



## Spread of the fascioliasis endemic area assessed by seasonal follow-up of rDNA ITS-2 sequenced lymnaeid populations in Cajamarca, Peru<sup>☆</sup>

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### ABSTRACT

Fascioliasis is a worldwide emerging snail-borne zoonotic trematodiasis with a great spreading capacity linked to animal and human movements, climate change, and anthropogenic modifications of freshwater environments. South America is the continent with more human endemic areas caused by *Fasciola hepatica*, mainly in high altitude areas of Andean regions. The Peruvian Cajamarca area presents the highest human prevalences reported, only lower than those in the Bolivian Altiplano. Sequencing of the complete rDNA ITS-2 allowed for the specific and haplotype classification of lymnaeid snails collected in seasonal field surveys along a transect including 2007–3473 m altitudes. The species *Galba truncatula* (one haplotype preferentially in higher altitudes) and *Pseudosuccinea columella* (one haplotype in an isolated population), and the non-transmitting species *Lymnaea schirazensis* (two haplotypes mainly in lower altitudes) were found. Climatic seasonality proved to influence *G. truncatula* populations in temporarily dried habitats, whereas *L. schirazensis* appeared to be more climatologically independent due to its extreme amphibious ecology. Along the southeastern transect from Cajamarca city, *G. truncatula* and *L. schirazensis* shared the same site in 7 localities (46.7% of the water collections studied). The detection of *G. truncatula* in 11 new foci (73.3%), predominantly in northern localities closer to the city, demonstrate that the Cajamarca transmission risk area is markedly wider than previously considered. *Lymnaea schirazensis* progressively increases its presence when moving away from the city. Results highlight the usefulness of lymnaeid surveys to assess borders of the endemic area and inner distribution of transmission foci. Similar lymnaeid surveys are still in need to be performed in the wide northern and western zones of the Cajamarca city. The coexistence of more than one lymnaeid transmitting species, together with a morphologically indistinguishable non-transmitting species and livestock movements inside the area, conform a complex scenario which poses difficulties for the needed One Health control intervention.

### 1. Introduction

Among the diseases included in the so-called Neglected Tropical Diseases (NTDs) by the World Health Organization [1], trematodiases have entered in the main focus due to their high susceptibility to the

impacts of climate and global changes. Besides a geographical spread of the disease, other type of influences have also been observed in fascioliasis, the disease more dependent on such changes within the NTD group of the food-borne trematodiases [2], including not only modifications of prevalences and intensities in humans and animals, but also

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changes in the disease transmission and human and livestock reservoir incidence seasonality [3]. The anthropogenic capacity to spread fascioliasis from the original palaeobiogeographical areas of the two causal fasciolid species *Fasciola hepatica* and *F. gigantica* up to reaching the worldwide distribution of this disease at present has been already analyzed in detail [4,5].

Human fascioliasis was considered of secondary interest in public health until the 1990s [6], in which the scenario changed completely after the progressive description of many human endemic areas in different continents [7]. In humans, this disease shows an unexpected complexity regarding both epidemiological situations and transmission patterns [4,7]. Aspects in great part linked to the human infection sources in both developing and developed countries [8,9] underlie pronounced differences regarding animal fascioliasis, which continues to worldwide cause big veterinary losses in livestock husbandry [10,11].

Latin America is the world zone where more human fascioliasis endemic areas have been reported. Although such situations have been described in Central America [12], it is the Andean region where most public health problems have been reported. Peru is the country where the highest number of people both infected or at high risk is estimated to live [4], covering the whole wide Andean zone and including numerous human hyperendemic areas. Both human and animal fascioliasis were proved to have a very high impact on the economy of the zones affected by the disease [13].

Among these areas of the Peruvian Andes, local prevalences of human fascioliasis in the northern Department of Cajamarca ranged from 6.7 to 47.7% (mean 24.4%) by coprology and proved to be the highest so far recorded throughout [14], only lower than those found in the Northern Bolivian Altiplano. Several studies have analyzed epidemiological aspects of fascioliasis in humans but also livestock in Cajamarca [15–23], including the analysis of infection risk factors [14,24]. It should be highlighted that fascioliasis in the Cajamarca area follows the so-called “valley pattern of transmission” [4], which is characterized by disease transmission seasonality [25] and so differs from the “altiplanic transmission pattern” in which seasonality is absent or negligible [26]. Additional studies have focused on strategies for the control of fascioliasis in the Cajamarca area [27] and a One-Health integrated approach has even been proposed for such a purpose [28]. Such a control action becomes appropriate for Cajamarca mainly due to the appearance of resistance to triclabendazole in this hyperendemic area [29], the actual drug of choice [30] on which the worldwide control initiative of the World Health Organization relies [31].

Unfortunately, in Cajamarca the knowledge available is still far from the multidisciplinary knowledge needed. Crucial aspects such as the geographical extent of the endemic area and the lymnaeid species and strains involved in the transmission of the disease still show many gaps. The presently ongoing One Health initiative for the fascioliasis control in the Northern Bolivian Altiplano, under coordination by the World Health Organization (WHO Headquarters Geneva) and the Pan American Health Organization (PAHO Headquarters Washington DC), has recently demonstrated the importance of efforts towards defining the latitudinal, longitudinal and altitudinal boundaries and inner patchy distribution of the lymnaeid snails acting as intermediate host (the term “vector” is also used) assuring the transmission of the disease [32]. The aim of the present study is to increase the knowledge about the outline of fascioliasis transmission by assessing the geographical distribution and seasonality of lymnaeid snail populations in the Department of Cajamarca. Owing to the presence of different morphologically indistinguishable species belonging to the *Galba/Fossaria* group of lymnaeids [33], already previously detected in the Cajamarca area [34], the second transcribed spacer of the nuclear ribosomal DNA (rDNA ITS-2) has been selected. The ITS-2 has proved to be the DNA marker of choice for the aforementioned purpose of species classification applied to lymnaeid snails in general [35] and also throughout South America [36].

## 2. Materials and methods

### 2.1. Lymnaeid snail surveys

Field surveys for the collection of lymnaeid snails were performed in 15 localities of the Department of Cajamarca, northern Peru. Locality names, respective districts and provinces, and corresponding coordinates and altitudes are included in Table 1. The geographical distribution of these localities is noted in Fig. 1. The selection of the zones to be surveyed was made by considering the following criteria: (i) zones never prospected regarding the presence of lymnaeid snails, to contribute new knowledge about their distribution; (ii) zones where livestock was present, indicating the possibility for the life cycle of the liver fluke to occur; (iii) zones close to human dwellings or where human activities were developed, enabling human infection risk. The surveys followed a south-eastern transect between the neighborhood of Cajamarca city and the village of Cajabamba, the two extreme zones where lymnaeids have been previously reported (Fig. 1) [34]. Efforts were made to cover a wide range of altitudes, from 2007 m a.s.l. up to 3473 m a.s.l. (Table 1), owing to the relationship between altitude and *F. hepatica* prevalences previously observed in this hyperendemic area [14].

Snails were searched for in sites presenting small, permanent or temporary freshwater collections such as irrigation canals but also marshes, ditches, stagnant water, streams, ponds, and also on the ground and plants near freshwater collections, which are known to be typically inhabited by the amphibious lymnaeid snails. Sampling sites were selected also considering the characteristics of animal fascioliasis infection foci, based on the information provided by local veterinarians. 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. Snails were collected during sunshine hours, by means of flat clamps. In the freshwater habitat of each locality, snail search and collection was performed by three persons during 30 min, according to the timed-search method [37].

Because of the seasonality of the transmission of fascioliasis in the Cajamarca area, each sampling site was surveyed four times during a period of one year, with the aim to cover the four seasons according to local availabilities, namely in September 2015 (winter), November 2015 (spring), March 2016 (summer), and May 2016 (autumn).

Many local ecological characteristics were measured and appropriately noted in each seasonal survey for future ecological analyses of the “valley transmission pattern”, in the way to compare with the same features of the “altiplanic transmission pattern” reported from the Northern Bolivian Altiplano human hyperendemic area [26,32]. Once collected, the snails were fixed in properly labelled containers with 96% ethanol and transported to the laboratory for subsequent study.

### 2.2. Molecular techniques

#### 2.2.1. Lymnaeid specimens

Localities furnishing the snail materials for sequencing are noted in Table 1. The molecular characterization of the snails has been made by DNA sequencing of the rDNA ITS-1 marker in the complete length of this intergenic spacer. This marker has already shown its usefulness for the classification of the lymnaeid species and the assessment of their biogeographic distribution throughout South America by comparative analyses [5,38–41].

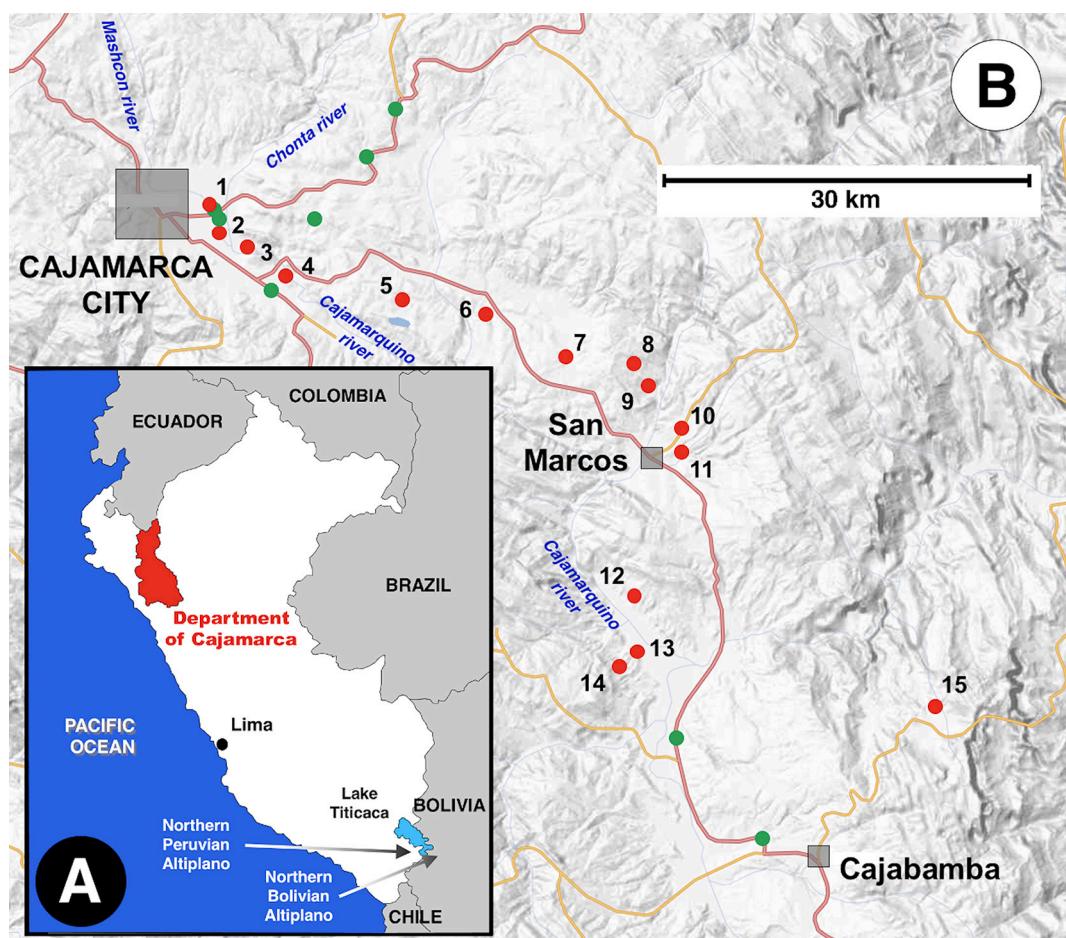
#### 2.2.2. ITS-2 sequencing

Ethanol-fixed tissue of the snail head-foot was used for DNA extraction and individually processed, as noted in other similar studies [42]. The phenol-chloroform extraction and ethanol precipitation method was used for total DNA isolation, which was subsequently stored at  $-20^{\circ}\text{C}$  until use.

**Table 1**

Geographical origin of the populations of Lymnaeidae snails prospected in the Department of Cajamarca, Peru. For localities in map see numbers in Fig. 1.

No.in Map	Locality	District	Province	Latitude	Longitude	Altitude m.a.s.l.
1	Tartar Chico	Baños del Inca	Cajamarca	S 07°09'05.1"	W 078°27'41.8"	2706
2	La Victoria	Baños del Inca	Cajamarca	S 07°07'42.6"	W 078°26'54.1"	2737
3	Huayrapongo Grande	Llacanora	Cajamarca	S 07°11'00.1'	W 078°26'48.8"	2627
4	El Llimbe	Llacanora	Cajamarca	S 07°12'15.6"	W 078°23'35.2"	2871
5	Namora - Dirección Regional de la Producción	Namora	Cajamarca	S 07°12'05.9"	W 078°19'15.9"	2729
6	Pampa Larga	Matara	Cajamarca	S 07°14'28.8"	W 078°15'51.2"	2776
7	Tinajones Bajo - Laparpupio	Matara	Cajamarca	S 07°15'34.8"	W 078°13'20.8"	2840
8	Sondor	Gregorio Pita	San Marcos	S 07°13'32.0"	W 078°13'47.1"	2872
9	La Manzanilla	Gregorio Pita	San Marcos	S 07°15'32.0"	W 078°12'07.7"	3091
10	Chuquiamo	Pedro Gálvez	San Marcos	S 07°19'05.0"	W 078°09'44.1"	2344
11	Rancho Grande	Pedro Gálvez	San Marcos	S 07°20'06.3"	W 078°01'34.8"	3473
12	Colpón	Eduardo Villanueva	San Marcos	S 07°26'18.0"	W 078°10'52.7"	2043
13	Cholocal	Cachachi	Cajabamba	S 07°28'35.9"	W 078°10'13.8"	2010
14	El Olivo	Cachachi	Cajabamba	S 07°28'27.3"	W 078°09'22.1"	2022
15	El Tingo	Sitacocha	Cajabamba	S 07°28'15.1"	W 078°07'57.8"	2007



**Fig. 1.** Maps showing location of the Department of Cajamarca within Peru (A) and fascioliasis endemic area surveyed along a southeastern transect from Cajamarca city (B). Lymnaeid snail sampling localities (red circles): a) Cajamarca province: 1 = Tartar Chico; 2 = La Victoria; 3 = Huayrapongo Grande; 4 = El Llimbe; 5 = Namora - Dirección Regional de la Producción; 6 = Pampa Larga; 7 = Tinajones Bajo - Laparpupio. b) San Marcos province: 8 = Sondor; 9 = La Manzanilla; 10 = Chuquiamo; 11 = Rancho Grande; 12 = Colpón. c) Cajabamba province: 13 = Cholocal; 14 = El Olivo; 15 = El Tingo. Green circles = localities where lymnaeid snails were previously reported [38]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The second transcribed spacer was PCR amplified independently for each specimen and each PCR product was sequenced for a bona-fide haplotype characterization. The complete rDNA ITS-2 was amplified using primers previously described [5]. Amplification procedures and thermal cycler conditions for that DNA marker were carried out in a Mastercycle epgradient (Eppendorf, Hamburg, Germany), according to previous descriptions [40].

PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) following 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 a Eppendorf Bio-Photometer (Hamburg, Germany).

The sequencing of each molecular marker was performed on both

**Table 2**

Lymnaeid species and rDNA ITS-2 haplotypes found and numbers of specimens collected according to localities and seasons, in the Department of Cajamarca, Peru. *G. tru* = *Galba truncatula*; *L.schi* = *Lymnaea schirazensis*; *P.col* = *Pseudosuccinea columella*. H = haplotype.

Locality	No. lymnaeids collected and species//haplotypes found				
	Winter (September 2015)	Spring (November 2015)	Summer (March 2016)	Autumn (May 2016)	TOTAL spp. per localities
Tartar Chico	13 <i>L.schi</i> H2	19 <i>L.schi</i> H2	8 <i>G.tru</i> H1	2 <i>L.schi</i> H2	42 <i>G.tru</i> / <i>L.schi</i>
La Victoria	1 <i>G.tru</i> H1	0 –	2 <i>G.tru</i> H1	3 <i>G.tru</i> / <i>L.schi</i> H1	6 <i>G.tru</i> / <i>L.schi</i>
Huayrapongo Grande	0 –	17 <i>G.tru</i> H1	7 <i>G.tru</i> H1	1 <i>G.tru</i> H1	25 <i>G.tru</i>
El Llimbe	226 <i>G.tru</i> H1	54 <i>G.tru</i> H1	211 <i>G.tru</i> H1	13 <i>L.schi</i> H1	504 <i>G.tru</i> / <i>L.schi</i>
Namora - Dirección Regional de la Producción	0 –	0 –	96 <i>G.tru</i> H1	102 <i>G.tru</i> H1	198 <i>G.tru</i>
Pampa Larga	28 <i>G.tru</i> H1	15 <i>G.tru</i> H1	141 <i>G.tru</i> H1	556 <i>L.schi</i> H1	740 <i>G.tru</i> / <i>L.schi</i>
Tinajones Bajo - Laparpuquio	33 <i>G.tru</i> H1 / <i>P.col</i> H1	3 <i>G.tru</i> H1	1 <i>G.tru</i> H1	14 <i>L.schi</i> H1	51 <i>G.tru</i> / <i>L.schi</i> / <i>P.col</i>
Sondor	7 <i>G.tru</i> H1	51 <i>G.tru</i> H1	32 <i>G.tru</i> H1	74 <i>G.tru</i> H1	164 <i>G.tru</i>
La Manzanilla	0 –	0 –	31 <i>G.tru</i> H1	0 –	31 <i>G.tru</i>
Chuquiamo	12 <i>L.schi</i> H2	36 <i>L.schi</i> H2	23 <i>L.schi</i> H2	18 <i>L.schi</i> H2	89 <i>L.schi</i>
Rancho Grande	76 <i>L.schi</i> H1	145 <i>G.tru</i> H1	7 <i>L.schi</i> H1	21 <i>G.tru</i> H1	249 <i>G.tru</i> / <i>L.schi</i>
Colpón	42 <i>L.schi</i> H2	116 <i>L.schi</i> H1	91 <i>L.schi</i> H2	140 <i>G.tru</i> H1	389 <i>G.tru</i> / <i>L.schi</i>
Cholocal	85 <i>L.schi</i> H1	277 <i>L.schi</i> H2	109 <i>L.schi</i> H1	81 <i>L.schi</i> H1	552 <i>L.schi</i>
El Olivo	50 <i>L.schi</i> H1	80 <i>L.schi</i> H1	68 <i>L.schi</i> H1	166 <i>L.schi</i> H1	364 <i>L.schi</i>
El Tingo	83 <i>L.schi</i> H2	57 <i>L.schi</i> H2	43 <i>L.schi</i> H2	26 <i>L.schi</i> H1	573 <i>L.schi</i>
TOTAL spp. per seasons	656 <i>G.tru</i> in 5 loc. <i>L.schi</i> in 7 loc <i>P.col</i> in 1 loc	870 <i>G.tru</i> in 6 loc <i>L.schi</i> in 6 loc	871 <i>G.tru</i> in 9 loc <i>L.schi</i> in 6 loc	1217 <i>G.tru</i> in 6 loc <i>L.schi</i> in 8 loc	3977 <i>G.tru</i> / <i>L.schi</i> / <i>P.col</i>

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.

#### 2.2.3. Sequence analyses

Sequences were edited by using Sequencher v5.4.6. (Gene Codes Co.) and aligned using CLUSTALW2 [43] in MEGA 6.0.6 [44], using default settings. Minor corrections for a better fit of nucleotide or indel correspondences were made, mainly due to the presence of microsatellite repeats. Homologies were performed using the BLASTN programme from the National Centre 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.

#### 2.2.4. DNA haplotype nomenclature

The haplotype (H) terminology used for the sequences obtained follows the standard nomenclature proposed for lymnaeid snails previously described [4,35]. 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 the ITS-2.

#### 2.3. Statistical analysis

Statistical analyses were done using “R Statistical Software 4.0.2 (“R: A language and environment for statistical computing”, [www.r-project.org](http://www.r-project.org))” for Windows. For the analysis of correlation between altitude of

the foci surveyed and seasonal presence/absence of each lymnaeid snail species, a Spearman’s correlation was used because of non-Gaussian data, complemented by a Poisson distribution lineal model for slope assessment. A *P* value less than 0.05 was considered significant.

### 3. Results

#### 3.1. Classification of species and haplotypes

Three species of Lymnaeidae could be identified by ITS-2 sequences: *Galba truncatula*, *Lymnaea schirazensis*, and *Pseudosuccinea columella*. The distribution of these species according to the localities surveyed is noted in Table 2.

All specimens classified as *G. truncatula* showed the same ITS-2 sequence, with a length of 401 nucleotides and a GC content of 59.10%. The comparison alignment of the sequence of the Cajamarca specimens with those of the three known ITS-2 haplotypes of this lymnaeid species available in GenBank (H1,H2,H3) demonstrated that it concerns the ITS-2 haplotype 1 (H1) of *G. truncatula* [GenBank: AJ243017].

There were two different sequences in the specimens of *L. schirazensis*. One was 436-bp long and its GC content was 53.90%. When compared to the two ITS-2 haplotypes of *L. schirazensis* available in GenBank, this sequence proved to be identical to that of the haplotype *L.schi*-H1 (GenBank: JF272601). The other ITS-2 sequence found proved to be 444-bp long, with a GC content of 53.8%, and proved to agree with haplotype *L.schi*-H2 (GenBank: JF272602). The pairwise comparison of these two ITS-2 sequences demonstrated that the difference in length

was due to the presence of a TGCT tetranucleotide microsatellite between positions 128 and 135 of the alignment. This microsatellite is repeated two times in L.schi-H1 but it is absent in L.schi-H2.

Only one sequence of the ITS-2 was found in specimens of the only *P. columella* population detected. This sequence showed a length of 404 nucleotides and a biased GC content of 60.89%. The appropriate alignments with sequences of the different *P. columella* haplotypes available in the GenBank proved the Peruvian ITS-2 to be identical to that of the haplotype P.col-H1 of this species (GenBank: FN598155).

### 3.2. Distribution analyses

The geographical, altitudinal and seasonal distributions of the lymnaeid species and their respective haplotypes found are noted in Table 2.

The species *G. truncatula* has been found in 11 out of 15 localities surveyed (73.3%) and *L. schirazensis* also in 11 out of 15 localities. Despite the wide distribution of these two species, *G. truncatula* appears more often in the northern foci whereas *L. schirazensis* shows an increasing southeastern trend. The species *P. columella* was only found in one locality.

The two species *G. truncatula* and *L. schirazensis* also show different altitudinal trends, the first being more common in the localities of higher altitude up to 3473 m a.s.l., whereas the second more linked to lower altitudes down to 2007 m a.s.l. (Table 1). The respective correlation analyses showed statistically significant results, with  $P = 0.005405$  and  $P = 0.005132$ , respectively (Fig. 2). The species *P. columella* was only sporadically collected at 2840 m a.s.l. in the locality of Tinajones Bajo.

The species *G. truncatula* proved to be the only lymnaeid species present in 4 out of 15 localities surveyed (26.6%), in which it was found in all seasons only in one site (Sondor), whereas it could not be found in one (winter), two (winter and spring), or three (winter, spring and autumn) seasons in the remaining three, namely Huayrapongo Grande, Namora and La Manzanilla, respectively. Summer was the season in which it was collected in these four localities.

The lymnaeid *L. schirazensis* was also detected alone in 4 localities and, opposite to the discontinuous appearance of *G. truncatula* in its localities, *L. schirazensis* showed a continuous presence throughout all seasons. The two haplotypes of *L. schirazensis* showed an irregular

distribution. There were localities in which only one haplotype was found (L.schi-H1 in La Victoria, El Llimbe, Pampa Larga, Tinajones Bajo, Rancho Grande, and El Olivo; L.schi-H2 in Tartar Chico and Chuquiamo), whereas the two haplotypes could be found in other localities (Colpon, Cholocal and El Tingo).

These two lymnaeids shared the same site in 7 localities (46.7%), with *G. truncatula* as the seasonally predominant species in northern localities (except in Tartar Chico) and *L. schirazensis* seasonally prevailing in the southern localities. Interestingly, *G. truncatula* and *L. schirazensis* were both detected at the same time in the autumn sampling in the locality of La Victoria.

The species *P. columella* was only detected in the winter season, simultaneously sharing the same habitat with *G. truncatula* in Tinajones Bajo.

## 4. Discussion

### 4.1. Distribution of lymnaeid species and respective haplotypes

Results allowed to detect one haplotype of *G. truncatula*, two haplotypes of *L. schirazensis*, and one haplotype of *P. columella*.

The haplotype H1 of *G. truncatula* was already found in the Cajamarca area [34] and is widely distributed throughout Europe [36,45,46]. The haplotype H1 of *L. schirazensis* is known from Asia (Iran), Africa (Egypt), Europe (Spain), and the Caribbean (Dominican Republic) [33], and has been also reported in Venezuela [38] and Peru, namely in the locality of Baños del Inca in the Cajamarca area [34]. The haplotype H2 of *L. schirazensis* has been found in Mexico and Ecuador [33] and its finding in the Cajamarca area represents its first report in Peru. The haplotype H1 of *P. columella* was described from Puerto Rico and Venezuela [39], and its detection in the Cajamarca area is its first report in Peru. Interestingly, *Lymnaea neotropica*, previously reported close to Cajabamba (Fig. 1) [34], has not been found in the present study.

Results show the presence of lymnaeids inhabiting suitable biotopes according to a continuous transect between Cajamarca city in the North and the southeastern village of Cajabamba. Whereas *G. truncatula* and *L. schirazensis* prove to be widely spread, *P. columella* appears restricted to

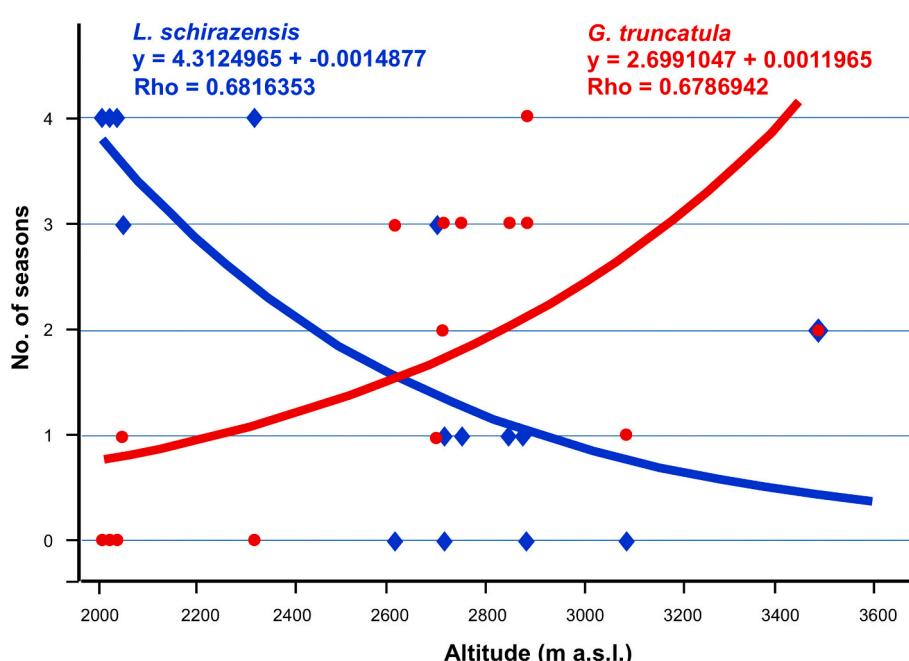


Fig. 2. Significant correlations between altitude of the foci surveyed and seasonal presence/absence of *Galba truncatula* and *Lymnaea schirazensis* in Cajamarca province.

an isolated temporary population, similarly as previously observed in the case of *L. neotropica* [34].

The differences in altitudinal distribution ascertained between *G. truncatula* and *L. schirazensis* are worth mentioning. Indeed, human infection by *F. hepatica* proved to show a statistically significant link with this orographic factor, human prevalences in the Cajamarca hyperendemic area being higher at higher altitudes [14]. The high altitudes of this endemic area may also underlie the sporadic detection of *P. columella*, a species pronouncedly less amphibious than *G. truncatula* and *L. schirazensis* and preferentially linked to lowlands [5,38]. However, the finding of *P. columella* in the winter season in the locality of Tinajones Bajo, at 2840 m a.s.l., agrees with its findings in Laguna Ubaque at 2070 m a.s.l. in Colombia [39] and in Lago San Pablo at 2665 m a.s.l. in Ecuador [47]. Hence, *P. columella* may reach higher altitudes in latitudes close to the equator, where seasonal, average higher temperatures do not vary as pronouncedly as in higher latitudes.

The seasonality involved in the “valley transmission pattern” followed by fascioliasis in the Cajamarca area [25,26], shows an evident influence on the population dynamics of *G. truncatula* and *L. schirazensis*. Local populations of *G. truncatula* were permanently active and thus detected in biotopes where surface freshwater was available during all seasons (as in Sondor and perhaps also in El Llimbe, Pampa Larga, and Tinajones Bajo), but disappeared in situations of seasonal dryness (as in given seasonal surveys in La Victoria, Huayrapongo Grande, Namora, and La Manzanilla), i.e. absence of available water leading them to hibernation buried into the drying soil.

The extreme amphibious characteristics of *L. schirazensis* allows its survival even far away from freshwater collections and remain long time fully active on humid soil, under conditions of total absence of surface freshwaters [33]. This indicates that this species should not, or less, be influenced by seasonality, i.e. disappearance of surface water availability. This explains local detection of specimens in all seasons on the localities surveyed at lower altitudes.

#### 4.2. Applied repercussions in fascioliasis epidemiology and control

The two species presenting only sporadically detectable populations in this area, *L. neotropica* [34] and *P. columella* (present study), are lymnaeids typically linked to animal fascioliasis transmission in lowlands where human infection cases appear isolated, as in Venezuela [38], Uruguay and Brazil [5]. *Lymnaea neotropica* has been involved in the disease transmission in a human endemic area only at intermediate altitudes of 1630–2825 m a.s.l. in Catamarca, Argentina, so far the only exception of human endemic area lacking *G. truncatula* in South America [40].

*G. truncatula* is the lymnaeid hitherto known to have the highest capacity to transmit *F. hepatica* throughout [48], and its marked anthropophilic behavior underlies its involvement in human infection. Such a high transmission capacity appears enhanced at the very high altitude [46], with a fasciolid cercarial shedding period and production [5] longer and higher than those shown by the other lymnaeid species considered of big transmission importance [49].

The fourth lymnaeid species, *L. schirazensis*, has been verified to not transmit *F. hepatica*, both in many experimental infection assays and in nature by analyzing thousands of specimens collected in different continents [33,50].

On the previous lymnaeid survey in the Cajamarca area, the transmitting species *G. truncatula* and *L. neotropica* were found close to Cajabamba, far away from Cajamarca city [34]. These findings stimulated subsequent human surveys which detected *F. hepatica* infection in 4.9% of schoolchildren in Cauday (2807 m a.s.l.) and 8.6% in Ogosón (2807 m a.s.l.) [23]. This demonstrates the usefulness of previous lymnaeid surveys and confirmed that the human endemic area was pronouncedly more spread than previously thought. In human endemic areas at high altitude, schoolchildren appear to be the most affected [4,7,8], including Peru even since long time [51–56] and also the

Cajamarca area [14–16,18–22]. Recent studies have demonstrated this disease to be more pathogenic than previously considered, mainly regarding the chronic phase of the disease in which children are diagnosed in these high altitude rural areas [7,55].

The coexistence of four lymnaeid species in the fascioliasis area of Cajamarca is not an exception. Two or more lymnaeid species have been reported from other human endemic areas in Andean countries: *G. truncatula*, *L. cubensis*, *L. schirazensis* and *P. columella* in Venezuela [38]; *L. cousinsi*, *L. neotropica*, *L. cubensis* and *P. columella* in Ecuador [39,57]; *L. neotropica* and *L. viator* in Argentina [40,58]; and *G. truncatula* and *L. viator* in Chile [41].

The epidemiological characteristics of an endemic area presenting two or more lymnaeid species is very complex. Ecology, ethology, population dynamics, seasonality, anthropophilia and fasciolid transmission capacity of a lymnaeid species define the transmission pattern and epidemiological scenario of fascioliasis in an endemic area [4,8,32,40]. Even the presence of a non-transmitting species as *L. schirazensis* poses additional problems, because its morphological similarity with other *Galba/Fossaria* transmitting species easily leads to confusion between foci involved and not involved in the disease transmission, and this gives rise to misunderstandings in the results of field surveys.

#### 5. Concluding remarks

In diseases transmitted by specific intermediate hosts or vectors linked to freshwater, the geographical distribution of infected human subjects manifests this spatial restriction. In onchocerciasis or river blindness, human prevalences gradually decrease with an increasing distance from the river where the larval stages of the simulid vectors develop. In schistosomiasis, similarly as in fascioliasis, human infection shows a patchy distribution depending on the location of the freshwater collections inhabited by the planorbid snails [59]. However, the opposite is observed in another disease also transmitted by snails, namely angiostrongyliasis or eosinophilic meningoencephalitis, because these snails are terrestrial and less specific. In fascioliasis, infection of schoolchildren has been verified to be patchily distributed around the transmission foci, i.e. freshwater collections inhabited by the transmitting lymnaeids [60], whereas livestock infection does not show such a marked distributional dependence in areas where the domestic ruminants run freely, i.e. they may become infected in a transmission focus but afterwards, with time, move more or less far away from that focus. This occurs in rural areas of Andean highlands where animals are not kept inside fenced land [61], as it is the case of all human endemic Andean areas, including the Cajamarca area.

The two frequent lymnaeid species found are useful biomarkers regarding the aforementioned aspects. *Galba truncatula* and *L. schirazensis* are amphibious snails that share the capacity of being passively transported when they remain in dried mud stuck to the feet of ruminants, then go into hibernation or estivation during the drought, and be able to reactivate once in a new location following contact with water [4,33]. The detection of *Galba truncatula* indicates the freshwater collection to be of risk for liver fluke infection, as it has proved to transmit liver fluke isolates from the different mammal host species in the highlands [61–63]. The non-transmitting species *L. schirazensis* has proved to be a useful biomarker for the follow-up of livestock movements and fascioliasis spread, inter- and intra-continentially and also locally [33]. The finding of this species in almost all localities surveyed indicates its passive spread together with movements of ruminants. A passive transport by other means (e.g., floods, birds, other animals) is negligible when compared to the daily free movements and the water collection frequenting behavior of mainly sheep and cattle. The possibility for human activities to underlie lymnaeid transport from one place to another cannot be disregarded, although in such very high altitude areas it uses to be easily distinguishable (road construction, man-made irrigation canals, plant cultures, etc.).

The present study demonstrates a fascioliasis transmission risk throughout the Cajamarca area markedly more extended geographically than previously considered. These results show the usefulness of field surveys for lymnaeid presence detection to assess the borders of the endemic area and establish the inner patchy distribution of transmission foci, a crucial initial step for an appropriate multidisciplinary One Health control action [32]. It is evident that the outer and inner extent of the Cajamarca endemic area is far from sufficiently known. Similar lymnaeid prospection surveys are still in need to be performed in the wide northern and western zones of the Cajamarca city.

## Ethics statement

No ethics approval nor consent was needed for snail collections done on public land. When in farms, previous consent from owners was obtained. The study was moreover performed after agreement with the Dirección Regional de Agricultura Cajamarca, Gobierno Regional de Cajamarca, whose veterinarian members personally collaborated in the field surveys. The initial protocol for the research initiative was further approved by the Universidad Nacional de Cajamarca, Cajamarca city, and the molecular study was performed in agreement with the Universidad Peruana de Ciencias Aplicadas, Lima.

Laboratory research was performed with the approval of the Evaluation of Projects concerning Animal Research at University of Valencia (Organo Habilitado para la Evaluación de Proyectos de Experimentación Animal de la Universidad de Valencia) (A1263 915,389,140), strictly following the institution's guidelines based on Directive 2010/63/EU. Permission for animal research was additionally obtained from the Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (No. 2015/VSC/PEA/00001 tipo 2).

## Consent for publication

All authors read and approved the final version for publication consideration.

## Availability of supporting data

The datasets generated for this study are available on request to the corresponding author. The data that support the findings are openly available in the GenBank database at <https://www.ncbi.nlm.nih.gov/genbank>, under accession numbers AJ243017, JF272601, JF272602, and FN598155.

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## Author contributions

Study concept and design: SMC; participated in field work: JNBV, CBB, CGP; participated in experimental work: JNBV, MDB, JHV; local coordination, protocols, and logistics: HBZ, JdVM, PO; analysis and

interpretation of data: MDB, PO, SMC; drafting of the manuscript: SMC; critical revision of the manuscript for important intellectual content and for final approval: MDB, JdVM, PO; obtained project funding: JdVM, PO, MDB, SMC; principal investigator: SMC.

## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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