

One Health initiative in the Bolivian Altiplano human fascioliasis hyperendemic area: Lymnaeid biology, population dynamics, microecology and climatic factor influences

Aplicação do conceito One Health na área hiperendêmica de fasciolíase humana do Altiplano Boliviano: biologia dos limneídeos, dinâmica populacional, microecologia e influência de fatores climáticos

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Abstract

Fascioliasis is a freshwater snail-borne zoonotic disease. The Northern Bolivian Altiplano is a very high altitude endemic area where the highest human prevalences and intensities have been reported. Preventive chemotherapy by treatment campaigns is yearly applied. However, liver fluke infection of cattle, sheep, pigs and donkeys assures endemicity and consequent human infection and re-infection risks. A One Health action has therefore been implemented. Activity concerns lymnaeid vectors and environment diversity. Studies included growth, egg-laying and life span in laboratory-reared lymnaeids. Different habitat types and influencing factors were assessed. All populations proved to belong to *Galba truncatula* by rDNA sequencing. Analyses comprised physico-chemical characteristics and monthly follow-up of water temperature, pH and quantity, and lymnaeid abundance and density. Population dynamics in the transmission foci differed. Mean environmental temperature was lower than fluke development minimum temperature threshold, but water temperature was higher, except during winter. A two generations/year pattern appeared in permanent water habitats, and one generation/year pattern in habitats drying out for months. The multidisciplinary control measures can be extended from one part of the endemic area to another. These studies, made for the first time at very high altitude, constitute a baseline useful for fascioliasis control in other countries.

Keywords: Human and animal fascioliasis, lymnaeid vectors, experimental biology, population dynamics, climatic factors, Bolivian Altiplano.

Resumo

A fasciolíase é uma doença zoonótica transmitida para os humanos por formas evolucionárias de *Fasciola hepatica* oriundas de limneídeos infectados. O Altiplano Boliviano Norte é uma área endêmica de altitude muito alta, onde foram relatadas as maiores prevalências e intensidades em infecções humanas. A quimioterapia preventiva por campanhas de tratamento é aplicada anualmente. No entanto, infecção por *Fasciola hepatica* em bovinos, ovelhas, suínos e asininos garante endemicidade e consequentes riscos de infecção e reinfecção humana. Portanto, ações norteadas no conceito “One Health” foram implementadas. As atividades envolveram os limneídeos e a avaliação da diversidade de ambientes. Os estudos incluíram o crescimento, postura de ovos e expectativa de

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vida de limneídeos criados em laboratório, bem como a avaliação da influência dos diferentes habitats. Todas as populações foram identificadas como *Galba truncatula* por meio do sequenciamento de rDNA. As análises incluíram características físico-químicas e acompanhamento mensal da temperatura, pH, quantidade da água, abundância e densidade de limneídeos. A dinâmica populacional nos focos de transmissão diferiu. A temperatura ambiente média foi mais baixa do que o limite mínimo de temperatura do desenvolvimento do helminto, mas a temperatura da água foi mais alta, exceto durante o inverno. Um padrão de duas gerações/ano apareceu em habitats com água permanente, enquanto um padrão de uma geração/ano foi observado em habitats que ficam secos durante meses. Os resultados permitem concluir que as medidas multidisciplinares de controle podem ser estendidas de uma parte da área endêmica para outra. Esses estudos, realizados pela primeira vez em altitudes muito elevadas, constituem uma base útil e extrapolável para o controle da fasciolíase.

Palavras-chave: Fasciolíase humana e animal, limneídeos vetores, biologia experimental, dinâmica populacional, fatores climáticos, Altiplano boliviano.

Introduction

Fascioliasis is a worldwide freshwater snail-borne zoonotic parasitic disease caused by *F. hepatica*, distributed in Europe, Asia, Africa, the Americas and Oceania, and *F. gigantica* restricted to Africa and Asia (Mas-Coma et al., 2009a). Its veterinary importance is due to the great losses it causes in livestock husbandry (Mehmood et al., 2017). The importance of human fascioliasis was recognized from the 1990 decade onwards (Chen & Mott, 1990; Mas-Coma et al., 2014a). Its high pathogenicity (Mas-Coma et al., 2014b; González-Miguel et al., 2019) in both the acute and chronic phases (Valero et al., 2006, 2008, 2016) led the World Health Organization (WHO) to include fascioliasis within the list of main neglected tropical diseases (WHO, 2013). The large diversity of human infection sources partly explain the large heterogeneity and complexity in transmission patterns and epidemiological situations of this disease (Mas-Coma et al., 2018).

The dependence of fascioliasis transmission on climatic factors (Ollerenshaw, 1959) underlie the marked influences of climate change on this disease. The impact of climate change has recently been demonstrated in human fascioliasis (Afshan et al., 2014), and also in animal fascioliasis (Mas-Coma et al., 2009b; Fox et al., 2011).

Latin America is the region in which more human fascioliasis endemic areas have been described. All these areas are at high altitude, as in Mexico (Zumaquero-Ríos et al., 2013), Peru (González et al., 2011; Valero et al., 2012a), Chile (Apt et al., 1993; Artigas et al., 2011), and Argentina (Malandrini et al., 2009; Mera y Sierra et al., 2011; Bargues et al., 2016). Many human cases have also been reported from altitude areas of Venezuela (Bargues et al., 2011a) and Colombia (Bargues et al., 2011b).

Bolivia stands out because of presenting the hyperendemic area where the highest prevalences (up to 72% by coprology) and intensities (up to more than 8,000 eggs of *Fasciola* per g of faeces) in humans have been reported (Hillyer et al., 1992; Bjorland et al., 1995; Esteban et al., 1997; Mas-Coma et al., 1999). The human fascioliasis endemic area is located in the Northern Bolivian Altiplano, between the Lake Titicaca, at 3,820 m altitude, and the city of El Alto, at around 4,100 m altitude, besides the valley of La Paz city, the capital of Bolivia. In 2007-2008, WHO decided to launch a preventive chemotherapy initiative by means of yearly triclabendazole monodose treatment campaigns focusing mainly on children (Villegas et al., 2012; Valero et al., 2012b).

Local efficacy monitorings performed in-between annual campaigns allowed for the detection of new infections and reinfections in children. Results proved a high infection risk, a great problem when considering the absence of premunition in *Fasciola* infection in humans and its immunological consequences (Gironés et al., 2007; Valero et al., 2017). In the Altiplano rural endemic area, livestock species, including cattle, sheep, pigs and donkeys, are crucial for community sustainability, because plant cultures are rarely practiced due to the extreme altitude conditions. Thus, liver fluke infection of these domestic animals assures fascioliasis endemicity and consequent human infection and re-infection risks.

The decision was taken to go for a One Health control action to decrease this infection risk. However, the very wide spread of the endemic area is not adequate for a One Health initiative, because of the impossibility to appropriately assess the so many multidisciplinary factors interacting in fascioliasis transmission. The One Health pilot initiative was therefore restricted to a small zone allowing for the correct monitoring of the different multidisciplinary interventions covering five main axes according to modern standards for such initiatives (Rinaldi et al., 2012; Webster et al., 2016): (i) lymnaeid snail vector populations; (ii) animal reservoirs; (iii) environment and its changing trends including climate change and anthropogenic modifications; (iv) human host; and (v) social, tradition and behavioural aspects.

Such a locally restricted One Health strategy is in need to verify that results obtained can be extended to the rest of the endemic area. The present work is part of these studies concerning the lymnaeid vectors, their environment

diversity and changing trends. Therefore, after the necessary classification of the lymnaeid populations by DNA marker sequencing, combined experimental and field studies were performed. Laboratory studies included growth, egg-laying and life span in laboratory-reared snail cultures. Field studies focused on selected transmission foci, comprising physico-chemical characteristics of the freshwater collections, shell measurements in natural populations, and monthly follow-up of lymnaeid snail population abundance and density, and temperature, pH and quantity of the water collections. Air climatic variables were analyzed after their obtaining by interpolation from data from the closest meteorological stations.

This is the first time that such experimental and field studies are made with lymnaeid vectors from very high altitude. The importance of these studies should be highlighted because in Latin America most of the human fascioliasis endemic areas are located in high altitude zones. The selected objectives, the methodologies used and the way of presenting the results were designed considering their usefulness for health workers in charge for the implementation of control activities in the endemic area, a personnel usually lacking a background in this scientific field. The present work does therefore constitute a baseline useful for fascioliasis control in other countries.

Materials and Methods

Snail habitat types studied

The Northern Bolivian Altiplano human hyperendemic area includes three neighboring parallel, physiographically uniform, flatlands so-called “corridors” expanding between mountainous hill chains (Fuentes et al., 2001): (i) the most northern one extends from El Alto westward up to the Lake and including the villages of Pucarani and Batallas, and northward through Huarina up to Achacachi; (ii) the second “corridor” extends from the El Alto plain in the east up to the Lake Titicaca in the west and includes Tambillo and Huacullani; (iii) a southern “corridor” which extends east-west up to the Lake and including Tiwanaku and Guaqui (Figure 1).



Figure 1. Northern Bolivian Altiplano human fascioliasis hyperendemic area: Map of altiplanic corridors between Lake Titicaca and the cities of El Alto and La Paz. Transmission foci monthly surveyed: 1) Kallutaca, 2) Tambillo 1, 3) Tambillo 2, 4) Quiripujo, 5) Rio Achicala, 6) Chirapaca 2, 7) Chirapaca 1, 8) Batallas, 9) Chijipata Alto. Other localities furnishing lymnaeids for additional experimental procedures: (A) Huacullani, (B) Ancocagua.

The Lake Titicaca influences the climatic characteristics of its neighboring zones by moderating the extreme conditions of the very high altitude (Mas-Coma et al., 1999; Fuentes et al., 1999). Anthropogenic activities modifying the environmental characteristics are observed, usually close to small human communities or villages (Bargues et al., 2020).

The implementation of a One Health action to decrease the human risk of liver fluke infection was decided at a PAHO-WHO meeting in La Paz in 2014. Working axes of research and control, including experimental and field studies on the Altipanic lymnaeids, were designed in 2015 and implemented in the subsequent years 2016-2019 according to the availability of funds. A total of nine transmission foci were selected for a monthly follow-up of the lymnaeid snail vector population dynamics in different types of lymnaeid habitats and influencing factors, in all cases in localities where human and animal fascioliasis showed high prevalences and intensities. These foci were selected among the 57 lymnaeid-inhabited freshwater sites detected in past surveys, and in which around 14,000 specimens were collected and studied in different years and seasons (Bargues et al., 2020). The two very close Tambillo 1 and 2 habitats were prospected to assess the transmission characteristics in a human community. The two very close Chirapaca 1 and 2 foci were prospected to compare two neighboring habitats with temporary water collections. The geographical location and characteristics of each focus are detailed in Table 1 and in Figures 1 and 2.

Table 1. Characteristics of the nine fascioliasis transmission foci of the Northern Bolivian Altiplano human hyperendemic area in which the dynamics of the lymnaeid populations was followed by monthly standardized surveys.

Transmission foci studied	Habitat characteristics	Proximity to livestock and/or humans	Distance from Lake Titicaca	Permanent/seasonal presence of water	Impact of human activity
Kallutaca	canal with neighboring flooding pastureland	besides cattle farm	far away	permanent	none
Tambillo 1	small stream with running polluted water inside village	inside small human community	far away	permanent	seasonal water pollution due to human waste
Tambillo 2	same small stream with cleaner running water	around 30 m outside village	far away	permanent	remaining pollution from close village
Quiripujo	stagnant water in lateral ditch besides non-paved road with water derived from canal	close to human community	close	seasonal	none
Rio Achicala	river bankmarsh with abundant vegetation	many livestock, close to the large human village of Viacha	far away	permanent	none
Chirapaca 2	canal besides paved road	close to human community, isolated ruminants	close	seasonal	none
Chirapaca 1	flooding grassland	close to human community, isolated ruminants	close	seasonal	none
Batallas	man-controlled small irrigation canal	in front of slaughterhouse, close to large human community	close	permanent	human management of canal irrigation
Chijipata Alto	small canal with neighboring flooding area	in front of school with very high prevalence in children	close	permanent	flooding man-made fountain

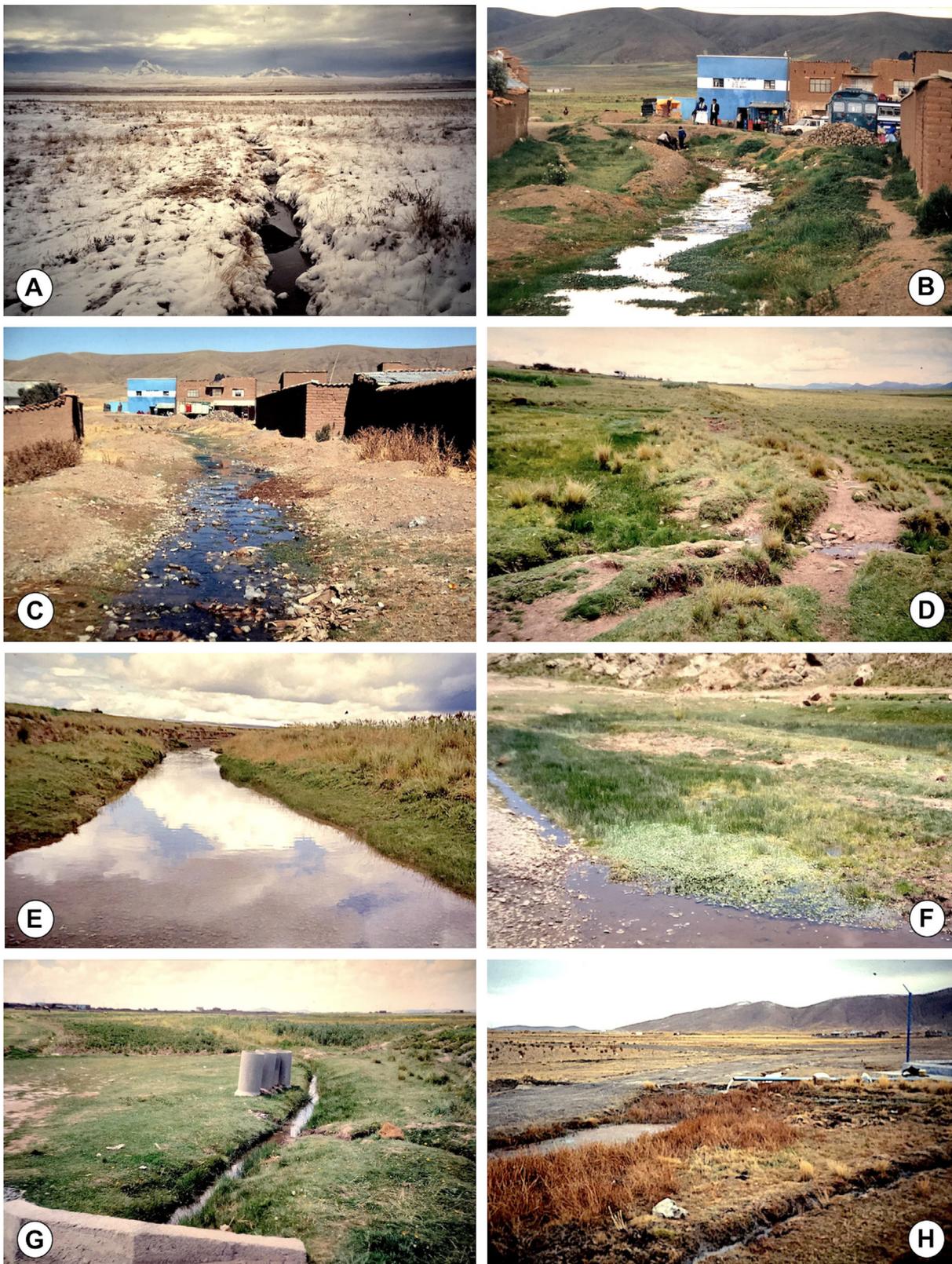


Figure 2. Monthly studied habitats inhabited by *Galba truncatula* in the Northern Bolivian Altiplano human fascioliasis hyperendemic area: (A) Kallutaca: canal with neighbouring flooding pastureland, besides cattle farm, covered by snow in July; (B) Tambillo 1: stream inside village (note aquatic vegetation) in March; (C) same stream (note absence of aquatic vegetation and abundant accumulated human waste) in June; (D) Quiripujo: stagnant water in lateral ditch with water derived from canal, in January; (E) Rio Achicala: river bankmarsh with abundant vegetation, in March; (F) Chirapaca 1: flooding zone, in March; (G) Batallas: man-controlled small irrigation canal in front of slaughterhouse, in February; (H) Chijipata Alto: small canal with neighbouring flooding area by artificial pipe, in June.

Snail studies were conducted in freshwater sites between Lake Titicaca and the city of El Alto, because the water of Lake Titicaca is slightly saline (Boulangé & Aquize Jaen, 1981; Dejoux & Ittis, 1992) and lymnaeid vectors do not survive in a minimum presence of salts. All habitats surveyed were located on public land.

These field activities included monthly surveys which covered a complete twelve month period. They were designed to avoid cutting a climatic season, starting at the beginning of the dry season, i.e. in April, and finishing in March at the end of the rainy season.

Molecular techniques

Three lymnaeid specimens from each snail population studied were molecularly analyzed for species classification, including those followed up in field studies on the nine localities of Kallutaca, Tambillo 1, Tambillo 2, Quiripujo, Rio Achicala, Chirapaca 1, Chirapaca 2, Batallas, and Chijipata Alto, and additionally those used for experimental studies from Huacullani and Ancocagua (Figure 1). Two DNA markers useful for lymnaeid classification were sequenced: the complete nuclear ribosomal DNA (rDNA) spacers ITS-2 and ITS-1 (Bargues et al., 2007, 2011c).

Snail head-foot tissue fixed on ethanol 70% was used for DNA extraction, according to methods previously described (Bargues et al., 1997, 2017). The phenol-chloroform extraction and ethanol precipitation method was applied for total DNA isolation. This isolated DNA was later stored at -20 °C until use.

The ITS markers were PCR amplified independently for each specimen and each PCR product was sequenced for a bona-fide haplotype characterization. ITS-2 and ITS-1 were amplified using previously described primers (Bargues et al., 2003, 2007). Amplification procedures and thermal cycler conditions for each one of the DNA markers were carried out in a Mastercycle *eppgradient* (Eppendorf, Hamburg, Germany) (Mas-Coma et al., 2009a; Bargues et al., 2011c).

The Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) was used for the purification of the PCR products, following the instructions of the manufacturer's protocol. They were subsequently resuspended in 50 µl of 10 mM TE buffer (pH 7.6). The absorbance at 260 and 280 nm on a Eppendorf BioPhotometer (Hamburg, Germany) was measured to determine the final DNA concentration. The dideoxy chain-termination method was used for the sequencing on both strands with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

The complete sequences of ITS-2 and ITS-1 were edited and assembled by using Sequencher v5.4.6. (Gene Codes Co.). The alignments were made by using CLUSTALW2 (Larkin et al., 2007) in MEGA 6.0.6 (Tamura et al., 2013), applying default settings. Minor corrections for a better fit of nucleotide or indel correspondences were made. The BLASTN programme from the National Center for Biotechnology information website (NCBI, 2020) was used for the analysis of homologies. Analyses by sequence comparisons and haplotype identification of lymnaeids were made using data available in the GenBank.

The haplotype (H) terminology used for the ITS-2 and ITS-1 sequences follows the standard nomenclature proposed for these snails (Mas-Coma et al., 2009a).

Shell measurements

A lymnaeid population from Batallas (Figure 1) was used to assess the shell morphometry variability in 60 specimens collected in the field. Another 60 specimens collected from Quiripujo (Figure 1) in the same day were used for comparison purposes (Samadi et al., 2000). These two localities are well known transmission foci and are representative of the two most important endemic corridors. Snails were assured to be free of infection by trematodes by verifying absence of cercarial shedding in Petri dishes containing natural water during 24 h.

Altiplanic lymnaeid specimens studied have been deposited in the parasite/vector collection of the WHO Collaborating Centre on Fascioliasis and its snail Vectors at the Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Spain (WHO CC SPA-37 Collection voucher specimen references: G.tru/Bol/15.04.07.01-60 for material from Batallas; G.tru/Bol/15.04.07.61-120 for material from Quiripujo).

Shell measurements were made by means of a computer image analysis system (CIAS) (Valero et al., 2005). Measurements were taken using a stereo-microscope (Figure 3A). and images captured by a digital camera (Nikon Coolpix), and analyzed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, Maryland, USA). Measurements were made according to a standardized proposal generally followed for lymnaeid snails (Hubendick, 1951). They included only the bare minimum useful for field work: shell length

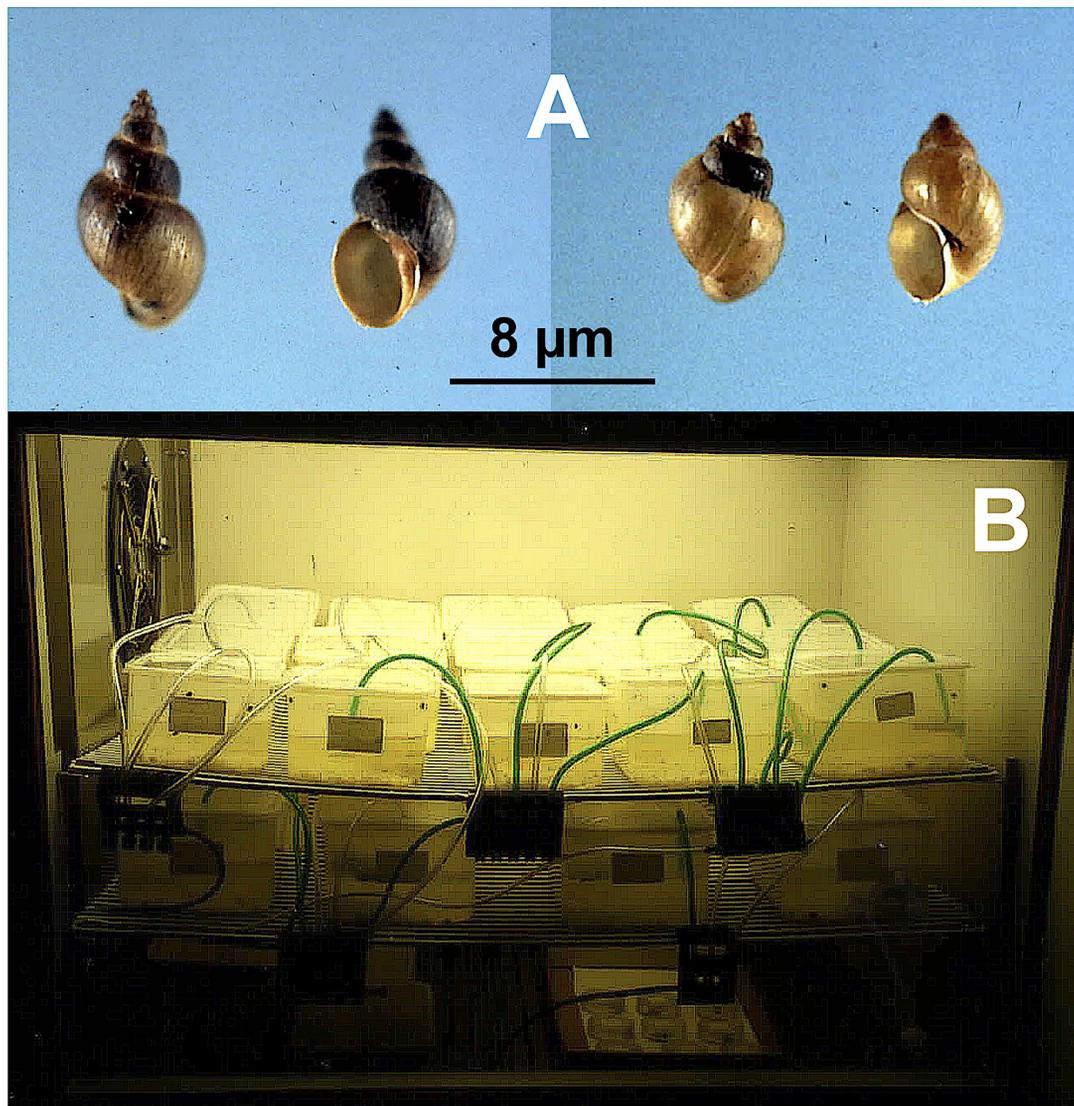


Figure 3. Morphology and culture methods of lymnaeid snails. (A) shells of *Galba truncatula* of the Northern Bolivian Altiplano showing intraspecific morphological variability of extreme morphs, in ventral and dorsal views; (B) breeding boxes containing 2000 ml fresh water for the maintained of lymnaeids under experimentally controlled conditions of 20° C, 90% relative humidity and a 12 h/12 h light/darkness photoperiod in precision climatic chambers in which day sunlight was of 3500 lux.

(SL), shell width (SW), aperture length (AL), aperture width (AW), and body whorl length (BWL) (Table 2). All of the material used was measured by the same person. Absolute measurements are noted to illustrate the size of snail vectors to health workers in charge for field control activities.

Snail laboratory cultures for the assessment of growth, egg-laying and life span

Living lymnaeids collected in Batallas (Table 2) and other localities (Table 3) were used for experimental procedures, always after discarding a possible natural infection by fasciolids or other trematodes. Non-infected lymnaeids were arranged in breeding boxes containing 2000 ml fresh water and maintained under controlled conditions of 20° C, 90% relative humidity and a 12 h/12 h light/darkness photoperiod in precision climatic chambers in which day sunlight was of 3500 lux (Heraeus-Vötsch VB-0714 and HPS-500) (Figure 3B). The water was changed weekly and lettuce added *ad libitum* (Bargues et al., 2011c).

Follow up studies were made in the first laboratory-born generation to avoid potential ecological and behavioral modifications induced by laboratory adaptation (i.e., to keep origin environmental effects as much as possible). A total of 48 eggs laid on the same day, very early after initial laboratory adaptation of field-collected snails, and the

Table 2. Shell morphometry of laboratory-reared and natural lymnaeid populations in two different localities of the Northern Bolivian Altiplano human fascioliasis hyperendemic area. Measurements in mm (mean values \pm standard deviation).

Shell characteristics	Abbreviations	Laboratory-reared population				Natural populations	
		Batallas				Batallas	Quiripujo
		n = 48				n = 60	n = 60
		Optimum period for miracidial infection			Maximum size	different ages	different ages
at week 4	at week 5	at week 6	at week 12				
Shell length	SL	4.39 \pm 0.34	4.73 \pm 0.37	4.98 \pm 0.41	6.19 \pm 0.55	4.78 \pm 1.81	6.57 \pm 2.31
Shell width	SW	2.58 \pm 0.16	2.75 \pm 0.18	2.88 \pm 0.22	3.58 \pm 0.28	3.02 \pm 1.18	3.65 \pm 1.28
Aperture length	AL	2.27 \pm 0.14	2.36 \pm 0.22	2.47 \pm 0.25	2.92 \pm 0.26	2.62 \pm 1.13	3.38 \pm 1.22
Aperture width	AW	1.47 \pm 0.15	1.59 \pm 0.16	1.64 \pm 0.17	1.89 \pm 0.18	1.90 \pm 0.77	2.53 \pm 0.96
Body whorl length	BWL	3.45 \pm 0.21	3.72 \pm 0.27	3.90 \pm 0.33	4.72 \pm 0.40	3.78 \pm 1.20	4.90 \pm 1.73

n = number of specimens.

Table 3. Total longevity (extreme and mean values) of lymnaeids from the Northern Bolivian Altiplano human fascioliasis hyperendemic area (days elapsed from birth) under experimental controlled conditions in the follow-up study in breeding boxes.

Experiment No.	Experimental conditions in climate chamber			No. of snails	Locality origin of snails	Total longevity		
	Temperature (°C day/°C night)	Relative humidity (% +/- 5%)	Photoperiod (h day/h night)			Extreme values (days)	Mean (days)	Values (months)
1	20/20	90	12/12	25	Ancocagua	79-226	100.5 (a)	3-4
2	/	.	/	30	Ancocagua	82-167	115.8 (a)	3-4
3	/	.	/	12	Huacullani	128-274	186.4 (b)	6-7
4	/	.	/	12	Huacullani	143-225	185.3 (b)	6-7
5	/	.	/	15	Huacullani	145-254	190.0 (b)	6-7
6	/	.	/	14	Huacullani	143-225	185.3 (b)	6-7
7	/	.	/	9	Huacullani	121-274	194.5 (b)	6-7
8	25/10	90	12/12	22	Huacullani	41-69	52.8 ©	1-2
9	16/9	65	12/12	14	Huacullani	161-285	242.5 (d)	8
10	/	.	/	21	Huacullani	171-275	242.1 (d)	8
11	22/5	65	12/12	18	Huacullani	146-211	182.9 (b)	6-7
12	/	.	/	21	Kallutaca	223-368	310.4 (e)	10-12
13	/	.	/	38	Batallas	71-203	150.8 (f)	5-6.5
14	/	.	/	35	Chijipata Alto	98-203	147.3 (f)	5-6.5

(a) = experiments with significant similar results ($P < 0.05$); (b) = experiments with significant similar results ($P < 0.05$); (c) = experiment with a result significantly different from (b) ($P > 0.05$); (d) = experiments with significant similar results ($P < 0.05$); (e) = experiment with a result significantly different from those of all other ($P > 0.05$); (f) = experiments with significant similar results ($P < 0.05$).

subsequent 48 living specimens born from these eggs, could be used for a follow-up period appropriately long as to assess the main epidemiological characteristics, including (i) time needed to reach the appropriate size for potential infection by *F. hepatica*, (ii) size ranges to enable the assessment of approximate snail age in field specimens, and (iii) size/age when egg laying starts. This number of 48 specimens was considered sufficient because of their successful complete follow-up (none died) and considering that this number fits the specimen quantity ranges traditionally used in experimental studies with this kind of snails. Well known problems posed by the laboratory keeping of living lymnaeids, to which the large problematics posed by their very high altitude strains should be added, underlie difficulties leading to experimental studies with low number of specimens (Rondelaud et al., 2009; Mas-Coma et al., 2020). Studies were as follows:

- Egg development: Egg clusters laid by lymnaeids from Batallas inside the breeding containers were isolated in Petri dishes, i.e. one cluster per Petri dish, and kept inside the climatic chambers under the same environmental conditions. Development of 48 eggs was daily followed-up to assess the embryonic period (from day of egg cluster laying to day of egg hatching);
- Shell growth: It was assessed in 48 specimens from Batallas. Immediately after egg hatching, each newborn snail specimen was isolated in another small Petri dish provided with a thin freshwater layer and microalgae (*Oscillatoria formosa*) as food. Growth and egg cluster laying produced by selfing were thereafter followed on a daily basis. Small portions of fresh and boiled lettuce were added to complement the food diet when lymnaeid size was sufficient. This follow-up was up to 12 weeks. Shell measurements were as noted above for field collected snails;
- Life span: To assess the total longevity until snail death and life-long egg cluster laying capacity beyond the first 12 weeks, the daily follow up was made with other snail specimens isolated in breeding boxes. The size of the breeding boxes allowed the snails to come out from and re-enter into water, an important option for amphibious lymnaeids not available inside the Petri dishes due to the short height of their lateral walls. Snail number, their localities of origin and varying experimental conditions of temperature and relative humidity in each experimental follow-up are noted in Table 3. This follow-up in the breeding boxes was continued until snail death.

The following characteristics were assessed (Bargues et al., 2011c): (i) life span (= from day of hatching to day of death); (ii) pre-laying period (=from day of hatching to day when first cluster was laid); (iii) laying period (= from first to last day when clusters were laid by a snail specimen); (iv) number of eggs per cluster; and (v) egg laying capacity rate given as mean egg/lymnaeid specimen/month throughout its laying period.

Environmental factors

Freshwater and air were characterized for each transmission focus, including abiotic factors influencing liver fluke transmission (Mas-Coma et al., 1999; Fuentes et al., 1999):

- Physico-chemical characteristics of the freshwater collections were studied to assess potential relationships with lymnaeid population dynamics. Water samples were collected during the day in the February-March period. Analytical methods used were: American Public Health Association (APHA) methods for pH and electric conductivity (Baird et al., 2017); Hach methods for colour, FTU (Formazine-Nephelometric Units) turbidity, solids in suspension, phosphates, sulphates, nitrites, nitrates, and ammoniacal nitrogen; flame emission for sodium and potassium; and atomic absorption for calcium and magnesium (Hach Company, 2012);
- Special focus was given to water temperature and pH. A field thermometer and pH-meter were therefore used in each monthly survey. Water presence/absence and its level fluctuations (= depth) were noted;
- Monthly air climatic variables were obtained by interpolation based on data furnished by the closest surrounding meteorological stations. The interpolation method was by nearest neighbour by using the New LocClim 1.10 software from FAO with a temporal resolution of days (Bargues et al., 2016). Climatic data registered in the meteorological stations were provided by the Servicio Nacional de Meteorología e Hidrología del Ministerio de Transportes, Comunicaciones y Aeronáutica Civil (La Paz, Bolivia): Ayo Ayo (17° 05' 39" S; 68° 00' 30" W; 3892 m), Chirapaca (16° 17' 46" S; 68° 29' 47" W; 3889 m), El Alto (16° 30' 17" S; 68° 11' 55" W; 4034 m), Hichucota (16° 10' 36" S; 68° 22' 52" W; 4460 m), Huarina (16° 10' 34" S; 68° 37' 50" W; 3838 m), Huayrocondo (16° 20' 35" S; 68° 29' 49" W; 3875 m), San Calixto (16° 29' 53" S; 68° 07' 57" W; 3658 m), Tiwanaku (16° 34' 07" S; 68° 40' 42" W; 3863 m), and Viacha (16° 39' 30" S; 68° 16' 55" W; 3850 m). Monthly data analyzed included mean environmental temperature (MET), mean maximum temperature (MMT), and mean minimum temperature (MmT) in °C, precipitation (Pt) in mm, number of days with precipitation (rD), relative

humidity (RH) in %, total evaporation (TEv) and potential evapotranspiration (PET) in mm (Fuentes et al., 1999).

Field studies on lymnaeid snail population abundance and density

A standard method of collection was used to allow for meaningful comparisons between and inside the nine transmission foci. Freshwater snails were collected using the man-time collection method (Relf et al., 2011; Charlier et al., 2014). Abundance was estimated by the total number of lymnaeids collected in a given site during a 30-minute time. To reflect aggregation/dispersion, density was established as the mean number of lymnaeid specimens collected during 1 minute, calculated from an uninterrupted 15-minute period (within the aforementioned 30-minute period to establish the abundance) on a searching circle of a radius of a maximum of 5 m (Figure 4). Such a circle allowed to follow population movements within the same focus (Figure 4). Surveys were always made by the same person. Snail collection was made between 10.00 h a.m. and 01.00 h p.m. of sunny days, in each collection site, using appropriate forceps with flat clamps.

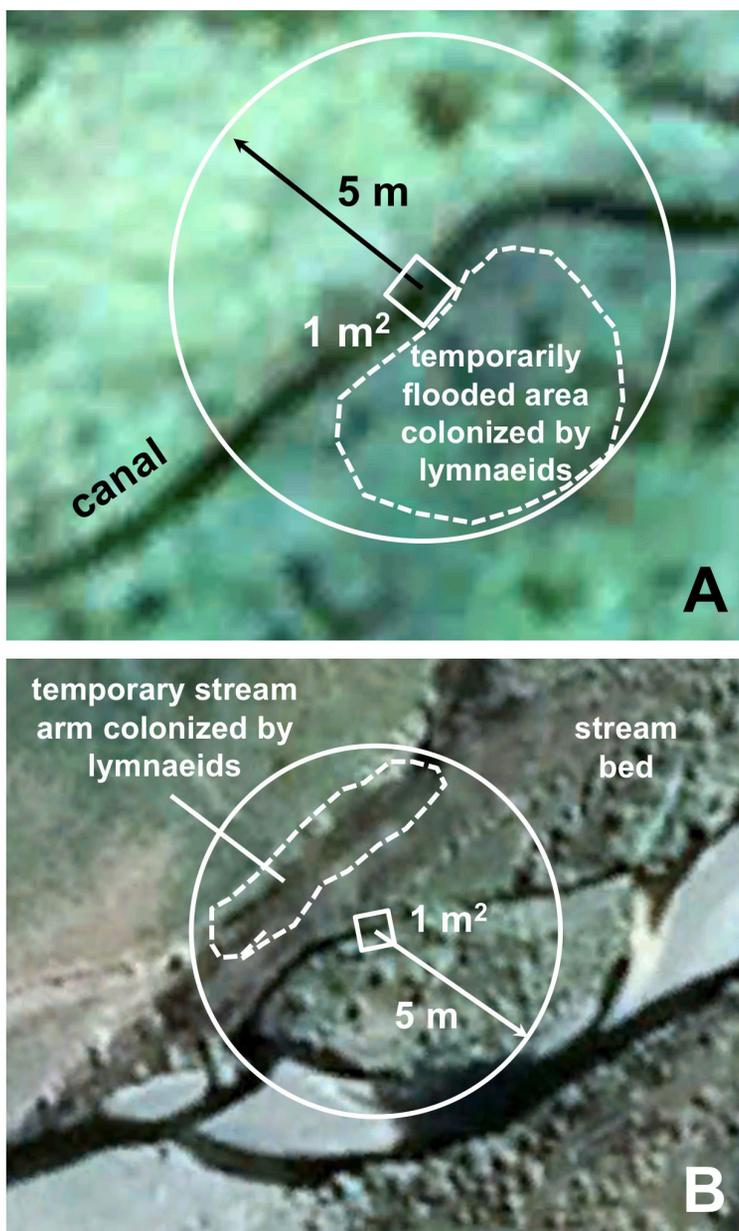


Figure 4. Standardized methodology for lymnaeid snail collection and population abundance and density estimates by monthly surveys, allowing for the follow up of population movements within the same fascioliasis transmission focus. Examples of (A) temporarily flooded area colonized by lymnaeids from neighboring water canal, and (B) temporary stream arm colonized by lymnaeids from another close water arm in wide flat stream bed. See text for further details.

Collected snails were put in a clean plastic container to differentiate lymnaeids from other snails by their typical small, smooth and dextral conical shell, and their pair of triangular tentacles with darkly pigmented eyes at their bases. There are no other freshwater snails in the Northern Altiplano which may be confused with lymnaeids. After lymnaeid counting, they were released in the same place to avoid disturbing their population dynamics.

Pronounced changes of water quantity and of the extent and density of the vegetation coverage, mainly by Ranunculaceae, Juncaceae and many other freshwater plants (Mas-Coma et al., 1999), did not allow for accurate abundance and density studies of small specimens, because many of which remain undetected under dense vegetation. Therefore, a distinction of age groups according to size, as made in other similar lymnaeid population studies in farms, flat grasslands, rice cultures or controlled pasturelands (Relf et al., 2011; Charlier et al., 2014), could not be performed. The same problem was already highlighted in field surveys on *Galba/Fossaria* species in Europe (Simon Vicente, 1968).

In the last 12th monthly survey, ten lymnaeid specimens from each followed-up population were fixed in 96% ethanol, of which three were used for DNA sequencing.

Statistical analyses

The Student's t-test and non-parametric Mann-Whitney test were used to compare the experimental life spans of lymnaeids between different localities and between different experimental conditions. Bivariant correlations (Pearson's correlation) were calculated for lymnaeid population dynamics (monthly values of abundance and density) versus mean monthly climatic variables of the transmission foci, also versus the values of the physico-chemical characteristics of the freshwater collections, and finally also versus the monthly values of freshwater characteristics (presence/absence, temperature and pH). Data processing was carried out with SPSS version 6.1 for Macintosh (SPSS Inc., Chicago, IL).

Results

DNA characterization of lymnaeid specimens

Ribosomal DNA ITS-2 and ITS-1 markers allowed for the verification of the systematic classification of the populations studied.

All 33 specimens from the eleven lymnaeid populations showed an identical ITS-2 sequence of a length of 401 bp and a 58.60% GC content. This sequence shows no nucleotide difference with the haplotype G.tru-H3 (GenBank AJ272051) of *G. truncatula*. This haplotype has already been reported from Bolivia, Chile and Argentina.

Moreover, all specimens presented the same 504-bp-long ITS-1 sequence, with a 57.53% GC content. This ITS-1 shows no nucleotide difference with the haplotype G.tru-HC (GenBank AJ272052) of the same species. Similarly, this ITS-1 haplotype has been previously reported in Bolivia, Chile and Argentina.

Experimental results

The growth curve obtained by following 48 specimens from Batallas agrees with the shell size ranges of lymnaeids in the field (Table 2, Figure 5A, B). After an embryonic period of 11-14 days (mean 12.1 days), lymnaeid growth shows a very fast development of around 1 mm increase in length per week. This leads the lymnaeids to reach the optimum size (4 mm length) for liver fluke miracidial infection 3 days before week 4 (Table 2, Figure 5A, B).

As a consequence of self-fertilization, lymnaeids individually isolated in Petri dishes began to lay the first egg clusters during week 4, in several specimens even 1-2 days before (pre-laying period). The total laying period, followed up in breeding boxes, proved to be very long and continuous until almost the death. At the beginning of the egg laying period the rounded-oval small clusters included a reduced number of eggs (2-4) measuring around 700 x 600 µm. However, with time these clusters increased in size, became more oval and slightly elongate, and the number of eggs per cluster increased considerably. So, a high egg laying capacity of 9-33 eggs per cluster (mean 17.5 eggs/cluster) appeared and a mean of 87.1 eggs/snail/month was reached and maintained throughout a whole year.

Regarding experiments for lymnaeid longevity assessment, similar results were obtained when using lymnaeids from the same locality, e.g. Huacullani, under the same environmental conditions (assays Nos. 3-7 in Table 3), sometimes even when under day-night changing conditions (assay No. 11 in Table 3), although this was not always

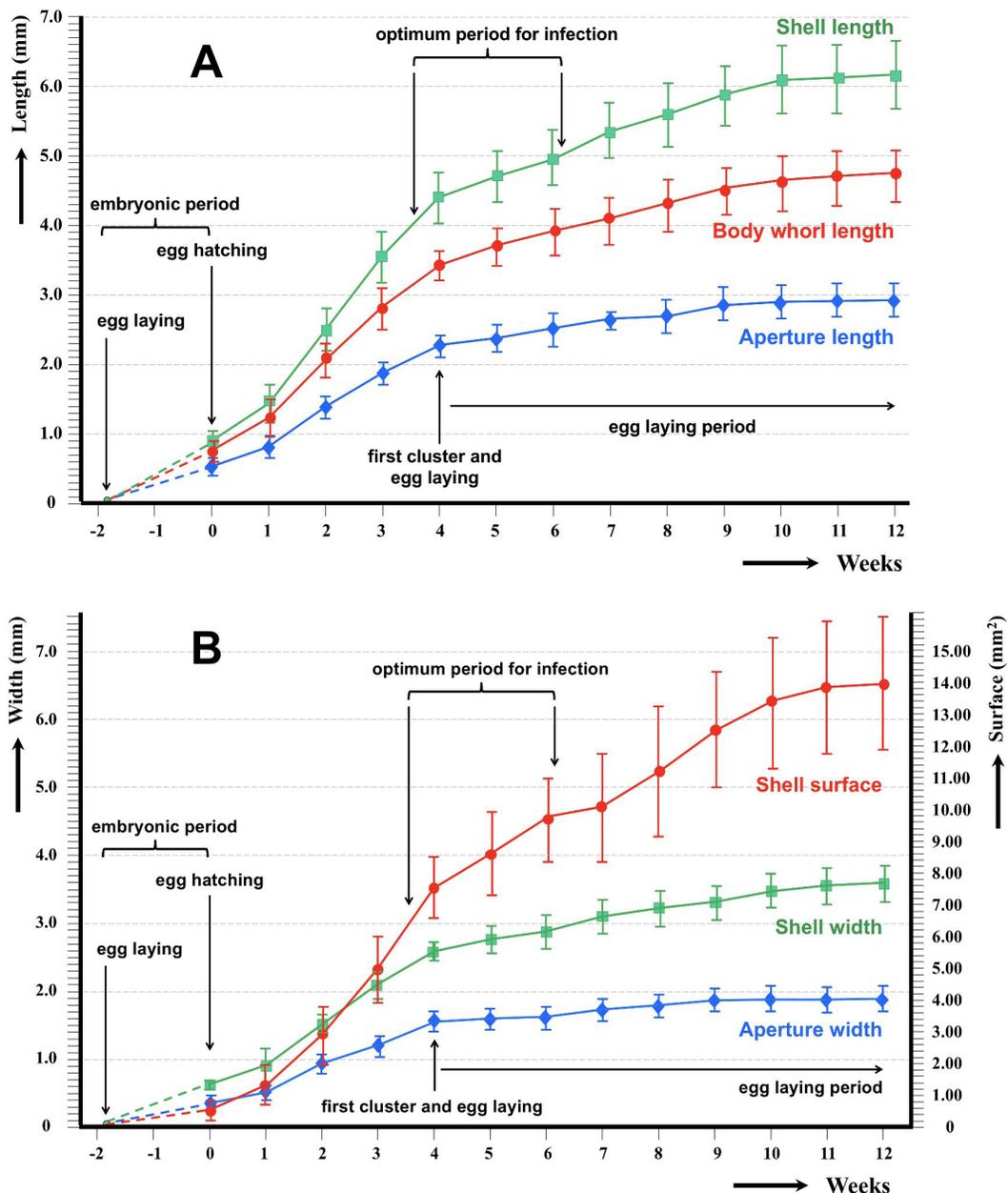


Figure 5. Experimentally followed shell growth (mean values ± standard deviation SD) of *Galba truncatula* from the Northern Bolivian Altiplano human fascioliasis hyperendemic area: (A) lengths of shell, body whorl and aperture; (B) widths of shell and aperture and shell surface. Note embryonic period, optimum period for infection by *Fasciola hepatica*, and egg laying period.

the case (assay No. 8 in Table 3). A similar life span was observed in the two experiments with lymnaeids from Ancocagua (assays Nos. 1 and 2 in Table 3), and also in lymnaeids from Batallas and Chijipata Alto (assays Nos. 13 and 14 in Table 3), the latter two localities geographically very close one another (see Figure 1). Life spans of lymnaeids from different localities differed significantly ($P < 0.05$).

Worth mentioning are assays carried out under temperatures differing during day and night hours. In lymnaeids from Huacullani under 22 °C/5 °C day/night temperatures (assay No. 11 in Table 3), the life span was similar to that obtained under a 24h-constant temperature of 20 °C (assays Nos. 3-7 in Table 3). Huacullani lymnaeids analyzed under 16 °C/9 °C day/night (assays Nos. 9 and 10 in Table 3) showed an unexpected high longevity, whereas when under 25 °C/10 °C day/night (assay No. 8 in Table 3) lymnaeids from this locality provided a markedly shorter longevity which proved to be statistically significant ($P < 0.05$). According to what happens in the Bolivian Altiplano in winter, the experimental relative humidity was decreased from 90% to 65% when applying colder temperatures of 16 °C/9

°C and 22 °C/5 °C day/night (assays 9-14 in Table 3), and the lymnaeid life spans obtained were pronouncedly longer than that obtained at 90% relative humidity but under 25 °C/10 °C day/night (assay No. 8 in Table 3).

The life span was of 10-12 months depending on locality origins and experimental conditions (Table 3). The longest was observed in lymnaeids from Kallutaca under temperatures of 22 °C/5 °C day/night (assay No. 12 in Table 3). It should be remembered here that all these results concern lymnaeids of the first laboratory-reared generation. After years of adaptation to standard laboratory conditions in breeding boxes at 20 °C/20 °C day/night and 90% + 5% relative humidity, lymnaeids descending from field collected specimens from the Altiplano were sometimes observed to survive up to 13-14 months.

Climatic characteristics of the transmission foci surveyed

Local climatic factor curves slightly differed from one transmission focus to another (Figures 6 and 7). However, due to geographic closeness, no differences were observed between Tambillo 1 and Tambillo 2 (Figure 6), between Chirapaca 1 and Chirapaca 2 (Figure 7), and between Batallas and Chijipata Alto (Figure 7).

No marked seasonality appears according to the temperatures, monthly fluctuations being relatively scarce except in the mean minimum temperature during winter. The mean environmental temperature is kept below 10 °C throughout the whole 12 month period and the mean maximum temperature does not reach the level of 20 °C in any of the transmission foci studied. The mean minimum temperature reaches its lowest level, below 0 °C

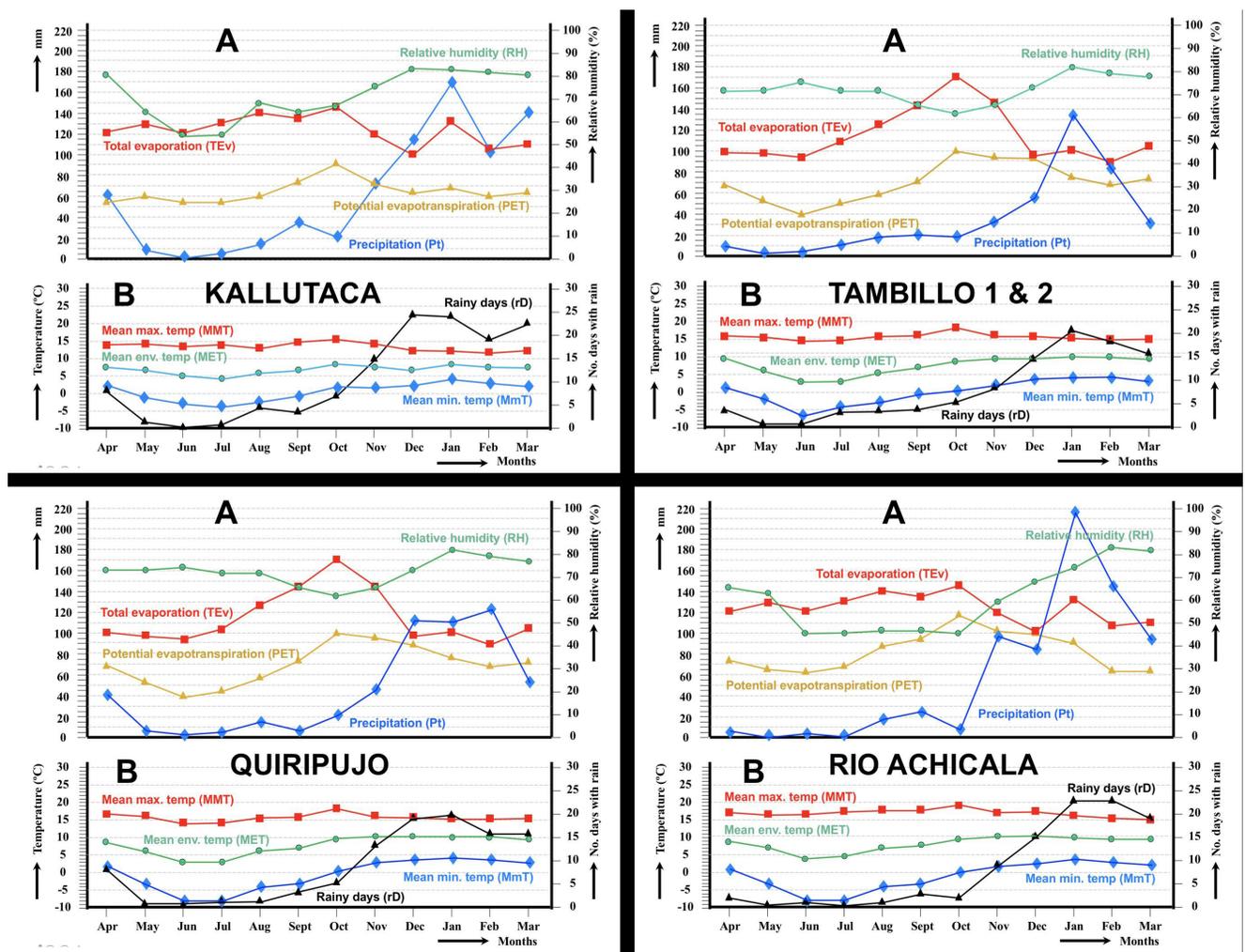


Figure 6. Mean monthly data of climatic factors in the transmission foci of Kallutaca, Tambillo 1, Tambillo 2, Quiripujo and Rio Achicala: (A) precipitation (Pt), relative humidity (RH), total evaporation (TEv) and potential evapotranspiration PET); (B) mean environmental temperature (MET), mean maximum temperature (MMT), mean minimum temperature (MmT), and number of days with precipitation (rD).

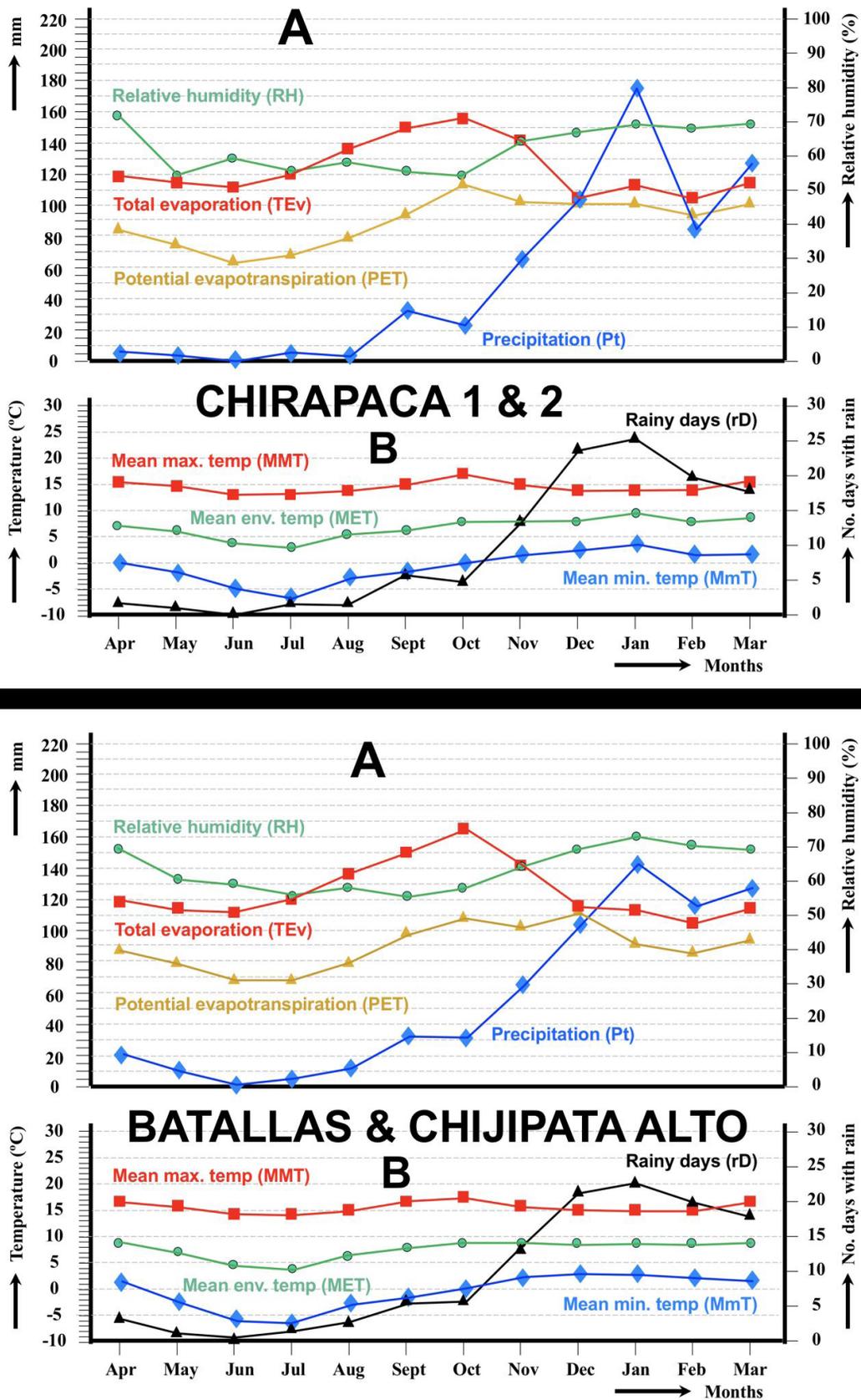


Figure 7. Mean monthly data of climatic factors in the transmission foci of Chirapaca 1 and 2, Batallas and Chijipata Alto: (A) precipitation (Pt), relative humidity (RH), total evaporation (TEv) and potential evapotranspiration PET); (B) mean environmental temperature (MET), mean maximum temperature (MMT), mean minimum temperature (MmT), and number of days with precipitation (rD).

and in given foci even lower than $-5\text{ }^{\circ}\text{C}$ and close to $-10\text{ }^{\circ}\text{C}$ as in Quiripujo and Rio Achicala (Figure 6), in the winter months of June and July, that is in the peak of the dry season.

Precipitation and number of days with precipitation allow to distinguish between a dry season from May to September and a rainy season from October to April, with a precipitation peak in January. Total evaporation and potential evapotranspiration are very high and show a peak in October in all transmission foci studied (Figures 6 and 7).

Characteristics of the water collections surveyed

No significant relationship appeared between each one of the physico-chemical characteristics of water (Table 4) and the lymnaeid population dynamics in each focus, not even when separately grouping foci with permanent water presence and foci with temporary absence of water (Figures 8A and 9A).

The monthly values of water pH throughout the twelve month period varied between 5.5 and 8.5, although extreme values were only sporadically obtained, and an acid trend was found in most of the habitats (Figures 8B and 9B). Water temperature showed evident fluctuations in given foci (Kallutaca, Tambillo and Batallas), whereas fluctuations appeared to be minor in others (Quiripujo, Chirapaca 1, Chirapaca 2 and Chijipata Alto). Monthly values of water temperature reached the minimum of $0\text{ }^{\circ}\text{C}$ only in July in Kallutaca, whereas a maximum of $25\text{ }^{\circ}\text{C}$ was only reached in September in Tambillo (Figure 8B and 9B).

Abundance and density of the lymnaeid populations

There were habitats in which lymnaeid snails were found in each monthly survey, such as Kallutaca, Rio Achicala (Figure 8C), Batallas and Chijipata Alto (Figure 9C). In other foci, no lymnaeids were found during several months,

Table 4. Physico-chemical characteristics of water samples obtained in the selected transmission foci of the Northern Bolivian Altiplano human fascioliasis hyperendemic area in which the lymnaeid population dynamics were followed up. Measurements performed during the day in the February-March period. Tambillo 1 and Tambillo 2 concerned two collecting sites in the same stream only separated 30 m one another and differing only by their distance from human dwellings.

Characteristics	Units	Kallutaca	Tambillo 1 & 2	Quiripujo	Rio Achicala	Chirapaca 2	Chirapaca 1	Batallas	Chijipata Alto
Conductivity	$\mu\text{S}/\text{cm}$	199	332	600	244.00	78.00	363.00	102.00	57.00
Apparent color	Pt/Co*	300.00	58.00	>500	70.00	27.00	470.00	18.00	17.00
True color	Pt/Co*	140.00	4.00	58.00	9.00	17.00	63.00	0.00	6.00
Turbidity	FTU†	50.00	0.00	240.00	15.00	7.00	80.00	0.00	0.00
Solids in suspension	mg/l	20.00	0.00	360.00	10.00	0.00	160.00	0.00	0.00
Total hardness	$^{\circ}\text{dH}^{\S}$	3.30	6.80	indet.	5.50	1.70	6.50	1.90	1.20
Alkalinity	meq/l	<<20	indet.	indet.	<<20	indet.	indet.	<<20	<<20
Phosphates	mg PO_4/l	0.79	0.06	1.23	0.00	0.02	0.47	0.05	0.00
Sulphates	mg SO_4/l	15.00	57.0	28.00	0.00	14.00	45.00	13.00	18.00
Nitrites	mg NO_2/l	---	---	---	0.000	---	0.000	---	---
Nitrates	mg NO_3/l	0.00	0.04	0.08	0.03	0.00	0.03	0.02	0.01
Ammoniacal nitrogen	mg NH_4/l	0.43	0.03	0.30	0.10	0.08	0.29	0.03	0.02
Sodium	mg Na/l	30.10	20.70	36.40	36.40	4.70	33.80	6.30	2.90
Potassium	mg K/l	20.10	10.40	27.80	41.00	1.90	27.80	3.70	2.80
Calcium	mg Ca/l	10.80	49.10	94.60	24.10	7.10	29.60	8.50	5.70
Magnesium	mg Mg/l	8.10	6.30	16.90	8.30	2.40	11.10	3.30	1.30

*Pt/Co = units of Chloroplatinate; † FTU = Formacine-Turbidity Units; § $^{\circ}\text{dH}$ = German hardness degrees; indet. = could not be determined.

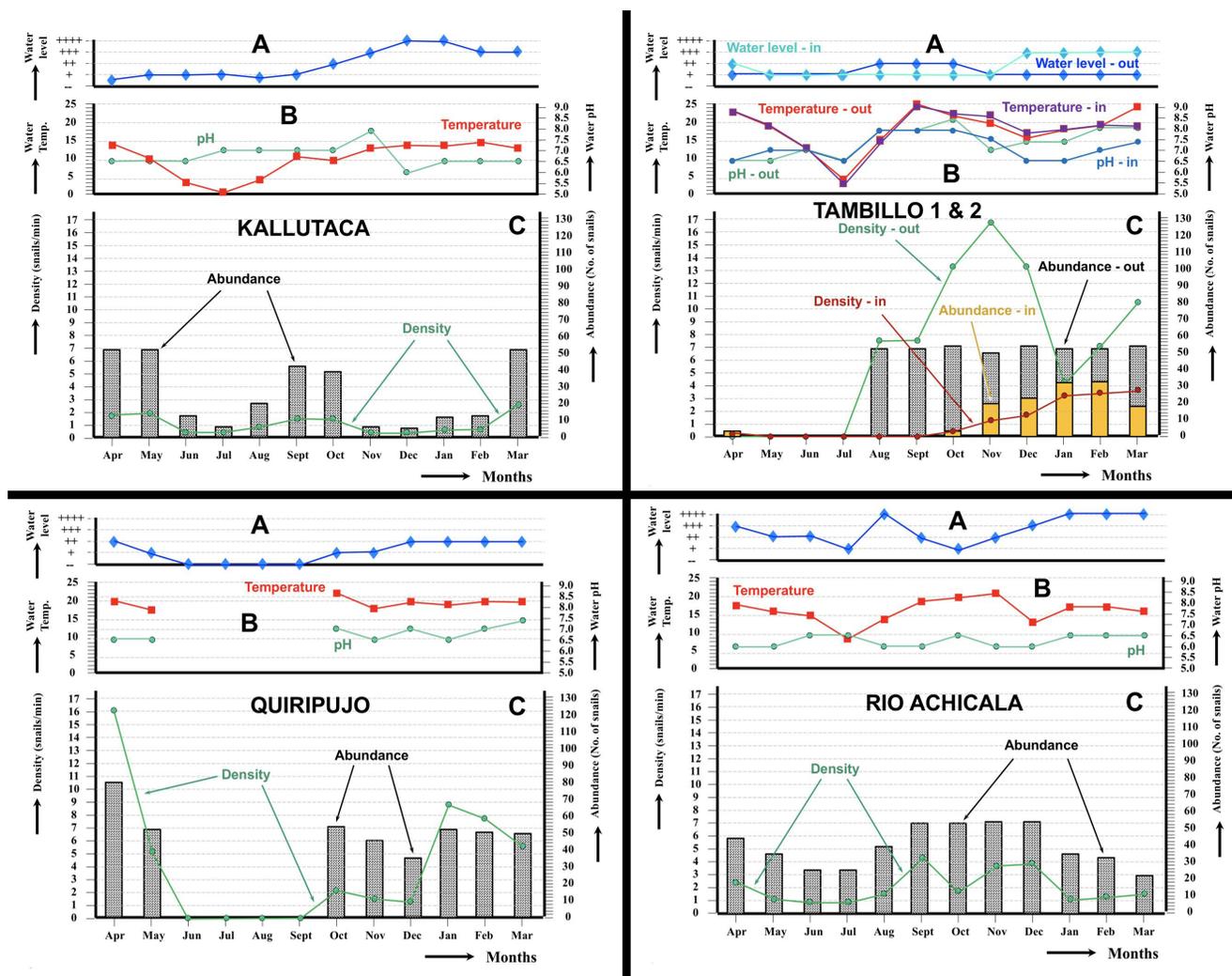


Figure 8. Monthly characteristics of the local freshwater habitats and dynamics of their respective inhabiting *Galba truncatula* populations in the transmission foci of Kallutaca, Tambillo 1 and 2 (in = inside village; out = outside village), Quiripujo and Rio Achicala: (A) water presence/absence and level; (B) water temperature and pH; (C) abundance and density of the lymnaeid populations.

as in Tambillo 1 and Tambillo 2, Quiripujo (Figure 7C), Chirapaca 1 and Chirapaca 2 (Figure 9C), where lymnaeid presence/absence significantly correlated with the presence/absence of available surface freshwater, except in Tambillo. In Tambillo, no snails were found during several months despite water presence, both inside and outside the village (Figure 8C). In Batallas, high numbers of lymnaeids were collected on mud in three discontinuous months in which no water was present (Figure 9C).

Abundance and density of the lymnaeid populations significantly correlated in transmission foci such as Kallutaca, Quiripujo, Rio Achicala (Figure 8C), Chirapaca 1, Batallas and Chijipata Alto (Figure 9C). However, in Tambillo 2 outside the village (Figure 8C) and in Chirapaca 2 (Figure 9C) the snails appeared more aggregated, i.e. with a higher density, in given months.

Discussion

Molecular classification of lymnaeid populations

The ITS-2 and ITS-1 markers proved that all snail populations studied belong to the species *Galba truncatula*. The two respective sequences were identical in all the transmission foci surveyed and correspond to those already reported from the same area before (Mas-Coma et al., 2001; Bargues et al., 2020).

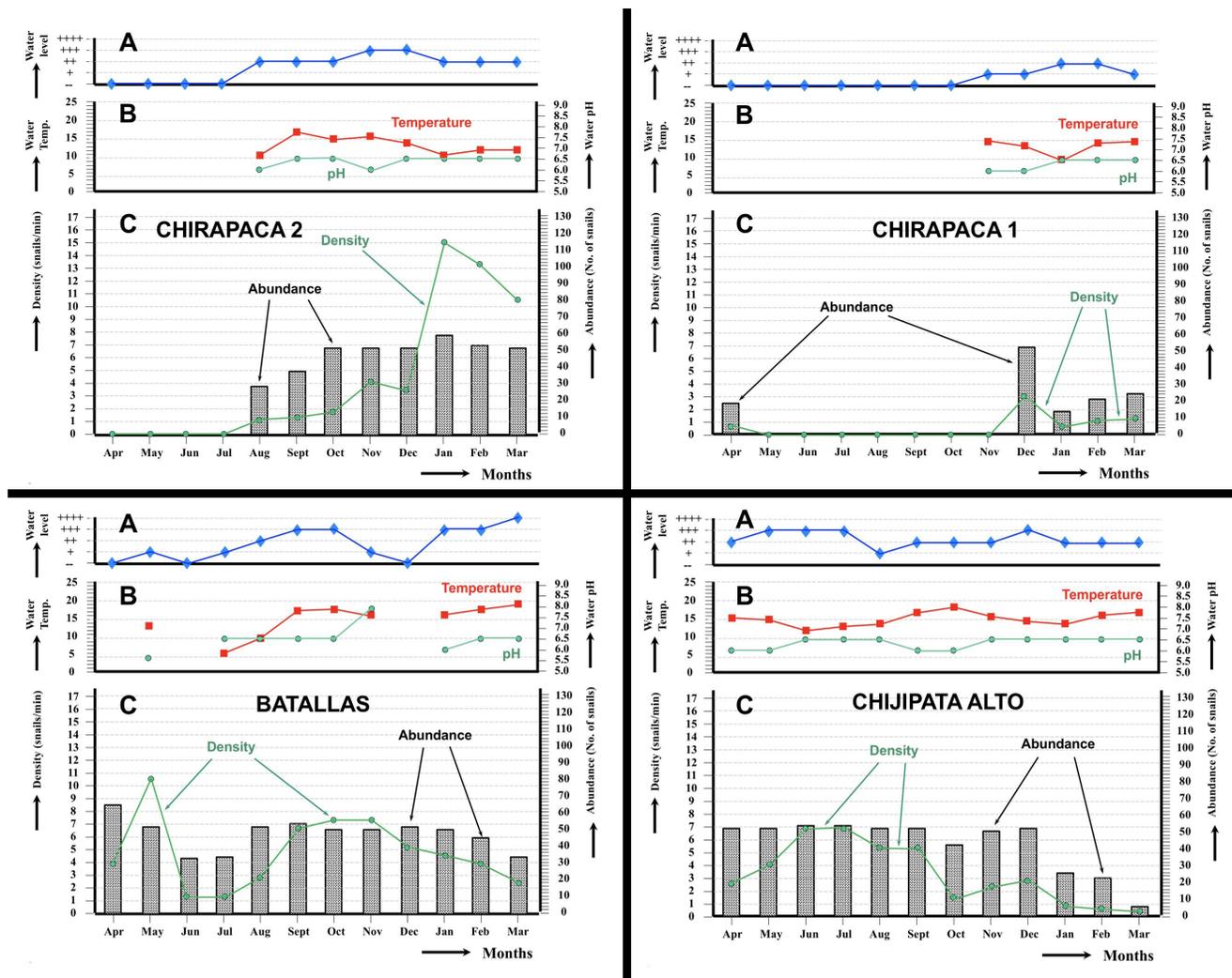


Figure 9. Monthly characteristics of the local freshwater habitats and dynamics of their respective inhabiting *Galba truncatula* populations in the transmission foci of Chirapaca 1, Chirapaca 2, Batallas and Chijipata Alto: (A) water presence/absence and its level (= depth); (B) water temperature and pH; (C) abundance and density of the lymnaeid populations.

In South America, *F. hepatica* is transmitted by different species of the co-called *Galba/Fossaria* group (Bargues et al., 2007, 2011c). Interestingly, more than one *Galba/Fossaria* species co-exist in other human endemic areas: *G. truncatula*, *L. schirazensis* and *L. neotropica* in Cajamarca, Peru (Bargues et al., 2012); *L. cousini*, *L. neotropica*, *L. schirazensis* and *L. cubensis* in Ecuador (Bargues et al., 2011b; Villavicencio et al., 2019); *G. truncatula*, *L. cubensis* and *L. schirazensis* in Venezuela (Bargues et al., 2011a); *L. neotropica* and *L. viator* in Argentina (Bargues et al., 2016); and *G. truncatula* and *L. viator* in Chile (Artigas et al., 2011). This high altitude scenario differs from fascioliasis in the lowlands, where fascioliasis is a veterinary problem in great part linked to the presence of a lymnaeid vector species not belonging to the *Galba/Fossaria* group as *Pseudosuccinea columella*, and human infection only appears in isolated cases, such as in Brazil (Maure et al., 1998; D’Almeida et al., 2016) and Uruguay (Bargues et al., 2017).

Given the well known different ecological requirements, ethological characteristics and liver fluke transmission capacities of the different *Galba/Fossaria* species (Bargues et al., 2017), dealing with only one snail species pronouncedly simplifies population comparisons, and the obtaining of significant results.

Embryonation, growth, morphometry, fecundity and longevity

The embryonic period of 11-14 days at 20 °C (Figure 5) fits well with what is known in *G. truncatula*. This period depends on the environmental temperature (Kendall, 1953): 32 days at 10-11 °C; 12-18 days at 16-21 °C; 11-12 days at 21-30 °C. According to the environmental (Figures 6 and 7) and water temperatures (Figures 8 and 9) of the foci, the embryonic period may vary between a longer period in the coldest winter to a shorter one in the other months.

Shell growth in *G. truncatula* depends on the kind and quantity of nourishment (Kendall, 1953), although evident growth differences only appear in specimens from a size of 7 mm onwards (Rondelaud et al., 2009). In altiplanic *G. truncatula* maintained at 20 °C, the 6-mm length is reached between 9 and 10 weeks of age, with a faster growth up to week 4 (0.971 mm/week) than between week 4 and week 10 (0.280 mm/week), before entering in a very slowly increasing plateau from week 11 (Figure 5). The growing speed of altiplanic lymnaeids allowing them to reach a 4-mm length before week 4 is as fast as the experimental growth obtained when furnishing a supplementary diet (Rondelaud et al., 2009). This has epidemiological repercussions, given that the optimum period for infection by *F. hepatica* begins when lymnaeids reach the 4 mm size.

Measurements show that lymnaeids may reach a longer size in nature, although specimens longer than 10 mm are very rarely found in the Altiplano. This size may lead to confusion with the morphologically similar *Succinea* sp., a terrestrial species usually found in humid habitats and close to freshwater, which presents less dark-brownish shell, and eyes at the tip of tentacles (opposite to lymnaeids in which tentacles are triangular and the darkly pigmented eyes are located at the bases of tentacles). *Succinea* spp. have been sometimes confused with lymnaeids in fascioliasis areas (Lepe-López et al., 2020).

Egg laying begins in 4.0-4.5-mm-long *G. truncatula* (Walton & Jones, 1926; Kendall, 1953; Smith, 1981). Thus, altiplanic *G. truncatula* are among the most precocious populations starting egg cluster laying, in several specimens even 1-2 days before week 4. Moreover, the egg laying capacity of 9-33 eggs/cluster (mean 17.5 eggs/cluster) of Bolivian *G. truncatula* is very high when compared to means of 12, 15 or 8.4 eggs/cluster reported by other authors (Kendall, 1953; Euzéby, 1971; Rondelaud et al., 2009). Similarly occurs with the mean of 87.1 eggs/snail/month of altiplanic lymnaeids. Quality and quantity of food have been argued to explain such differences in fecundity (Kendall, 1953). Owing to the fact that temperature also underlies fecundity differences (Kendall, 1953), such very high fecundity capacity of altiplanic *G. truncatula* in the laboratory should not be expected to occur in the Altiplano where air and water temperatures are lower than the standard permanent 20 °C applied in our experimental procedures.

Longevity in *G. truncatula* usually varies between 6 and 12 months, although up to 17 months have been reported (Mehl, 1932). Altiplanic *G. truncatula* fit well within the aforementioned extremes. Experiments under temperatures differing during day and night hours (Table 3) indicate that the higher day temperatures compensate the a priori negatively influencing low night temperatures. Indeed, in the Altiplano mean temperatures do not fluctuate pronouncedly throughout a year, and the temperatures show marked differences between the maximum during sunshine hours and the minimum at night (Fuentes et al., 1999). This allows to understand that the longer life span of up to 12 months was observed in lymnaeids under temperatures of 22 °C/5 °C day/night (Table 3).

In the Altiplano, *G. truncatula* may enter aestivation due to the lack of surface freshwater, and in hibernation due to cold temperature. Aestivation appears to be better supported by young than by adult specimens (Kendall, 1949; Roberts, 1950). Under natural conditions, *G. truncatula* can survive from 6 weeks up to 4.5 months in mud without water, and in the laboratory a whole year survival in a Petri dish without water has been reported (Kendall, 1949). Lymnaeids buried into the drying soil are more numerous in altitude habitats than in lowlands (Goumgghar et al., 2001). Concerning hibernation, *G. truncatula* may be active up to a low temperature of 1.5 °C, although with reduced activity at low temperatures (Kendall, 1953). The appearance of ice reduces lymnaeid movements under the ice layer, as observed in Kallutaca. Aestivation and hibernation modify lymnaeid longevity and explain the longer longevities of up to 13-14 months observed in long-term laboratory adapted altiplanic *G. truncatula* kept in breeding boxes. Interestingly, altiplanic lymnaeids proved to survive longer to *F. hepatica* infection than lowland specimens (Bargues et al., 1995).

Unfortunately, the influence of the relative humidity cannot be assessed independently from temperature after the longevity experiments carried out (Table 3). However, the comparison of life spans at 16 °C/9 °C and 22 °C/5 °C day/night and 65% relative humidity (assays 9-14 in Table 3) with those under 25 °C/10 °C day/night and 90% relative humidity (assay No. 8 in Table 3), suggests that a decreasing relative humidity accompanying the cold night temperatures may compose a better environment for altiplanic lymnaeids. Indeed, these are the climatic characteristics of the Bolivian Altiplano in winter (see Figures 6 and 7).

Lymnaeid population dynamics, freshwater characteristics and climatic variables

Differences of lymnaeid population dynamics were detected between the transmission foci (Figures 8C and 9C). Presence/absence and level (= depth) of freshwater on soil surface (Simon Vicente, 1968), differences between soil

surface temperature and air temperature (Smith & Wilson, 1980), soil humidity (Cruz-Mendoza et al., 2004), and to a lesser extent soil and water pH (Euzeby, 1971), are habitat microecology factors influencing lymnaeid population abundance, density and dynamics. In farms, different water bodies (e.g., pond, ditch, trench, furrow, moist area) show pronounced differences in *G. truncatula* population dynamics (Charlier et al., 2014).

The mean environmental temperature (MET) in the Northern Altiplano is lower than 10 °C throughout a complete year (Fuentes et al., 1999). This was already highlighted because the intramolluscan larval stages of *F. hepatica* do not develop at temperatures lower than 9-10 °C (Ollerenshaw, 1959; Fuentes et al., 1999). The present study shows that MET was lower than this minimum temperature threshold in all the months at the nine transmission foci (Figures 6B and 7B). However, local water temperature was always higher than that threshold in all these foci, excepting the winter months of June, July and August in a few of them. This explains why *F. hepatica* larval development goes on despite the low yearly MET. On the other side, neither MET nor local water temperature in the nine foci reached the maximum temperature threshold of *F. hepatica*, which is usually put at 26 °C to allow for intramolluscan larval development and cercarial shedding (Afshan et al., 2014).

Optimum pH for lymnaeids ranges between 6.6 and 8.6 (Euzeby, 1971), although *G. truncatula* has also been found in acid soils with a pH of only 5.1 and in calcareous habitats with a pH of up to 9.0 (Euzeby, 1971). The abundance of *G. truncatula* is associated with soil and water pH (Charlier et al., 2014). Water pH measurements detected acid pHs in all monthly samples in Rio Achicala, Chirapaca 1, Chirapaca 2, Batallas and Chijipata Alto, whereas fluctuations below and above the neutral pH in Kallutaca, Tambillo 1, Tambillo 2 and only sporadically above in Quiripujo (Figures 8B and 9B). This suggests the more successful *G. truncatula* populations to prefer altiplanic acid habitats.

The local availability of freshwater or sufficient soil surface humidity to maintain appropriate mud are main factors for *Galba/Fossaria* species. Rainfall and relative humidity are therefore key variables for fascioliasis transmission (Fuentes et al., 1999; Afshan et al., 2014; Fox et al., 2011). On the contrary, the very high evaporation and potential evapotranspiration at the very high altitude of the Altiplano eliminate temporary small water collections and surface humidity derived from rainfall quite rapidly. Such temporary habitats are therefore never inhabited by lymnaeids in the Bolivian Altiplano (Mas-Coma et al., 1999; Fuentes et al., 1999), i.e. in the Altiplano local rainfall has a negligible impact on fascioliasis transmission. In this area, the freshwater sources are either waters directly coming from the thaw and rain in the Eastern Andean Chain by means of rivers and streams, or on natural spring pools whose waters are provided from quite superficial phreatic layers leading the underground waters from the Eastern Andean Chain (Mas-Coma et al., 1999). Among the foci surveyed, neither the habitats presenting non-permanent water, nor habitats presenting permanent water collections, depend on rainfall. Rainfall water appeared to be of some additional value only in the Quiripujo focus. Natural spring water pools from phreatic layers give rise to fascioliasis foci even far from the Eastern Andean Chain, as in the zone westward from Tiwanaku (Figure 1) (Mas-Coma et al., 1999).

Transmission foci

The intra- and interpopulation monthly variation comparisons of the 12-month curves of abundance and density of the altiplanic *G. truncatula* populations show differences between the transmission foci surveyed. Several aspects should be highlighted:

- There are habitats in which active lymnaeids are always present, e.g. those including permanent water collections (Kallutaca, Rio Achicala, Chijipata Alto) but also habitats in which water disappears for a short time during which lymnaeids keep activity on mud (Batallas);
- There are other habitats in which lymnaeids could not be found in several monthly surveys, e.g. in water absence (Quiripujo, Chirapaca 1, Chirapaca 2), but also others where water was always available (Tambillo 1, Tambillo 2). In the former, the abundant lymnaeids in the first month with water after the dry months suggests that lymnaeids were hibernating buried in soil;
- The concentrated presence of human activity waste chronologically correlates with the absence of lymnaeids despite the permanent availability of water, in the focus inside Tambillo village during a five month period (May to September). A slow population recovery occurs afterwards (October-November) and a progressive decline later (March-April) with increasing waste concentration after the higher water level period. This differs from the only 30 m distant focus outside Tambillo;
- Marked differences in abundance and density were found in neighboring foci (Chirapaca 1, Chirapaca 2) depending on habitat type and the period without water. In these close foci, the different snail size indicates

different generation moments (e.g. many adults in one site whereas only scarce small specimens in the other site);

- Nothing suggests the distance between the transmission foci and the Lake Titicaca to have an influence on the population dynamics.

One, two or three generation patterns have been reported to occur in *G. truncatula* populations throughout a whole year, depending on the characteristics of the transmission focus (Rondelaud et al., 2009). Moreover, egg-laying dates vary according to local climatic conditions independently of the generation pattern (Smith & Wilson, 1980). In the Altiplano, the coexistence of small juveniles and large adult specimens in the same habitat are mainly detected in November-December (Tambillo 1, Rio Achicala, Chirapaca 2, Batallas, Chijipata Alto) and February-March (Kallutaca, Tambillo 2, Quiripujo, Chirapaca 1), that is, beginning and end of the rainy season which also correspond to the year period in which the mean minimum air temperatures (MmT) are less low and water temperatures are higher. Results suggest that, in the Altiplano, two patterns may occur: (i) a two generations/year pattern in habitats with permanent water and sufficiently humid short period assuring mud availability, and (ii) a one generation/year pattern in habitats with lack of water availability during several months. Abundance and density curves in habitats with permanent populations (Kallutaca, Rio Achicala, Batallas, Chijipata Alto) illustrate the aforementioned two generations/year pattern (Figures 8C and 9C).

These results fit well with epidemiological data indicating that the infection risk for humans and livestock covers the whole year. This is due to the infectivity of metacercariae, which lasts up to 31 weeks, i.e. almost 8 months (Valero & Mas-Coma, 2000; Valero et al., 2002) and defines the so-called “altiplanic transmission pattern” of fascioliasis (Mas-Coma et al., 2009a, 2014a).

Concluding Remarks

From the point of view of the One Health initiative in the Northern Bolivian Altiplano hyperendemic area, experimental and field results allow to conclude that the multidisciplinary control measures can be extended from one part of the endemic area to another. It should be remembered that, in this endemic area, all lymnaeid populations are molecularly clonic, i.e. genetically identical due to the fecundation of *Galba truncatula* by selfing, an evolutionary strategy enhanced under the extreme conditions of the very high altitude conditions (Bargues et al., 2020). It is evident that local microecological conditions differing from one transmission focus to another may give rise to some field variability in the characteristics analyzed in the experimental growth and the field follow-up studies, but the physiographic and climatic uniformity of the endemic corridors at the Altiplano endemic area, together with the genetic monomorphism of the snails, suggest the extrapolation potential of the data obtained.

The diversity of habitat types and differing respective population dynamics of the only one lymnaeid species found, *G. truncatula*, agree with present knowledge on these aspects in situations where this lymnaeid species is the only transmitting snail present, as in Europe. Indeed, the singularity detected in given characteristics are linked to the extreme conditions of the very high altitude, which are similar throughout the endemic area.

The experimental and field studies here reported concern a very high altitude endemic area of human and animal fascioliasis for the first time. They offer the necessary methodological baseline and an overview example of the lymnaeid vector scenario in a human hyperendemic area for such kind of needed studies still to be implemented in high altitude fascioliasis endemic areas in other tropical and subtropical countries of Latin America.

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