

Insights on the Presence and Economic Impact of Water Related Trematodes in Natural Environments from Patagonia, Southern Argentina

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Abstract

In this cross-sectional epidemiological survey, we assessed the diversity and frequency of water related trematodes in Futaleufu Department, a region of Southern Argentina with ecological importance due its value as natural fresh water resource and flora and fauna sanctuary. Snails, Aquatic vegetables and Faecal samples from domestic and wild animals were collected between February 2012 and March 2014. In addition, the official records of authorized slaughterhouses located in the area were analysed in order to estimate the specific rate of liver condemnation due to fascioliasis in livestock and its derived direct costs. The collected snails were identified as *Lymnaea spp.*, *Chilina spp.* and *Physa spp.*, 20% of the snails proved to be infected at the larval stages of the *Fasciola hepatica* and Notocotylidae-like family *V. anagallis-aquatica* was positive for the presence of the metacercarial stage of both species. The 23.3% of samples of deposition from domestic and wild animals were positive to *F. hepatica* eggs. The prevalence of fascioliasis in slaughtered animals was 31.4% (CI95%: 31.1%, 31.8%) in bovines and 6.0% (CI95%: 5.9%, 6.2%) in ovines, representing an economic loss of 210,807.5 USD. In consequence, Futaleufu Department should be regarded as an endemic region for animal fascioliasis. Our findings contribute to expand knowledge about the parasitological fauna of freshwater natural environments in the Southern Andean Patagonia. Additionally, they would be useful for the application of more effective control strategies against fascioliasis and other water related trematodiasis in the studied region.

Keywords

Water related trematodiasis; Aquatics vegetables; Wildlife; Economic losses; Argentina

Introduction

Humans, domestic and wild animals can be infected by water related trematodes helminthes, whose infective larval stages occur in animals or aquatic plants. Almost eighty different species of these trematodes have been reported, fascioloids being the most important ones. Fascioliasis is considered by the World Health Organization to be one of the most significant helminth diseases, with a noteworthy impact on human development [1]. It has been emerging in various regions of the world including Latin American countries over the last two decades. This appears to be related to climate change and global warming considering the dependence of lymnaeid snails and fasciolid larval stages on environmental characteristics [2].

In Argentina this helminthiasis is caused by *Fasciola hepatica* (Trematoda: Digenea), with an indirect life cycle involving domestic and wild herbivorous mammals and humans as definitive hosts, and freshwater gastropods as vector [3]. In the Southern Patagonian region the status of trematodiasis in natural environments was studied in a limited number of epidemiological surveys [4]. With the aim of increasing knowledge about the parasitological situation of natural environments in the Andean region of Southern Patagonia (Argentina), the principal aim of this study was to confirm the presence of trematode species of medical and/or veterinary importance as well as to give insights into the prevalence of fascioliasis in livestock and its derived economic impact.

Material and Methods**Study area and meteorological data**

Futaleufú Department is located in northwest of Chubut Province, Argentina (Figure 1) comprises several different ecosystems, including a protected area called Los Alerces National Park. Summers are generally dry and mild and winters are the rainiest season, with a snowy period from June to August. The region has permanent and temporary freshwater

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environments such as lakes, lagoons, rivers, streams, ponds and water-saturated plains (locally known as mallines). This Department is one of the most important tourist destinations in the country for both local and foreign travellers. During the studied periods, data related to meteorological conditions were obtained from the records of the National Meteorological Service at the local Esquel station. The average maximum and minimum temperature was 21.6°C and 16.5°C, the relative humidity (RH) was 56.7% and 47.5%, and the water temperature was 20°C and 14°C, respectively. The accumulated precipitations were 182 mm and 52 mm.

Trematode environmental presence study

The snails, aquatic plants and faecal samples from wild animals and livestock were collected from different biotopes (Table 1) during surveys conducted in three sampling periods, the first from February 2012 to March 2012, the second from December 2012 to March 2013, and the third from January 2014 to March 2014.

A total of 329 snails were hand-collected from each freshwater environment by means of an incidental sampling. They were transported alive to the laboratory in plastic containers hermetically closed at ambient temperature and containing water from the collection site. Upon arrival to the laboratory, snails were anesthetized with menthol [5] and they were identified at genus level by morphometric features of their shell. Snail shells were measured according to traditional malacological methods [4,6]. Moreover, the soft parts of the snails were dissected under a stereomicroscope and the anatomy of reproductive system analysed. For the presence of sporocysts and cercariae of trematodes snails were dissected by removing their shell and the internal organs were then inspected using conventional microscopy at 10x and 40x magnifications in a microscope LEICA DM500. Cercariae identification was carried out using morphological criteria [7,8]. Characteristics as number and position of body suckers, the presence of well visible eyespots, shape and relative dimensions of the cercarial tail, the presence or absence of various specialized surface structures like stylet and spiny collar were assessed.

The identification of one individual of *Physa acuta* was confirmed by PCR's analysis in the Unit of Bioassays Services and Ecotoxicological Diagnoses of the National University of La Plata. DNA extraction was carried out by means of the commercial kit Qiagen DN easy Blood & Tissue Kit. A fragment of approximately 700 bp was amplified by the use of universal primers described in FOLMER et al. (1994) [9]. The amplified fragment was purified by the AxyPrep PCR Clean-up Kit system and its sequencing in both directions was performed through the automatic sequencing service of Macrogen, Inc. (Seoul, Korea). The sequences achieved in both directions were optimized by visual inspection and manually edited; after eliminating the primers, the consensus sequence of 655 bp in length was obtained. Finally, the identity of this sequence was obtained using the BLASTN algorithm, based on its comparison with all the DNA sequences deposited in GenBank, NCBI.

Five hundred grams of specimens of aquatic wild plants usually used for human consumption were collected by means of an intentional sample in each biotope. The vegetal samples were washed twice with sterile distilled water and fixed with 5 ml of 5% formaldehyde. Next, 5 ml of sulfuric acid was added and the samples were incubated at room temperature for 8 hours. The sediment was centrifuged at 2000 rpm and inspected by microscopic analyses at 10x and 40x magnifications, to verify metacercarial presence. Metacercariae identification was carried out using morphological features as cyst darkness and color, shape and diameter, and cyst wall features. The vegetables were identified based on morphometric features [10].

Nineteen faecal samples of wildlife animals (*Sus scrofa*, *Hippocamelus bisulcus*, *Lama guanicoe*, *Lepus europaeus*, *Lycalopex culpaeus*), and forty-one from domestic animals (*Ovis aries*, *Bos taurus*, *Equus caballus*) were collected by means of a non-random incidental sampling method, from a radius of up to 50 m around the natural fresh water environment in each biotope. These faeces were

classified by means of its morphologic macroscopic characteristics and the identification of remains of own food of the diet of every animal species or by chasing to the animal when it was possible. Depositions were placed in formaldehyde 5% in sealed plastic bags and stored protected from light at room temperature. *F. hepatica* eggs were searched for in accordance with the sedimentation technique of Dennis-Stone and microscopically examined at 40x magnification in duplicate. A sample was considered as positive if *F. hepatica* eggs with the correct morphology of ellipsoidal and operculated structure were observed. Obtained frequency per faecal pellet was expressed as a proportion of positive samples to *F. hepatica* eggs.

Fasciola hepatica presence in slaughterhouse animals and estimation of associated economic losses

As a background, the official records of the three authorized slaughterhouses located in the Department were analysed in order to determine the condemnation rate due to fascioliasis during the 2009-2012 period. Collected data were summarized into a Microsoft Excel 7 spreadsheet (Microsoft Corporation, Redmond, WA, USA). Proportions were used to describe some of the data obtained. True prevalence was estimated using WinEpi 2.0 online open tool [11] with a confidence level of 95%. The total number of the bovine and ovine population by year was obtained from the official livestock census of the province of Chubut conducted by the National Institute of Agricultural Technology [12]. In addition, adult specimens of *F. hepatica* were obtained from bovine and ovine livers condemned at the main slaughterhouse of the Department. The total economic loss was calculated from adding up the livers condemned due to *F. hepatica* from each year and from the whole period 2009 to 2012. For this purpose, we used the following formula [13]:

$$TEL = n \times P \times W$$

TEL = Total economic loss

n = Total number of condemned livers due to *F. hepatica*

P = Liver price (dollar/kg),

W = Average liver weight (kg)

For the calculation, the local slaughterhouse's selling prices were considered. They were 2.5 USD for each kilogram of bovine liver and 4 USD for each kilogram of ovine liver and livers weighing of 5 kg in average in the case of cattle and of 1 kg in the case of sheep.

Results

Snails

Throughout the study period, snails were found repeatedly in all surveyed waterbodies. A total of 329 snails were collected from the natural environments and were identified as *Chilina spp.* (n = 202), *Physa spp.* (n = 104) and *Lymnaea spp.* (n=23) (Figure 2), being *Chilina spp.* founded in all biotope types (Table 1). Interestingly, the identity of the consensus sequence obtained from one individual of *Physa spp.* studied by molecular methods corresponds 99% with *Physa heterostrophia* (GenBank #AY651193), which according to the new Wethington & Lydeard [14] proposal is currently classified as *Physa acuta* Draparnaud, 1805. The percentage of identity mentioned is explained on the basis that both sequences differ in only three nucleotides of the 655 compared.

A total of 66/329 (20%) of the snails collected proved to be naturally infected with stages of developmental forms of trematodes varying the percentage of naturally parasited from 19.3% in *Chilina spp.* to 21.7% in *Lymnaea spp.* The intramolluscan larval trematode stages found belonged to the Notocotylidae-like family and to *F. hepatica* (Figure 3). Notocotylidae-like larval stages were found in *Chilina spp.* (n = 39), *Physa spp.* (n = 20) and *Lymnaea spp.* (n = 1) snails while *F. hepatica* larval stages were found in *Lymnaea spp.* (n = 4) as well as *Physa spp.* (n = 2). Notocotylidae-like monostome type cercarias were recognized stating the following characteristics: tail unlocked, finfolds on tail not present, ventral sucker absent, two or occasionally three eyespots present, a pair of adhesive organs present at posterior end of body and, numerous cystogenous glands

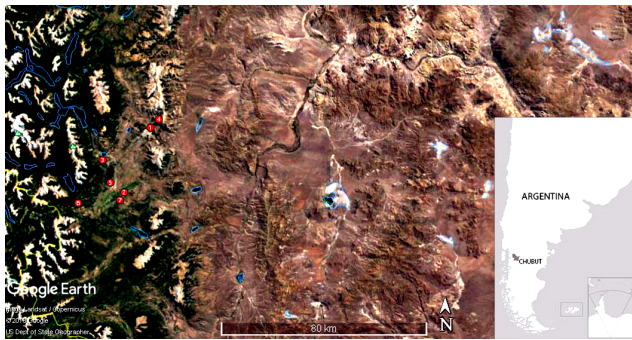


Figure 1: The Geographical Location of the Futaleufu Department inside the Chubut Province, Southern Argentina, Where the study has been conducted. Red circles indicate the sampling locations (Image from Google Earth).

Images used are in the public domain and have been downloaded from: National Geographic Information Center, Madrid, Spain.

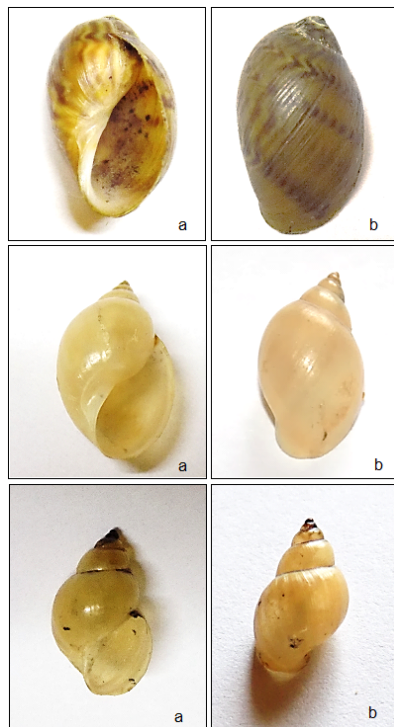


Figure 2: Shell of *Chilina* spp (up), *Physa* spp (middle) and *Lymnaea* spp (below) from Futaleufu Department, Southern Argentina. In each case: a) Ventral views, b) Dorsal views. Photographs credits: Débora Saunders.

in body. The *F. hepatica* cercariae were recognized by its large heart shaped body and simple long tail, and the tail length is nearly three times as the body, ventral sucker is larger than the oral sucker, body has a thick wall, and is surrounded by minute spines, body is full of numerous cystogenous glands.

Plants

The aquatic and semiaquatic plants studied were identified as *Veronica anagallis-aquatica* (locally known as berro, watercress), *Mimulus glabratus* (locally known as berro amarillo, yellow watercress) *Myriophyllum quitense* (locally known as hinojo, fennel), and *Dysphamia ambrosioides* (locally known as paico). Only *V. anagallis-aquatica* was found positive for the presence of the encysted metacercarial stage of Notocotylidae-like trematode family ($n = 3$) and *F. hepatica* ($n = 1$) (Table 1, Figure 3).

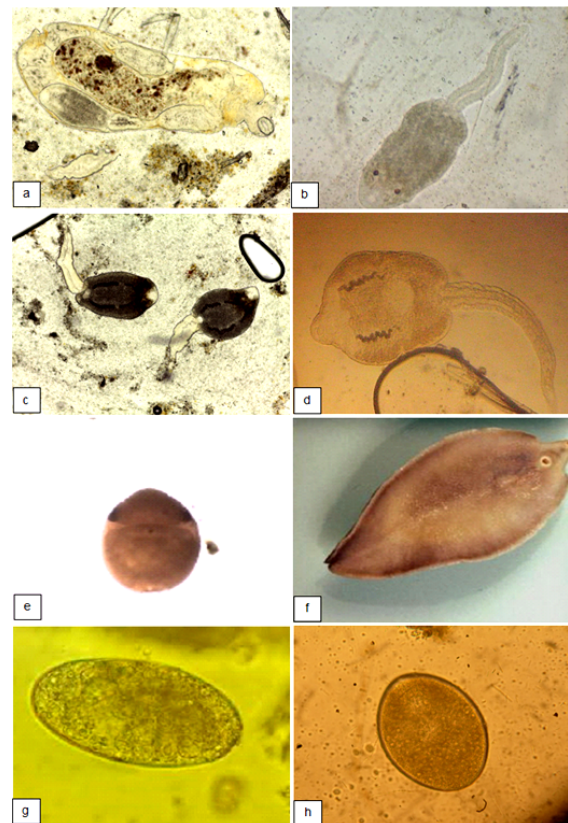


Figure 3: Stages of larval trematode founded in snails from Futaleufu Department, Southern Argentina.

- Redia with immature cercariae of Notocotylidae family in *Physa* spp.,
- Monostome type cercaria of Notocotylidae family in *Chilina* spp.,
- Monostome type cercaria Notocotylidae family in *Lymnaea* spp.,
- Fasciolidae type cercaria in *Physa* spp (40x Magnification). Photographs credits: Debora Saunders,
- Encysted metacercarial stages of Notocotylidae founded in watercress (10x Magnification). Photographs credits: Viviana Hechem,
- Adult *Fasciola hepatica* recovered from liver sheep (40x Magnification). Photographs credits: Elena Sanero,
- F. hepatica* egg recovery from faecal sample of hares,
- F. hepatica* egg recovery from faecal sample of cattle (40x Magnification). Photographs credits: Elena Sanero.

Stools

F. hepatica eggs were detected in 14/60 (23.3%) samples of faecal material from different domestic and wild animal species (Table 2, Figure 3).

Slaughterhouse records

As seen in Table 3, cattle were the type of animal most affected by fascioliasis over the 2009-2012 periods with a relative frequency of liver condemnation due to fascioliasis of 31.4% in cattle and 6.1% in sheep. Further to that, adult stages of the parasite were detected in condemned liver from both animal species (Figure 3). The direct costs derived from liver condemnation due to fascioliasis were estimated to be about USD 190,887.5 USD and USD 19,920 in bovine and ovine respectively.

Discussion

This study provides the first extensive description of the

A Biotope type	Studied environment	Location and meters above sea level	No. of snails collected	Species identified	No. of naturally infected snails	Presence of vegetables contaminated with metacercarial stages (species)	Presence of <i>F. hepatica</i> eggs in animal fecal samples
Stream	Arroyo Esquel	42° 53' S, 71° 18' W, 620 m.a.s.l.	124	<i>Chilina</i> spp. (18)	0	Notocotylidae <i>F. hepatica</i>	Yes
				<i>Physa</i> spp. (91)	22		
				<i>Lymnaea</i> spp (15)	2		
	Arroyo Miguens	43° 06' S, 71° 27' W, 200 m.a.s.l.	20	<i>Chilina</i> spp. (20)	1	No	Yes
Lagoon	Laguna Terraplen ¹	42° 59' S, 71° 31' W, 53 m.a.s.l.	1	<i>Chilina</i> spp. (69)	1	NR	No
	Laguna Willimanco	42° 53' S, 71° 16' W, 715 m.a.s.l.	45	<i>Chilina</i> spp. (39)	23	No	Yes
				<i>Lymnaea</i> spp (6)	3		
River	Rio Percy	43° 05' S, 71° 28' W, 376 m.a.s.l.	84	<i>Chilina</i> spp. (69)	8	Notocotylidae	Yes
				<i>Physa</i> spp. (13)	0		
				<i>Lymnaea</i> spp (2)	0		
	Rio Grande	43°10' S, 71°40' W, 329 m.a.s.l.	50	<i>Chilina</i> spp. (50)	3	NR	No
	Río Corinto	43° 07' S, 71° 26' W, 356 m.a.s.l.	5	<i>Chilina</i> spp. (5)	3	Notocotylidae	Yes

¹Located in Los Alerces National Park. m. a. s. l: meters above sea level, NR: not recorded

Table 1: Collection sites, location data, number of snails sampled and number of naturally infected specimens, presence of contaminated vegetables with metacercarial stages and presence of *Fasciola hepatica* eggs in animal feces in Patagonian freshwater biotopes of Futaleufu Department, Southern Argentina.

Animal category	Animal species	Number of samples (N)	Number of positive samples (n)	Percentage of positive samples (%)
Domestic	Sheep (<i>Ovis aries</i>)	19	5	26
	Cow (<i>Bos taurus</i>)	19	5	26
	Horse (<i>Equus caballus</i>)	3	0	0
Wild	Wild board (<i>Sus scrofa</i>)	4	0	0
	Deer (<i>Hippocamelus bisulcus</i>)	2	0	0
	Guanaco (<i>Lama guanicoe</i>)	2	0	0
	Hare (<i>Lepus europaeus</i>)	10	4	40
	Fox (<i>Lycalopex culpaeus</i>)	1	0	0
Total		60	14	23.3

Table 2: Frequency per faecal pellet of positive samples of domestic and wild animals to *Fasciola hepatica* eggs. Futaleufu Department, Southern Argentina.

diversity and frequency of trematodes from Futaleufu Department, including Los Alerces National Park, a region of Argentina with ecological importance due its value as natural fresh water resource and flora and fauna sanctuary. Ours results confirm the presence of *F. hepatica* and report the finding of Notocotylidae-like family in different natural environments from Patagonian. In consequence, this investigation complements and expands the limited data on the epidemiology of trematodes in Southern Andean Argentinian region. It's important pointing out that *F. hepatica*, as causal agent of water related diseases, inflict considerable morbidity on healthy immunocompetent people, and may cause life-threatening diseases

among immunocompromised and immunosuppressed populations [15]. On the other hand, it is noteworthy that the Notocotylidae is an intestinal parasite family of birds and mammals.

In terms of vectors, at least three genera of the aquatic pulmonate gastropod mollusks were identified and we documented the presence of naturally infected snails of *Chilina* spp., *Physa* spp. and *Lymnaea* spp. with Notocotylidae-like and, *Lymnaea* spp. as well as *Physa* spp. with *F. hepatica*. *Chilina* spp. has been the most ubiquitous genus. It has been established that fasciolid life cycle and lymnaeid population dynamics are markedly dependent on climate, mainly temperature and rainfall [2]. Ours findings suggest that the climate conditions of

Livestock	Year	N	Total number of animals slaughtered (n)	Number of liver condemned (n)	Proportion of liver condemned (%)	Number of liver condemned due to fascioliasis (n)	Proportion of liver condemned due to fascioliasis (%)	True Prevalence of fascioliasis (IC95%)
Cattle	2009	49599	14230	6543	45.9	5933	41.7	41.7% (41.0%, 42.4%)
	2010	48641	14230	5690	45.3	5248	41.8	41.8% (41.1%, 42.6%)
	2011	48641	11945	4034	33.8	3020	25.3	25.3% (24.6%, 26.0%)
	2012	49827	9865	1903	19.3	1070	10.8	10.8% (10.3%, 11.4%)
	Total	197986	48588	18170	37.4	15271	31.4	31.4% (31.1%, 31.8%) ¹
Sheep	2009	106964	23920	3978	16.6	1980	8.3	8.3% (7.9%, 8.6%)
	2010	110389	7098	2423	34.1	1935	27.3	27.3% (26.3%, 28.3%)
	2011	102001	26443	2024	7.6	1045	3.9	3.9% (3.7%, 4.1%)
	2012	101017	24912	431	1.7	24	0.9	0.09% (0.06%, 0.13%)
	Total	420371	82373	8856	10.7	4980	6.1	6.0% (5.9%, 6.2%) ¹

¹Total period prevalence 2009-2012.

Table 3: Annual records of livestock slaughtered in official slaughter houses during 2009-2012 period and related prevalence of fascioliasis. Futaleufu Department, Southern Argentina.

the region appear to be appropriate for the fluke's biological cycle development and for fascioliasis transmission, with maximum and minimum registers of 31.4 and 8°C of temperature, of 92% and 22% of RH and of 182 mm and 52 mm of accumulated precipitations.

One of the most intriguing finding of this study was the detection of some sporocysts of *F. hepatica* in *Physa* spp. Arguing in favor of the occurrence of *F. hepatica* in this intermediate host it was reported that *P. acuta* could be an unusual snail species involved in the transmission of this trematode in watercress beds in central France, with a prevalence of the snail infection less than 1% [16]. Even been *Physa* spp. a poor host for this fluke, it is interesting to carry out more exhaustive studies about its role in the transmission of this parasite under natural environmental Patagonian conditions. Nevertheless, this result should be confirmed in the future due to its rarity and the *F. hepatica* specify characteristics worldwide.

In Argentina, cattle and sheep are natural hosts for *F. hepatica* [3] and fascioliasis in wild animals such as *Lagomorpha*, *Cervidae* and *Rheidae* species has also been reported [17]. Our study corroborates the presence of *F. hepatica* eggs in faecal material belonging to cows, sheep and hares collected in different biotopes of the studied region. The finding of *F. hepatica* eggs in feces from hares (*Lepus europaeus*) supports the hypothesis that the liver fluke naturally infects the European brown hare also in Southern Patagonia [18]. Noteworthy, *L. europaeus* may play a significant role in the trematode transmission cycle given that it shares habitats with livestock and snails and its abundance is high.

Transmission of trematodes is enhanced by cultural practices of eating raw or inadequately cooked food, socio-economic factors, and the availability of wide zoonotic and sylvatic reservoirs [15]. The inhabitants of the area use all the plant species studied for human consumption. It is important to highlight the presence of the metacercariae stage of *F. hepatica* in specimens of *V. anagallis-aquatica* growing naturally along the river and streambeds. Ingestion of watercress carrying this infective form of the parasite is known to constitute the main fascioliasis infection source in humans worldwide. In particular, in Argentina previous watercress ingestion has been described in 214 patients [3]. To the best of our knowledge, just one study included the assessment of plant borne contamination in Patagonian region [19], been the present the first and Southernmost report of watercress contamination with metacercarial stages of *F. hepatica* and of Notocotylidae.

To the Chubut province, it has been reported prevalence's of 18.3% in bovines and 2.7% in ovines for the 2006-2009 period [3], using data from the Agriculture and Food Quality National Service. In our study the true prevalence of *F. hepatica* infection was estimated at 31.4% (CI95%: 31.1%, 31.8%) in bovines and 6.0% (CI95%: 5.9%, 6.2%). Moreover, *Fasciola* spp. is the most widespread liver fluke

found in cow and sheep and is more dominant in cows than sheep. Our abattoir survey generally reflected the infection situation in the Futaleufu Department. These results indicate that the prevalence in bovine and ovine fascioliasis is higher than previously reported to the whole province. These findings could be attributed to at least two causes, a real increase of the frequency of appearance of the infection in livestock or/and a notification bias from slaughterhouse records to governmental control authorities.

Argentina has a large livestock industry in which *F. hepatica* causes serious economic impact due to reduction in milk and meat production, liver condemnation, reproductive failure and mortality. We estimated that for the 2009-2012 period direct economic loss due to fascioliasis bovine and ovine condemnation amounts 210,807.5 USD in losses. In order to mitigate the effect of fascioliasis on regional economics, the control of the trematodiasis mainly relies on timely treatment with triclabendazole of livestock and veterinary slaughterhouses inspection. However, this timely treatment is the quickest way to control morbidity associated with fascioliasis, it should be complemented by implementing measures that aim to reduce transmission rates. Nevertheless, there is no a special control program of fascioliasis conducted by local or regional sanitary authorities and/or planned information about the disease that is accessible to the community. In addition, triclabendazole resistance detected in Argentinian cattle in the Patagonian province of Neuquen [20] supposes a serious question mark on disease transmission control in Argentina. Although the types of control measures to be complemented depend on the setting it would be recommendable to include measures at three different fields [1]. One would be education and communication promoting cultivation of vegetables in water free from faecal pollution as well as thorough cooking of vegetables before human consumption. Related to the veterinary field treating domestic animals and enforcing separation between husbandry and humans should be taken into account. Finally, environmental measures such as containment of the snail intermediate hosts and drainage of grazing land should be implemented. Our epidemiological survey is added to the evidence from other exhaustive studies that have been performed mainly in central and northern Patagonia [19,21-24]. This kind of findings regarding the ecology of definitive and intermediate hosts would not only help to understand the flow patterns of larval digeneans in Patagonian freshwater environments but also provide a valuable baseline on which to design adequate control strategies on fascioliasis in animal reservoirs and snails vectors in this very large and environmentally heterogeneous region of the country.

Our work has some limitations, we have no the possibility to perform experimental infections in laboratory animals to obtain the trematode adult stages and verify their systematic belonging and, molecular studies could not be carried out for specimen classification

purposes in snails, with the exception of *Physa* spp. In this last case we expanded the preliminary finding further. Thus, the presence of *Physa acuta* was assessed by molecular methods. More information of some life cycle stages and on the ecology of the intermediate hosts is needed to clarify the taxonomic status of the Notocotylidae like parasites. It is surprising that Paramphistomids, that are usual parasites of ruminants everywhere in the world, were not founded in this study. Considering that Paramphistomid larval stages also present eye spots in their larval stages, we used characteristic as ventral sucker absent and the presence of many cystogenous glands in body to differentiate with Notocotylidae type cercaria. Also, the operculate eggs of this specie of ruminants parasites could be confused with *F. hepatica* eggs. Again, the impossibility of carrying out molecular studies constitutes a limitation to our results. Therefore, they should be included in future research conducted in this region.

Conclusion

Water for life is the predominant natural resource, which is at the dangerous edge today and it is necessary to protect the resources for future generations. In this sense, we may conclude that this study is of sanitary importance as Futaleufu Department, a key freshwater resource at national level, may be regarded as an endemic region for animal fascioliasis. Ours results would be useful for the application of more effective control strategies against the mentioned water related trematodiasis in the studied region and, in consequence, decrease the economic losses due to condemnation of infected livers with *F. hepatica*. Likewise, it contributes to expanding the knowledge of the parasitic fauna in this important Southern Argentinean area.

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