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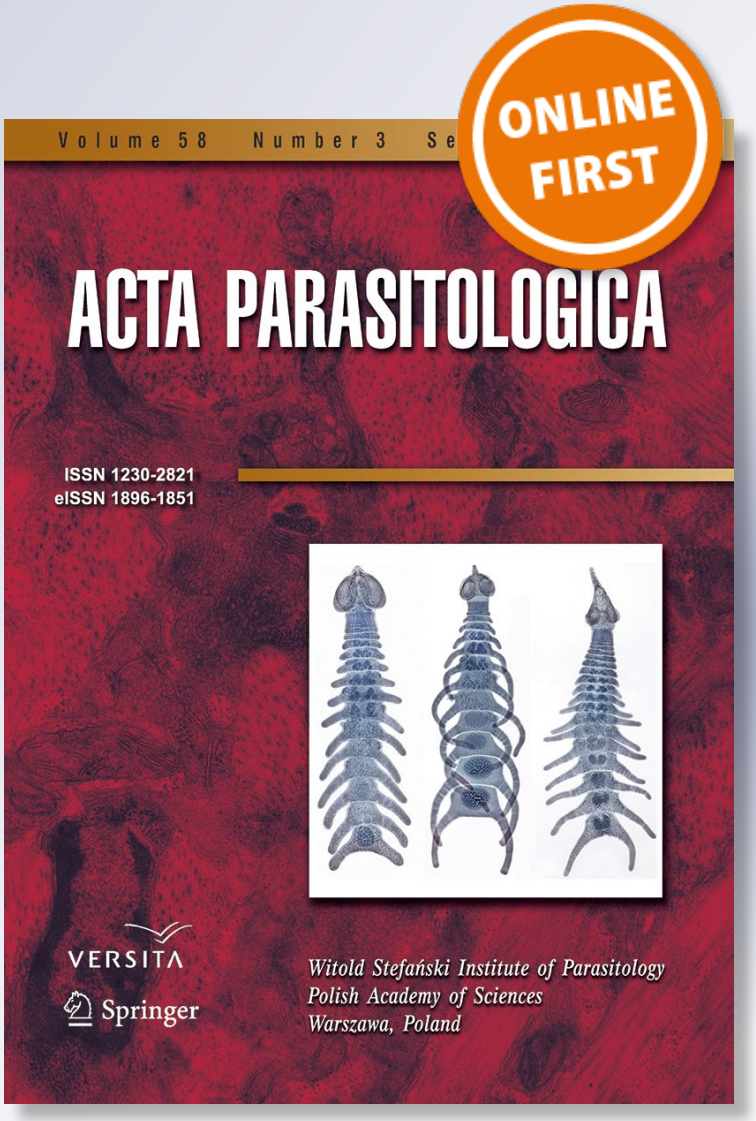

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ORIGINAL PAPER

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

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Abstract

Purpose Fascioliasis is a freshwater snail-borne zoonotic trematodiasis of high pathogenicity and wide veterinary repercussions. In South America, moreover, it causes serious public health problems, with high human infection rates in Andean countries. Ecuador offers a worrying risky scenario due to its physiography, including many human infection reports and animal endemicity throughout its Andean highlands.

Methods Endemic areas with increasing animal fascioliasis were surveyed for lymnaeid snails in the province of Loja, southern Ecuador, close to the border of Peru, the country known to present the widest human fascioliasis endemic zone. The altitude of the sampling sites ranged between 150 and 1770 m a.s.l., and their location was close to human villages. Biotopes surveyed were characterized according to fascioliasis transmission needs.

Results The species *Lymnaea schirazensis* and *L. neotropica* were identified by rDNA ITS-2 and ITS-1 sequencing. The non-transmitting *L. schirazensis* combined haplotype agreed with populations of this species previously reported from northern Ecuador. The finding of the efficient vector *L. neotropica* is reported for the first time in Ecuador and suggests a passive introduction from neighbouring Peru by uncontrolled livestock transport.

Conclusions Rice irrigation system implementation, lymnaeid finding on *Taraxacum* (dandelion) plants which are consumed fresh in salads by people, and *Saccharum* (sugarcane), whose bark is peeled off with the teeth, represent potential infection sources for humans. The closeness to the Cajamarca human hyperendemic area in northern Peru, where the same two lymnaeids have been also found and triclabendazole resistance reported, is an additional risk to be considered regarding the livestock transborder exchange.

Keywords Lymnaeidae · *Lymnaea neotropica* · *Lymnaea schirazensis* · rDNA combined haplotyping · Vectors · Fascioliasis epidemiology · *Fasciola hepatica* · Southern Ecuador · South America

Introduction

Digenean species belonging to the genus *Fasciola* cause fascioliasis, a freshwater snail-borne, zoonotic trematodiasis. Well known due to its wide repercussions in livestock husbandry [22, 48], it was only considered a disease of secondary importance in public health owing to the relatively low number of human infection reports in the 1970–1990 period [21]. However, the description of many human fascioliasis endemic areas in the Americas, Asia, and Africa and a progressive increase of human infection reports in numerous countries from the 1990 decade onwards designed a completely new scenario [45].

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The new scenario was considered of sufficient impact as to include fascioliasis within the list of main neglected tropical diseases by the World Health Organization [71]. This scenario is characterized by a large heterogeneity and complexity in transmission patterns and epidemiological situations [43]. Five aspects appear to underlie this trend:

1. Increasing evidences about the high pathogenic capacity of fasciolid liver flukes [36, 46, 68], not only including the acute phase as traditionally considered, but also the chronic phase [63–65] in which infected subjects are typically detected in human fascioliasis endemic areas [26, 27]. The immunosuppression induced by the liver fluke in the chronic phase underlies the usual coinfections of *Fasciola*-infected subjects with other pathogenic parasites [34].
2. The large diversity of human infection sources throughout the different countries of Europe, Asia, Africa, Oceania, and the Americas [47] partly explain the large heterogeneity and complexity in transmission patterns and epidemiological situations, and also the remarkable differences between human and animal fascioliasis.
3. The life cycle is highly dependent on the local characteristics of the climate [31] and allow for mathematical modelling as well as remote sensing and GIS assessments of the geographical distribution of the disease [32]. This pronounced dependence of fascioliasis transmission on climatic factors underlies the marked influences of climate change [44], similarly as it has been observed in other freshwater snail-borne trematodiases [17]. The impact of climate change has already been demonstrated in animal fascioliasis [29, 44] and recently also in human fascioliasis [1].
4. Many aspects comprised within the broad term of global change have also shown to greatly influence fascioliasis. Aspects to be considered in that sense are, for instance, anthropogenic modifications of the environment, importation/exportation of livestock between continents and countries, and also livestock movements inside countries, as well as livestock management according to local traditions and strategies [43]. Man-made constructions of the type of irrigation systems have been proved to cause biseasonal transmission and human infection in an area where the characteristics of the climatic factors would only allow for a monoseasonality [1].
5. Lymnaeidae is a freshwater gastropod snail family including numerous species covering the Old and New Worlds. The susceptibility to transmit *Fasciola* differs depending on the lymnaeid groups and the liver fluke species. *Fasciola hepatica* is mainly transmitted by amphibious species of the *Galba/Fossaria* group [10, 11] present in Europe, Asia, Africa, Oceania, and the Americas, whereas *F. gigantica* is mainly transmitted by the more aquatic species of the *Radix* group which is absent in Oceania and the Americas [9]. Fascioliasis is a typical vector-borne disease in which vector species are crucial in defining the geographic distribution, seasonality, transmission intensity, and epidemiological features of the disease according to the ecological and ethological characteristics of the lymnaeid species involved in each endemic area [9, 11, 12, 15, 16]. Assessing the lymnaeid transmitting species becomes, therefore, one of the essential baselines in each fascioliasis area.

South America highlights because of presenting more human endemic areas than any other region of the world [43, 45]. These human endemic areas are found in the highlands of the Andean countries [16]. Peru is the country with the largest human endemic zone, which extends mainly throughout the whole Andean chain, including valleys [6, 35, 42, 66] and Altiplano [28]. Bolivia is, however, the country in which the highest prevalences and intensities in humans have been described, namely in the Northern Altiplano [26, 27]. Other human endemic areas have been described in Chile [3, 5] and more recently in Argentina [15, 41, 50]. Many human infection cases have also been reported from northern South America, including Venezuela [12] and Colombia [13].

The geographically strategic situation of Ecuador, with physiographic characteristics dominated by an Andean highland chain running throughout the whole country along a north to south axis, makes Ecuador a potential wide zone for fascioliasis transmission affecting both livestock and humans [19, 37, 58]. In this country, moreover, the zone throughout the southernmost valleys is well known to livestock owners by the disease endemicity in cattle and goats. In addition, these southern Ecuadorian valleys are epidemiologically important from the international point of view due to the usual exchange of livestock with the neighbouring Peru, a country where fascioliasis causes large veterinary losses [25]. Despite of all this, research on lymnaeid snail vectors involved in the transmission of the disease in this wide southern zone of Ecuador has never been conducted. This has been the objective of the present study, by means of the DNA sequencing of selected nuclear ribosomal markers which have previously demonstrated their usefulness for the specific classification of snail specimens of Lymnaeidae, a gastropod family in which the shell morphology and soft part anatomy have repeatedly shown the uselessness of the traditional malacological methods [7, 9, 11, 12, 15, 16].

Materials and Methods

Field Surveys

Malacological surveys were conducted in livestock grazing lands located in the cantons of Vilcabamba (one sampling site), Gonzanamá (one site called Sta. Barbara and another site), Macará (three sites), and Zapotillo (two sites) of the Loja province, southern Ecuador, in March 2018 (Fig. 1). Snails were searched for in marshes, ditches, stagnant water, streams, ponds, shallow lakes, and also on the ground and plants near freshwater collections. Sampling sites were selected considering the characteristics of animal fascioliasis infection foci, based on the information provided by veterinarians responsible for the sanitary conditions at slaughterhouses in the studied area. Sampling sites had a moderate slope (5–10%) and a slightly undulated relief.

Lymnaeids were initially distinguished from other freshwater snails cohabiting in the same habitats mainly by their small, smooth and dextral conical shell, and their pair of triangular tentacles with darkly pigmented eyes at their bases. This was, however, not always affordable in insufficiently humid sites where the snails were not active. Snails were collected between 10.00 h a.m. and 02.00 h p.m., by means of flat clamps using the timed-search method (one person over 10 min per site). Once collected, they were fixed in properly labelled containers with 96% ethanol and transported to the laboratory for further analyses.

The following data were determined for each sampling site: geographic coordinates using GPS, altitude, canton, type of habitat, surface area, distance from the site to the closest village, number of lymnaeid specimens collected, co-occurring non-lymnaeids snails, presence of water, height of water column, air temperature, relative humidity, presence of vegetation, soil pH, and any additional relevant information (Table 1). Data were entered into an Excel file with the purpose of future mapping with ArcGIS software.

Molecular Techniques

Snail Materials

Localities furnishing the lymnaeid specimens sequenced are noted in Table 1. The molecular characterization of the snails has been made by DNA sequencing of the complete nuclear ribosomal DNA (rDNA) spacers ITS-2 and ITS-1. These markers have already shown their usefulness for the classification of the lymnaeid vector species and the

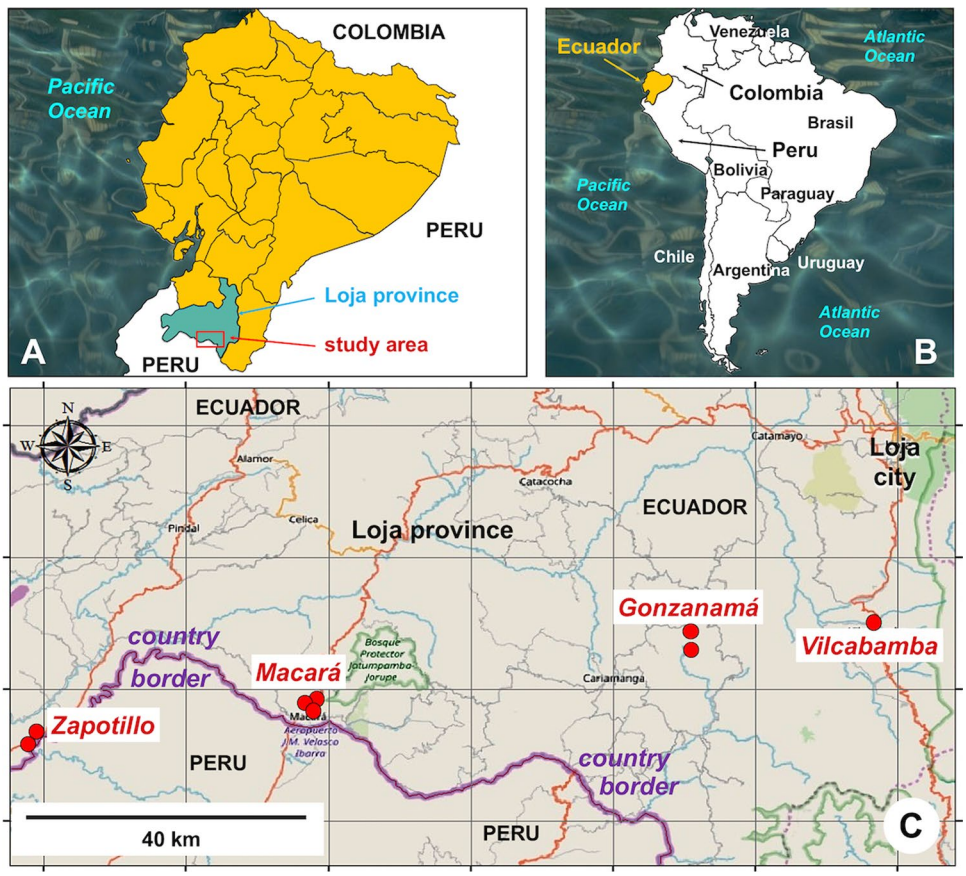

Fig. 1 Maps showing the location of the localities and lymnaeid snail sampling sites surveyed: **a** map of South America showing the location of Ecuador neighbouring northern Peru and southern Colombia; **b** map of Ecuador showing provinces, including the southern province of Loja and study area highlighted; **c** map of the southern zone of Loja province showing the localities and lymnaeid snail sampling sites surveyed close to the country border with Peru

Table 1 Distribution and characteristics of the sampling sites where lymnaeid snail specimens were collected in animal fascioliasis endemic areas of the province of Loja, southern Ecuador

Sampling site	Canton	Geographical coordinates	No. lymnaeids collected	Distance to the closest village (km)	Biotope area (m^2)	Altitude (m a.s.l.)	Air temperature (°C)	Relative humidity (%)
1	Vilcabamba	04°15′ 10.9″S 79°13′ 29.4″W	1	1	100	1523	28	64
2	Gonzanamá–Santa Barbara	04°16′ 01.0″S 79°27′ 37.9″W	11	6	36	1773	26	96
3	Gonzanamá	04°16′ 50.7″S 79°27′ 29.0″W	7	2	50	1694	25	96
4	Macará	04°21′ 37.6″S 79°56′ 08.4″W	3	2	226	494	31	47
5	Macará	04°21′ 39.9″S 79°56′ 08.5″W	9	1	4	504	31	47
6	Macará	04°22′ 08.7″S 79°56′ 18.9″W	3	1	500	480	33	47
7	Zapotillo	04°24′ 13.7″S 80°16′ 57.4″W	6	3	123	160	31	21
8	Zapotillo	04°25′ 01.1″S 80°17′ 36.2″W	7	0.1	528	153	38	21

For the geographical location of the biotopes, see Fig. 1c

assessment of their biogeographic distribution by comparative analyses [7, 9–13, 15, 16].

DNA Sequencing

For DNA extraction, the snail head–foot tissue fixed on ethanol 70% were used and processed individually, as previously described [8, 16]. Total DNA was isolated according to the phenol–chloroform extraction and ethanol precipitation method, and stored at − 20 °C until use.

Each one of the DNA markers was 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 [9, 10]. Amplification procedures and thermal cycler conditions for each one of the DNA markers were carried out in a Mastercycler ep *gradient* (Eppendorf, Hamburg, Germany), as previously described [11, 43].

PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, 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 on a 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 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using PCR primers.

Sequence Analyses

Sequences were edited and assembled using Sequencher v5.4.6. (Gene Codes Co.) and aligned using CLUSTALW2 [39] in MEGA 6.0.6 [57], using default settings. Minor corrections for a better fit of nucleotide or indel correspondences were made. Homologies were performed using the BLASTN program 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 [7, 43]. It shall be noted that haplotype codes are only definitive in the case of complete sequences of the marker in question, as it is here the case for both ITSs.

Results

Characteristics of Fascioliasis Foci

Lymnaeid snails were found in eight sampling sites, with different types of biotopes (Fig. 2), including ditches, marshes, ponds, and streams at approximately similar frequency of around 25%. The surface area of the aforementioned freshwater collections ranged from small ones of only 4 m^2 up to large ones reaching more than 525 m^2, with a mean of 196 m^2. The depth of the water column ranged between 2.3 and 20 cm, soil pH between 6 and 7, temperature between 25 and 38 °C, and relative humidity between 21 and 96%. The data according to each sampling site are noted in Table 1.

The most frequent plants occurring in the biotopes with lymnaeid snails belonged to the genera *Typha*, *Taraxacum*, *Brachiaria*, *Panicum,* and *Saccharum*. Trees shorter than 5 m were also present. Animal tracks, cattle faeces, and slightly turbid streams with slow-flowing water were recorded.

Lymnaeid snails were predominantly found on the ground, e.g., in six out of the eight sampling sites (75%), whereas they were collected on plants in the remaining 25%. Moreover, lymnaeids were also found in water. All the lymnaeid specimens collected were showing a small, smooth, and dextral conical shell typical of members of the *Galba/Fossaria* group. In all of the eight sampling sites, lymnaeids shared the habitat with snails of the family Physidae.

In addition, data of epidemiological interest to be considered regarding fascioliasis transmission included a large range of altitudes from the lowest biotope of Zapotillo at 153 m a.s.l. up to the highest of Gonzanamá–Santa Barbara at 1773 m a.s.l. The number of lymnaeid specimens collected per sampling site is noted in Table 1, and the estimations indicated a low mean number of six snails per biotope, which fits well to the conditions of high temperatures in the year dry season of the sampling.

An aspect which should be emphasized is the closeness of all these sampling sites from the nearest village, which ranged between 0.1 and 6 km, with a mean of only

Fig. 2 Biotopes where lymnaeid snails were found in Loja province, southern Ecuador: **a** typical site of the extremely amphibious *Lymnaea schirazensis* found under low growing vegetation in Vilcabamba; **b** cattle grassland with *L. schirazensis* besides lateral drainage canal in Gonzanamá; **c** small irrigation canal surrounding grazing plain with *L. neotropica* found in water, in cattle farm at Macará; **d** main and secondary canal system assuring water for irrigation sites where *L. neotropic*a was collected at Zapotillo

2 km (Table 1), indicating the potential risk for human infection.

Ribosomal DNA ITS-2 Sequencing

Two different sequences of the second internal transcribed spacer of the nuclear ribosomal DNA were obtained from the lymnaeid specimens sequenced (Table 2).

An ITS-2 sequence of a length of 436 bp and a 53.90% GC content was found in the localities of Zapotillo, Macará, Santa Barbara, and Vilcabamba. The BLASTN analysis demonstrated that lymnaeids presenting this sequence belong to the species *Lymnaea schirazensis*. Moreover, when compared to the two ITS-2 haplotypes of *L. schirazensis* available in the GenBank, the alignment comparisons probed that the ITS-2 sequence from these four south Ecuadorian localities was identical to the previously described haplotype L.schi-H2 (GenBank: JF272602). This haplotype differs from the other haplotype known L.schi-H1 in only 8 polymorphic sites, corresponding to 8 indels caused by a microsatellite tetranucleotide repeat (TGCT), being absent in the haplotype 2 and present twice in the haplotype 1 between positions 128 and 135 of the alignment.

The other ITS-2 sequence obtained showed a shorter nucleotide length of 417 bp and a GC content of 56.83%. This second ITS-2 sequence was only found in snail specimens collected from the localities of Zapotillo and Macará. The BLASTN analysis demonstrated that lymnaeids presenting this 417-bp-long ITS-2 sequence belong to the species *Lymnaea neotropica*. When compared with the ITS-2 haplotypes of *L. neotropica* available in GenBank (L.neo-H1 and L.neo-H2), the Ecuadorian sequence proved to be identical to the previously described ITS-2 haplotype 1 (H1) for *L. neotropica* from the type locality of this species in Peru (GenBank: AM412225). This haplotype L.neo-H1 differs from the other haplotype L.neo-H2 by one transition and two indels.

Ribosomal DNA ITS-1 Sequencing

Similarly as with the ITS-2 marker, two different ITS-1 sequences were found in the sequencing analyses of the same lymnaeid specimens used for molecular characterization (Table 2).

An ITS-1 sequence of a length of 531 bp and a 59.12% GC content was found in the localities of Zapotillo, Macará, Santa Barbara, and Vilcabamba. The BLASTN analysis of the ITS-1 spacer demonstrated that lymnaeids presenting this sequence belong to the species *L. schirazensis*. This ITS-1 sequence perfectly correlated to the aforementioned ITS-2 sequence of this lymnaeid species in the same specimens. This 531-bp-long sequence was compared with the two ITS-1 haplotypes of *L. schirazensis* available in the GenBank (L.schi-HA and L.schi-HB) and proved to be base-to-base identical to the previously described L.schi-HB (GenBank: JF272604). One transversion and two indels allow for the differentiation of these two haplotypes.

Another ITS-1 sequence was 533 bp long, showed a GC content of 56.66%, and was found in lymnaeid specimens from the localities of only Zapotillo and Macará. The corresponding BLASTN analysis of this ITS-1 spacer demonstrated that specimens presenting it belonged to the species *L. neotropica* and that these specimens were also the same showing the *L. neotropica* ITS-2. This sequence was compared with the ITS-1 haplotype of *L. neotropica* available in GenBank and proved to be identical to L.neo-HA from the type locality of this species originally described in Peru. The haplotype L.neo-HA differs from the other haplotype L.neo-HB by only two insertions in the latter.

Discussion

Epidemiological Analyses

In Ecuador, infections of livestock with *F. hepatica* have been reported from provinces along the inter-Andean corridor [38, 40, 51, 69], such as Azuay [55], Cañar [18],

Table 2 Freshwater snails collected according to localities surveyed in the southern part of Loja province, Ecuador, and respective lymnaeid species and haplotypes identified by nuclear ribosomal DNA ITS-2 and ITS-1 sequencing

Localities	Type of water bodies	Snails collected	Lymnaeid codes	Lymnaeid species	ITS-2 haplotype	ITS-1 haplotype
Vilcabamba	Small river	One lymnaeid, Physidae	LELV1	*L. schirazensis*	L.schi-2	L.schi-B
Gonzanamá–Santa Barbara	Irrigation ditch	Lymnaeidae, Physidae	LELSB1-5	*L. schirazensis*	L.schi-2	L.schi-B
Macará	Pond	Lymnaeidae, Physidae	LELM1	*L. schirazensis*	L.schi-2	L.schi-B
			LELM2-6	*L. neotropica*	L.neo-1	L.neo-A
Zapotillo	Irrigation ditch	Lymnaeidae, Physidae	LELZ4	*L. schirazensis*	L.schi-2	L.schi-B
			LELZ1-3	*L. neotropica*	L.neo-1	L.neo-A

Chimborazo [51], Cotopaxi [59], Tungurahua [52], Pichincha, Imbabura [56], Carchi [4], and Bolívar [24].

Human infection has been reported in Ecuador since long ago. The first human fascioliasis report in an Ecuadorian citizen was in the late 1960s [2], shortly followed by another [53], whereas the most recent has been reported 50 years later [19]. In between, more than 45 cases have been reported from Ecuador, mainly from the Andean highland communities [19].

According to veterinarians in charge of slaughterhouses in the studied areas of Loja province, there has been an increase of fascioliasis prevalence in bovine livestock over the last years. Only a few records were available 10 years ago, which were limited to the border with Peru. Such increase in fascioliasis prevalence may be due to the following aspects and corresponding risks:

1. Uncontrolled trade of infected cattle from the neighbouring country of Peru, which has been reported to show high infection rates [25].

    (a) Livestock movements, transport, and export/import have sufficiently proved their risk in spreading fascioliasis by two ways: (1) introduction of liver fluke strains from abroad and (2) hybridization phenomena with local strains owing to the capacity of fasciolid adults for cross reproduction when coinfecting in the liver of the same animal [43]. In the present case of southern Ecuador, this means a risk of spreading of resistance to triclabendazole, the drug of election nowadays for both humans and animals because of its high efficacy as the only drug able to kill both the liver fluke adult stage in the biliary canals and the juveniles migrating through the tissues, as well as the lack of colateral secondary effects [70]. The short distance from the human and animal hyperendemic area of Cajamarca [35], in the northern part of Peru, where triclabendazole treatment problems have been reported [54], may facilitate such a resistance introduction risk.

    (b) Moreover, movements, transport, and exportation/importation of livestock have also demonstrated their capacity to passively expand lymnaeid snails. Indeed, evidence suggests that these amphibious snails may remain in dried mud stuck to the feet of ruminants, then go into hibernation or estivation, and be able to reactivate once in a new location following contact with water or sufficient humidity [43]. The wide spread of several lymnaeid species of the *Galba*/*Fossaria* group argue about such a passive transport of lymnaeid snails, including well-documented examples for the species *G. truncatula* [43], *L. schirazensis* [11], and *L. neotropica* [16].

2. The implementation of irrigation systems for rice (*Oryza sativa*), which affects the microclimate of habitats by improving the proliferation of lymnaeid snails. Indeed, rice cultures are well known as appropriate habitats for lymnaeid populations [61] and the risk of such irrigation systems for fascioliasis transmission has already been highlighted in Peru [28] and specifically for human infection by *Fasciola* throughout [47].
3. Environmental factors associated with the climate change phenomenon, including modifications of mainly temperatures, rainfall, and humidity on soil surface, but also other climatic factors influencing both the amphibious lymnaeid population dynamics and the free living *Fasciola* larval stages of miracidium, cercaria and metacercaria, without forgetting the intramoluscan development of sporocyst and redial generations inside the poikilothermic freshwater snails [1, 31, 44]. Indeed, studies of this type have already focused on Ecuador [33], including reference to the impact of the El Niño–Southern Oscillation in this country which was suggested to underlie a fascioliasis increase in a subsequent 2-year period [30].

When considering all of the aforementioned aspects and the closeness of the disease transmission foci to human settlements, and taking into account the long-term viability of metacercariae and that fasciolid metacercariae from animal isolates are infective for humans [60, 62], a worrying risk for human infection in these rural areas becomes evident. Interestingly in that sense, besides on grasses used for cattle grazing (e.g. *Typha*, *Brachiaria,* and *Panicum*), some lymnaeids were found on plants such as *Taraxacum* (dandelion) which is consumed fresh in salads by people, and *Saccharum* (sugarcane), whose bark is peeled off with the teeth. These plants may represent potential infection sources for humans as already seen in other endemic areas [47].

Lymnaeid Species in Fascioliasis Transmission

The molecular analyses of the lymnaeid snails collected in southern Ecuador by means of the sequencing of two complete ribosomal DNA ITS markers of species resolution level demonstrate that two morphologically similar species are present in the animal fascioliasis endemic areas of the province of Loja: *L. schirazensis* and *L. neotropica*. Both species are first findings for this Ecuadorian province.

Regarding genus ascription of *L. schirazensis* and *L. neotropica*, molecular comparisons inside the *Galba*/*Fossaria* group, and maximum support values obtained for the internal branching nodes in the phylogenetic analyses of the

species of this group, demonstrated that these Neotropical species do not belong to the genus *Galba* defined by its Palaearctic type species *G. truncatula* [15]. Until sequence data from the very large number of *Galba*/*Fossaria* species known from the Nearctic region are obtained, prudence recommends to taxonomically keep *L. schirazensis* and *L. neotropica* in the genus *Lymnaea* sensu lato [11].

The species *L. schirazensis* has been proved to show a very wide geographical distribution, including Asia, Africa, Europe, the Caribbean, North America, and South America [11]. The molecular studies demonstrated a low genetic variability throughout, as a consequence of a recent passive transport by man-made movements of livestock, suggesting a palaeobiogeographical spreading origin in the Near East and subsequent wide spread from the Old World to the New World during the Spanish colonization, e.g., within the last 500 years [11].

Sequence comparison by alignment analyses demonstrated that the populations of *L. schirazensis* from Loja province are all base-to-base identical and correspond to the combined ITS haplotype L.schi-H2B, which is already known from more northern areas of Ecuador, such as in the localities of Guarandauco, Chillogallo (3158 m a.s.l.), La Buena Esperanza, Cayambe (2821 m a.s.l.), and Machachi, Santo Domingo (2810 m a.s.l.) [11]. The same combined haplotype has also been reported from other American countries such as Mexico and Peru [11]. The finding of *L. schirazensis* H2B does, therefore, fit perfectly with previous knowledge. Indeed, *L. schirazensis* has been catalogued as a very useful marker of livestock movements [11].

An important epidemiological aspect is the lack of capacity of *L. schirazensis* to transmit *Fasciola*. The wide multidisciplinary study performed to assess this crucial feature demonstrated that *L. schirazensis* is not a vector of fascioliasis [11]:

- analysis of natural populations: a long study on 8572 specimens belonging to 20 different populations from fascioliasis endemic areas in different continents and countries did not allow for the finding of any specimen shedding fasciolid cercariae [11];
- experimental infections of specimens: in the laboratory study of 338 experimentally infected specimens, it was verified that penetration by the *F. hepatica* miracidium may rarely occur, but never lead to cercarial production, suggesting that the small size of this species (it does not reach a length of 8 mm) might underlie this phenomenon [11]; similar experimental results were obtained by other experts [23];
- ecological characteristics: field studies in numerous fascioliasis endemic areas in different countries and continents demonstrated this lymnaeid to be an extremely amphibious species, sometimes inhabiting biotopes far from the nearest water collection, in sites where there is no way for the *Fasciola* miracidium to hatch from the egg and subsequently swim to contact a snail, suggesting this extreme ecological features to evolutionarily underlie the loss of susceptibility/compatibility [11];
- ethological behaviour in the laboratory: when specimens experimentally kept in containers with fresh natural water are forcedly submerged inside the water, they come out from water by climbing on the container walls immediately; this occurs each time such an attempt is repeated; similarly to the aforementioned ecological observation, this speaks about an only very sporadic contact of this lymnaeid with the water, which in its turn reduces a potential contact with a miracidium to an absolutely negligible probability [11].

In the present field surveys in Loja province, *L. schirazensis* has been the only lymnaeid found in the localities of Vilcabamba and Gonzanamá. This should, however, not be interpreted as the possibility of this species locally playing as a vector able to keep liver fluke endemicity, as has been suggested despite repeatedly finding similar results [20]. Our results more logically suggest that at least another not detected, well-efficient transmitting lymnaeid species should be present in these sites following seasonal population dynamics which may explain its absence (or detection difficulty) during the survey period.

The species *L. neotropica* has a geographical distribution restricted to South America, although it had never been found in Ecuador before [16]. The present study is, therefore, the first report of *L. neotropica* in Ecuador. Similarly to *L. schirazensis*, its low genetic variability also suggests a *L. neotropica* spread occurred recently in time, which has been historically documented to have taken place together with the impressive livestock movements conducted by the Spanish *conquistadores* through the Andean highlands and the eastern lowlands of the Neotropical region [16].

The *L. neotropica* combined ITS haplotype L.neo-H1A found in souther Ecuador has been previously reported in the type locality near Lima, in Peru [10], as well as in Argentina [49], and Uruguay [16]. The closeness of the Ecuadorian sampling sites surveyed to the Peru border suggests an introduction with livestock from this country. In this sense, *L. neotropica*, a lymnaeid adapted to both high and low altitudes [10, 12, 14, 16], should most probably be also present in the higher altitude localities of Vilcabamba and Gonzanamá, where it was not detected in these surveys probably due to the unappropriate season, as indicated by the very low number of lymnaeid specimens collected in each site. Indeed, this lymnaeid is a very efficient fascioliasis vector, confirmed experimentally [16] and also in the field [49].

These two species, *L. schirazensis* and *L. neotropica*, are already known to coexist in fascioliasis hyperendemic areas,

as it is the case of Cajamarca, in northern Peru [14], a Peruvian valley where very high liver fluke infection prevalences and intensities described in children [67] show increasing values with increasing altitudes [35]. Experimental studies have demonstrated the higher transmission capacity of lymnaeid populations from altitude [16] and allow understanding similar epidemiological situations in North America [72].

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Santo Domingo, 21 de julio del 2020

Firma

.....

Guamán Guamán, Rocío Noemí

C.C.: 1722987003

UNIVERSIDAD DE LAS FUERZAS ARMADAS
INNOVACIÓN PARA LA EXCELENCIA

DEPARTAMENTO DE CIENCIAS DE LA VIDA Y LA AGRICULTURA

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Santo Domingo, 21 de julio del 2020

Guamán Guamán Rocío Noemí

C.C. 1722987003

CERTIFICADO

Santo Domingo de los Tsáchilas, 05 de Febrero del 2020.

Por medio dela presente tengo a bien certificar que: la Srta. Guamán Guamán Rocío Noemí con cédula de identidad 1722987003, es autora y ha presentado a la revista Acta Parasitologica ISSN: 1230-2821 (Print) 1896-1851 (Online) (Witold Stefanski Institute of Parasitology, Polish Academy of Sciences 2019) el artículo científico titulado "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", esta contribución se encuentra publicada en el volumen 64, número 4 en las páginas 839-849 desde el 16 de Agosto de 2019. Se adjunta además el link de la revista donde consta la publicación online en Springer https://link.springer.com/article/10.2478/s11686-019-00104-1

Sin más por el momento me suscribo de usted no sin antes expresar mis más altas seguridades de estima.

Muy atentamente;

My. Angel Villavicencio A. PhD
Director de Investigación.
Universidad de las Fuerzas Armadas.