.	Japan[c]	(225)	*	*	*	*	*	*	—	C	C	C	T	T	C	—	T	A
Fasciola sp.	Japan I[a]	362	C	T	G	T	G	T	C	T	T	T	C	C	C	T	T	G
Fasciola sp.	Japan II[a]	362	C	T	G	T	G	T	C	C	C	C	T	T	C	T	—	A
Fasciola sp.	Japan[b]	362	C	T	G	T	G	T	C	C	T	C	T	T	C	T	—	A

Table 3. Nucleotide contents and differences found in the comparison of the sequences of ITS-1 and ITS-2 of lymnaeid populations from the Northern Bolivian Altiplano and Europe

	ITS-1 (504 bp long) Positions				ITS-2 (401 bp long) Positions		
Lymnaeid populations	% GC	74	75	132	% GC	55	149
Lymnaea sp. morph I Bolivia	57·5	G	T	T	58·6	T	T
Lymnaea sp. morph II Bolivia	57·5	G	T	T	58·6	T	T
L. truncatula Javalambre, Beira, Corsica	57·5	A	G	T	59·1	G	C
L. truncatula Sueca, Benicasim, Minho, Le Toulard	57·5	A	G	T	58·8	G	T
L. truncatula Morocco	57·7	A	G	C	58·8	G	T

with a 51·9 % GC content (see Table 1). As it is the first time that the sequence of this spacer of the liver fluke has been obtained, no comparison with populations from other areas can be made.

The ITS-2 sequence was also identical between the Northern Bolivian Altiplano and Spain. Its complete sequence was 364 bp long, with a 48·3 % GC content (see Table 1). The comparison with the ITS-2 sequences of *F. hepatica* from other geographic origins only showed a few nucleotide differences consisting in insertions/deletions and C/T transitions (Table 2). More substantial differences (mostly found at the 3′ end) were noted between our sequence and that reported by Michot *et al.* (1993), although the latter has been already criticised by different authors (i.e. Hashimoto *et al.* 1997). Interestingly, the ITS-2 sequence from Bolivia and Spain differ from all other presently known at least in one position (Table 2).

According to the ITS-2 sequence comparison obtained in the 364-bp-long alignment, the liver fluke from Bolivia and Spain both belong to the species *F. hepatica*. *F. hepatica* and *F. gigantica* differ at only 5 sites (positions 210, 234, 273, 279 and 337 in Table 2). The classification of the fasciolid designated Zambia I by Itagaki & Tsutsumi (1998) as belonging to *F. gigantica* becomes doubtful.

Lymnaeid snails. Length and GC content of both ITS-1 and ITS-2 sequences of all lymnaeid populations studied are noted in Table 3. No nucleotide difference between the two morphs I and II from the Northern Bolivian Altiplano in both spacers was found, but differ in a few nucleotide positions from all other populations studied from Europe and Morocco (Table 3).

At the level of ITS-1, all European populations appear identical and differ in only one nucleotide

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Table 4. Results obtained in infection experiments of Altiplanic *Lymnaea truncatula* with sheep and cattle isolates of *Fasciola hepatica* from the Northern Bolivian Altiplano (means in parentheses)

Host isolate	Sheep	Cattle
Day presenting the maximum % of eggs with advanced morula	12/88·9 %	8/86·7 %
Day in which the first outlined miracidium appeared inside egg	18	18
Day presenting the maximum % of eggs with outlined miracidium	36/37·3 %	38/56·2 %
Day in which the first developed miracidium appeared inside egg	38	24
Day presenting the maximum % of eggs with developed miracidium	58/16·4 %	46/24·9 %
Isolate infectivity (% snails infected)	69·2	39·1
Prepatent period (days)	48–58 (49·6)	49–58 (51·0)
Shedding period (days)	47–88 (71·5)	42–85 (74·1)
No. cercariae shed per individual snail	384–562 (451·8)	151–589 (446·2)
Snails surviving until shedding (% snails)	86·6	92·0
Snail survival after end of shedding period (days)	1–132 (44·5)	1–133 (55·4)
Postinfection longevity in shedding snails (days)	95–192 (157·1)	91–268 (165·0)
Longevity in non-infected snails (days post-infection)	98–182 (175·0)	100–209 (145·0)

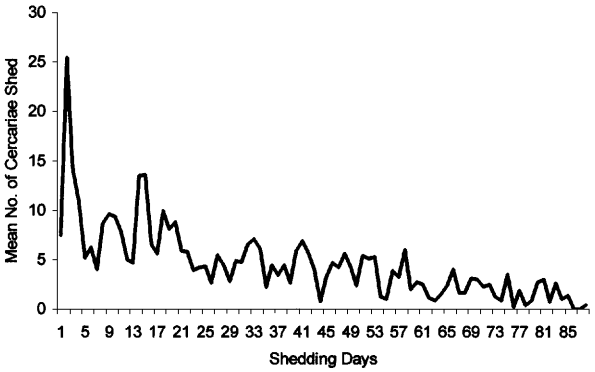

Fig. 1. Chronobiology of cercarial shedding of the sheep isolate of *Fasciola hepatica* by *Lymnaea truncatula*, both from the Bolivian Northern Altiplano. Shedding period analysed from the day of the emergence of the first cercaria by each snail.

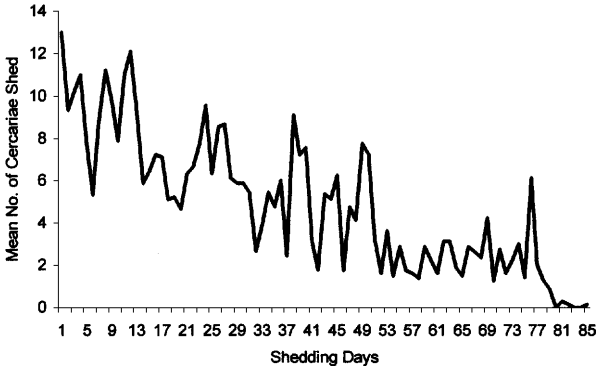

Fig. 2. Chronobiology of cercarial shedding of the cattle isolate of *Fasciola hepatica* by *Lymnaea truncatula*, both from the Bolivian Northern Altiplano. Shedding period analysed from the day of the emergence of the first cercaria by each snail.

position from that of Morocco. At the level of ITS-2, given European populations (Javalambre, Beira and Corsica) differ from the remaining from Europe and that of Morocco in one C/T transition (Table 3).

Life cycle characteristics of sheep and cattle isolates

Results of embryogenesis inside the egg, lymnaeid snail infection, intramolluscan parasite larval development and influences of the latter on snail survival are noted in Table 4. Embryogenesis followed a similar pattern in both sheep and cattle isolates from the Northern Bolivian Altiplano, although development in eggs of the cattle isolate was somewhat faster. The prepatent period in lymnaeid snails and the cercarial shedding period, as well as the number of cercariae shed per lymnaeid individual appeared similar in both sheep and cattle isolates, although the snail infectivity of the latter was lower than that of the sheep isolate. The host isolate did not appear to influence lymnaeid survival, results obtained before shedding started and after

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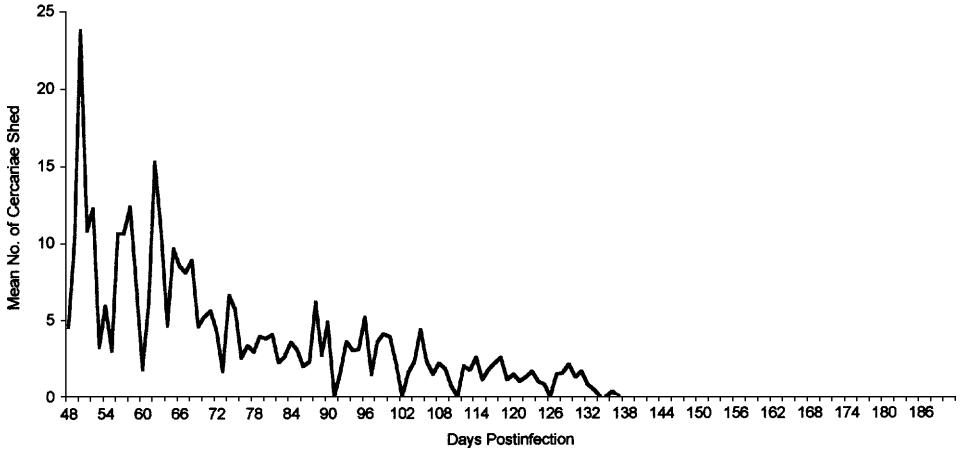

Fig. 3. Chronobiology of cercarial shedding of the sheep isolate of *Fasciola hepatica* by *Lymnaea truncatula*, both from the Bolivian Northern Altiplano. Shedding period analysed from the day of the miracidial infection. Prepatent period not shown. Curve followed up to the death of shedding snails.

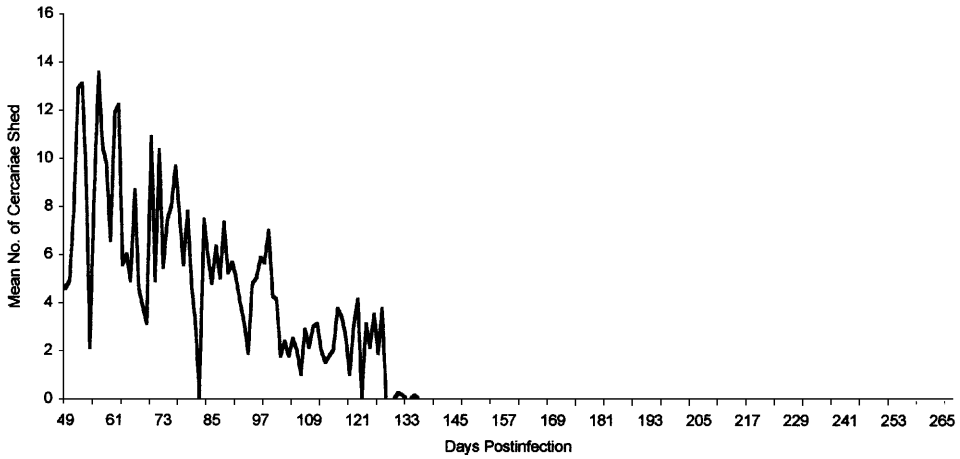

Fig. 4. Chronobiology of cercarial shedding of the cattle isolate of *Fasciola hepatica* by *Lymnaea truncatula*, both from the Bolivian Northern Altiplano. Shedding period analysed from the day of the miracidial infection. Prepatent period not shown. Curve followed up to the death of shedding snails.

shedding ended being similar in both isolates. Interestingly, longevity after the day of miracidial infection was also similar in both infected and non-infected lymnaeids.

The chronobiological patterns of cercarial emergence in the sheep and cattle isolates are shown in Figures 1 and 3, and 2 and 4, respectively. It appears to be similar in both isolates. When the shedding period is analysed from the day of the emergence of the first cercaria by each snail (Figs 1, 2), it appears that the shedding process appears as an irregular succession of waves in which a slowly decrease of the daily number of cercariae is envisaged, the higher acrophases taking place in the first days of the emergence period. When the shedding period is analysed from the day of the miracidial infection (Figs 3, 4), a similar decreasing multiwave pattern is detected. In these curves of Figs 3 and 4, days occurred in which all snails failed to shed any cercaria. When analysing the emergence curves per individual snail (figures not shown), a total of 6 shedding pauses are evident. In the sheep isolate there is a first pause between days 60 and 73 post-infection, a second between days 90 and 93, a third on days 100–103, a fourth on days 108–112, a fifth on days 117–121, and a last sixth one on days 125–126. In the cattle isolate there is a first pause on days 63–69 post-infection, a second on days 80–84, a third on days 92–96, a fourth on days 100–106, a fifth on days 111–113, and a final sixth one on days 117–119. Consequently, there are about 20–30 days between the two first pauses and afterwards the pauses become separated by an elapse which appears to follow a slowly progressive reduction from 10 to 6 days.

No significant difference between sheep and cattle isolates appeared in metacercarial infectivity assays (statistical comparisons with the Chi-Square test using SPSS 6·1; values considered statistically significant when $P < 0{\cdot}05$). In the sheep isolate, 11 from a total of 14 rats (78·6 %) became infected when inoculated with 20 metacercariae each, and between 1 and 8 adult worms were obtained per rat (mean 3·6). In the cattle isolate, 6 from a total of 8 rats

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(75%) became infected when inoculated with the same metacercarial dose, giving rise to 1–2 adult flukes per rat (mean 1·7).

DISCUSSION

ITS-1 and ITS-2 of the liver fluke

According to molecular clock estimations based on ITS sequences (see review in Bargues *et al*. 2000), the very few nucleotide differences between ITS sequences of liver flukes from different regions of the world suggest a very recent geographical dispersal of the parasite. This agrees with previous assumptions indicating that *F. hepatica* is a parasite transmitted by *L. truncatula* original of Europe (Oviedo *et al*. 1995), where it has even been found in prehistoric human populations living at the end of the Mesolithic period, 5000–5100 years ago (Aspöck, Auer & Picher, 1999). From this European geographical area it has spread by the exportation of European livestock to the five continents where it has adapted to other lymnaeid species (Mas-Coma & Bargues, 1997).

ITS sequence comparison confirms that the liver fluke of the Northern Bolivian Altiplano is *F. hepatica*. Moreover, the fact that ITS-1 and ITS-2 sequences of *F. hepatica* from the Altiplano are identical to those of *F. hepatica* from Spain and differ from those of other world regions suggests that the Bolivian liver fluke is derived from Iberian liver flukes, probably a recent introduction at the time of Spanish colonization or somewhat later.

Despite the absence of ITS sequence differences, phenotypical studies demonstrated that slight differences can be found in the allometry of body measurements between the *F. hepatica* adult from highland and that from lowland populations of Bolivian and Spanish sheep, respectively (Valero *et al*. 1999). Moreover, the Bolivian sheep and cattle liver fluke populations have a smaller uterus size than that of European populations (Valero, Panova & Mas-Coma, 2000). These uterus differences between the liver fluke population of highlands and lowlands have been tentatively related to high altitude influences, although it cannot be discounted that to some extent this may be attributable to intraspecific variability (Valero *et al*. 2000). As known, high altitude environmental factors exert an influence on vertebrates, and those born and living at high altitude show morphological and physiological characteristics different from those inhabiting low altitudes. The development of *F. hepatica*, mainly because of its tissue migration and haematophagous diet (Dawes & Hughes, 1964; Boray, 1969), may be influenced by changes such as hypoxia, alterations in immune response, elevated haematocrit levels, differences in blood oxygen pressure values and blood viscosity and elimination of dissolved gases, especially N_2, from the blood (Valero *et al*. 2000).

It is known that oxygen is still required for egg production in *F. hepatica* (Mansour, 1958; Bjorkman & Thorsell, 1963; McGonigle & Dalton, 1995). Thus, high altitude hypoxic conditions could be the origin of a reduced egg production by the flukes. Moreover, although the uterus in digeneans has traditionally not been considered as a storage organ but mainly an organ adapted to the developmental time of the eggs (in fasciolids, eggs are layed unembryonated, the miracidium beginning its development once the egg is deposited in freshwater), our recent experimental studies with rats have demonstrated that there is a direct relation between *F. hepatica* uterus size and the number of eggs shed per g of faeces (Valero *et al*., unpublished). In the Northern Bolivian Altiplano, climatic conditions, freshwater body characteristics and lymnaeid ecology enable fascioliasis transmission to take place throughout the year (Mas-Coma *et al*. 1999; Fuentes *et al*. 1999), so that egg storage is *a priori* not needed as in the northern hemisphere latitudes where fascioliasis transmission is typically seasonal (Valero *et al*. 2000).

ITS-1 and ITS-2 of the lymnaeid snails

ITS sequence analyses of the lymnaeid morphs I and II prove that there is only one species in the Northern Bolivian Altiplano. Moreover, the very few differences between the ITS sequences of the Bolivian lymnaeids and *L. truncatula* populations from Europe and Morocco indicate that the snail species involved in the transmission of human and animal fascioliasis in this Andean endemic area is *L. truncatula*. At any rate, the nucleotide differences in both ITS-1 and ITS-2 sequences allow us to differentiate the Altiplano population from all other European and African populations studied. As in the case of the liver fluke, ITS results suggest that fascioliasis transmitting snails of the Altiplano were introduced from Europe, most probably imported by Spanish colonizers.

These results do not agree with those obtained by Ueno *et al*. (1975), who mentioned two different lymnaeid forms transmitting fascioliasis in the Northern Bolivian Altiplano, which they classified as *Lymnaea viatrix* and *L. cubensis*. However, our results are in agreement with those obtained in more recent studies on shell morphology and visceral mass anatomy (Oviedo *et al*. 1995; Samadi *et al*. 2000), which suggested that what Ueno *et al*. (1975) classified as two different species are in fact only extreme morphs within a large intraspecific variability (Oviedo *et al*. 1995; Samadi *et al*. 2000), morphs I and II of Oviedo *et al*. (1995) corresponding to *L. viatrix* and *L. cubensis* of Ueno *et al*. (1975), respectively. Further molecular confirmation was obtained by the absence of nucleotide differences in the complete sequence of the 18S rDNA gene

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between both extreme morphs and between them and *L. truncatula* from Europe (Bargues & Mas-Coma, 1997; Bargues *et al*. 1997). At any rate, this gene is largely more conserved and is usually not very useful for the differentiation of species which are very similar, such as *L. viatrix*, *L. cubensis* and *L. truncatula* (Bargues *et al*., unpublished); moreover, its validity for lymnaeid taxonomy and phylogeny has recently been questioned owing to single nucleotide polymorphisms detected in *L. natalensis* and intraspecific *versus* interspecific divergence levels it shows in certain groups (Stothard *et al*. 2000), although further studies on the 18S rDNA gene of lymnaeids are evidently needed. Results obtained in isoenzyme comparison analyses also suggested the link between Altiplanic lymnaeids and *L. truncatula* from western Europe. Additionally, isoenzyme analyses proved that all lymnaeid populations inhabiting the Altiplanic endemic area are monomorphic, a clonicity related to selfing reproduction processes and a single founding population (Jabbour-Zahab *et al*. 1997). Recent studies on genetic diversity and population structure of lymnaeids by means of polymorphic DNA microsatellite analyses also confirmed the presence of a single *L. truncatula* genotype on the Bolivian Altiplano (Meunier *et al*. 2001). Interestingly, moreover, differences detected in all these comparative studies between the Altiplanic lymnaeids and *L. truncatula* from Europe were always non-significant or non-existent at all.

Life cycle characteristics of Altiplanic Fasciola hepatica *isolates*

The embryonation times observed in the sheep and cattle isolates of *F. hepatica* fit in the ranges mentioned by several authors when tested at 20 °C (Kendall, 1965; Diez-Baños & Rojo-Vazquez, 1976; Foreyt & Todd, 1978). As known, the development of the miracidium inside egg is arrested below 9 °C and above 37 °C and has a duration between 9 and 161 days depending upon the temperature, the range 20–25 °C offering the optimum for the hatching of a higher number of miracidia (Roberts, 1950; Rowcliffe & Ollerenshaw, 1960; Valenzuela, 1979; Wilson, Smith & Thomas, 1982).

Infection percentages and prepatent period in monomiracidial infections may also be considered as normal at 20 °C when compared to similar studies carried out with *F. hepatica* isolates and *L. truncatula* specimens from European zones (Roberts, 1950; Pantelouris, 1965; Kendall, 1965, 1970; Odening, 1971; Boray, 1982; Oviedo *et al*. 1996). The prepatent period detected in the Altiplanic material studied also fits in the known range (43·1–58·2 days) for the European *F. hepatica*/*L. truncatula* in the nature (Rondelaud & Dreyfuss, 1997).

Infectivity of metacercariae from Altiplanic *F. hepatica* does not significantly differ from that of the liver fluke in lowlands of other countries (Valero & Mas-Coma, 2000). However, the results obtained in the shedding period, the number of cercariae shed and the snail survival must be emphasized. The duration of the shedding period in the Bolivian *F. hepatica*/*L. truncatula* appears to be very long. In European *F. hepatica* experimentally infecting *L. truncatula* snails of the same size as ours under the same constant conditions of 20 °C and 12 h/12 h photoperiod, the patent period lasted only $46 \pm 27{\cdot}6$ days (Dreyfuss & Rondelaud, 1994). Similarly, results obtained in nature show that the patent period in Europe ranges between 5·0 and 9·3 days in the winter generation and 18·3–40·3 days in the summer generation (Rondelaud & Dreyfuss, 1997).

The cercarial shedding pattern detected in the Bolivian *F. hepatica*/*L. truncatula* model does not disagree with the patterns observed by other authors. Kendall & McCullough (1951), Bouix-Busson, Rondelaud & Combes (1985), Audousset *et al*. (1989) and Dreyfuss & Rondelaud (1994) reported that cercarial emission was discontinuous with shedding waves separated by periods of rest. The infradian-type rhythm with a periodicity of 7 days and 5–7 waves described by Audousset *et al*. (1989) when working under variable temperatures has been put into doubt by Dreyfuss & Rondelaud (1994) who worked under constant conditions of temperature and photoperiod. These latter authors observed that snails shed their cercariae in 1 to 14 waves, although the majority of snails produced their parasites in 4 and 5 waves. Consequently, the six pauses separating 8 waves is understandable taking into account the longer shedding period in the Bolivian fluke/snail model. The only discrepancy between our results and those of these authors is found at the level of the distribution of cercarial numbers throughout the shedding period, as in the Bolivian model the highest daily numbers of cercariae are clearly shed in the first weeks whereas in the European model acrophases appear delayed.

The number of cercariae shed per individual lymnaeid in the Bolivian material studied is also very high, a phenomenon related to the marked length of the shedding process. Working under the same experimental conditions, a mean of only 238·5 metacercariae per snail was found by Dreyfuss & Rondelaud (1994). Always working with the same European *F. hepatica*/*L. truncatula* model, Dreyfuss *et al*. (1999) obtained only $114{\cdot}9 \pm 80{\cdot}3$ metacercariae per snail infected monomiracidially, and they observed that the duration of the shedding and the number of metacercariae were independent of the number of miracidia used for the infection of each individual lymnaeid, although single-miracidium infections were most effective because of the much higher snail survival rate and despite the mean number of cercariae shed being the same as in multi-miracidial infections.

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Lymnaeid snail survival in the experiments carried out with *F. hepatica* and *L. truncatula* from the Northern Bolivian Altiplano are worth mentioning. Differences in survival of different geographical strains of *L. truncatula* to *F. hepatica* infection have already been described (Gold, 1980). The longevity of the experimentally infected Bolivian molluscs after the moment of the miracidial infection is usually longer than the same survival period observed in the European species *L. truncatula*, in which a postinfection longevity of 70 days postinfection is usually observed (Kendall, 1953; Hodasi, 1972*a*, *b*; Wilson & Denison, 1980; Rondelaud & Barthe, 1986), with a maximum of 16 weeks described once (Hodasi, 1972*a*, *b*), and even longer than that known in other American lymnaeids such as 119 days postinfection for *Lymnaea viatrix* (Venturini, 1978) and 113·4 days postinfection for *Lymnaea bulimoides* (Jay & Dronen, 1985). The capacity of Altiplanic lymnaeids to survive up to more than 4 months after the end of the shedding period is surprising, as in Europe, snails die either during the shedding period, immediately after shedding ends or shortly after it. Dreyfuss & Rondelaud (1994) found that of 102 snails shedding on the first day, the number drastically reduced to only 56 on the second day and subsequently decreased on day 76 to four snails.

CONCLUSIONS

The results on rDNA ITS-1 and ITS-2 sequences suggest that the human and animal fascioliasis high endemic area of the Northern Bolivian Altiplano is the consequence of a recent introduction of both *F. hepatica* and *L. truncatula* from Europe. In this double importation, parasite and lymnaeid, have followed a process of adaptation from the European lowlands to the Bolivian highlands. When analysing the different aspects of the life cycle of *F. hepatica* from the Northern Bolivian Altiplano by comparison with that of *F. hepatica* in *L. truncatula* of Europe, no phase is observed in which the parasite development appears to be negatively modified for the transmission. Several aspects can be shown to be similar to those in Europe, such as duration of miracidium development inside the egg, the lymnaeid infection capacity of miracidia, the duration of the prepatent period and the infectivity of metacercariae.

However, results obtained show that certain aspects favour transmission, such as the longer cercarial shedding period and the higher cercarial production, both related to the greater survival capacity of infected lymnaeid snails. The absence of survival differences between parasitised and non-parasitised molluscs suggests a better parasite-host adaptation in the Bolivian Northern Altiplano. This phenomenon may be related to the clonal characteristics of the *L. truncatula* populations of the Northern Bolivian Altiplano. The initial founding snail individual or few individuals imported from Europe that have given rise by selfing to the numerous monomorphic populations that today inhabit the Altiplano endemic area were most probably snails that were very susceptible for *F. hepatica* infection. These snails would have genetically transmitted their high susceptibility to their descendants by almost absolute predomination of autofecundation, suggesting a large and homogeneous susceptibility of the different Altiplanic *L. truncatula* populations to the liver fluke.

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