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Diphyllobothriidea in the north area of the Andean Patagonia: Epidemiology in urban dogs, morphometrical and molecular identification, with comments on wild carnivores

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ABSTRACT

Diphyllobothriidea are the principal agents of Diphyllobothriosis, a widespread food-borne cestodosis. Accurate identification of the species in samples is therefore crucial for diagnosis and epidemiology in wild and domestic animals, and also humans. We aim to identify at specific level the causative agent, and provide an observational, descriptive, and transversal study of the epidemiology of this zoonosis in urban dogs. Also data on wild carnivores from Northwestern Patagonia are presented. Dog feces were collected in thirteen neighborhoods of varying socioeconomic status, and stools were analyzed by two concentration methods. Adult worms were collected and identified by molecular methods. The population of free-roaming dogs in each neighborhood was estimated, and surveys were conducted at all veterinary clinics registered in the Veterinary Medical College of Bariloche city. A total of 36 wild carnivores road killed or found dead in three National Parks were analyzed. Molecular and morphometric analyses of proglottids and eggs from dogs indicate they are infected with *D. latum.* Twenty out of 118 dog feces were positive for *Diphyllobothrium*, from 9 out of 13 neighborhoods, with infection values between 10% and 66%. Percentage of infection was correlated positively with the number of free roaming dogs per block, and with Unsatisfied Basic Needs (UBN %), but not with distance to nearest water body. Infection by *D. latum* in dogs is widely distributed throughout the city. Not all local veterinarians know the occurrence of Diphyllobothriosis in the dogs of the city, and it is evident that this zoonosis is underdiagnosed in relation to the percentage of infection found in this study. None of the analyzed wild carnivores were positive for *Diphyllobothrium*.

Bullet points

- Occurrence of Diphyllobothriosis in urban dogs from Bariloche is directly related to the number of free-roaming dogs and the socio-economic status of the neighborhoods.
- Infection by *Diphyllobothrium latum* in urban dogs is widely distributed throughout an Andean Patagonian city (Bariloche).
- The disease is not well known among local veterinarians, and its diagnosis is underestimated due to the lack of routine copro-parasitological analysis. To improve diagnosis in veterinary clinics, a reliable database is also necessary, and control measures should be established for the deworming and management of stray dogs.

1. Introduction

Classification of Diphyllobothriidea has been controversial and complicated (Kuchta and Scholz, 2017), particularly in the genus *Diphyllobothrium,* which historically included around 30 species. Recent molecular studies have reduced this number and showed that there are three subgroups in the genus: (1) species from cetaceans, (2) species parasitizing pinnipeds, which form a polyphyletic *Diphyllobothrium* group, and (3) species from terrestrial mammals and birds (Kuchta and

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Scholz, 2017). One of the difficulties found in the identification of this group is the unvarying strobilar morphology seen across the taxa, and the high level of intraspecific and interindividual variation recorded for most morphometric and morphological characters across the order (Hernández-Orts et al., 2015). Molecular studies resurrected the genus *Dibothriocephalus* Lühe, 1899, which includes terrestrial and most human-infecting taxa, previously considered as belonging to the genus *Diphyllobothrium*. This resurrected genus includes the species *Dibothriocephalus latus* (Linnaeus, 1758 syn. *Diphyllobothrium latum*) and *Dibothriocephalus dendriticus* (Nitzsch, 1824 syn. *Diphyllobothrium dendriticum*). However, for reasons of use and custom, for the purposes of this study we will refer to the disease as Diphyllobothriosis, and the species involved as *Diphyllobothrium latum* and *Diphyllobothrium dendriticum.*

Diphyllobothriidea are the principal agents of Diphyllobothriosis, a widespread food-borne cestodosis, therefore accurate species identification of the clinical samples is crucial to the diagnosis and epidemiology of both humans and domestic, farmed or wild animals (Waeschenbach et al., 2017). In addition to molecular methods, the combination of morphological, morphometric, and ultrastructural data on eggs could also be useful for differentiation of the species affecting humans (Leštinová et al., 2016). Humans acquire this infection with the ingestion of undercooked or raw fish from gourmet dishes like sushi, sashimi or "ceviche" (Waeschenbach et al., 2017). The increase of consumption of these meals all over the world has provoked human infections in several countries, including some outside the natural distributional range of these parasite species (Semenas, 2006; Scholz and Kuchta, 2016).

The life cycle of *D. latum* and *D. dendriticum* includes copepods and fishes as intermediate hosts (von Bonsdorff, 1977). Two species of calanoid copepods (*Tumeodiaptomus diabolicus* and *Boeckella gracilipes*) were identified as first intermediate hosts in Chilean Patagonia (Torres et al., 2004), and introduced salmonids and native fishes were found to be second intermediate hosts in both Argentinean and Chilean Patagonia (Torres et al., 1989, 1991; Revenga and Semenas, 1991; Revenga, 1993; Ortubay et al., 1994; Viozzi et al., 2009). Also, *Larus dominicanus* has been cited as the definitive host for *D. dendriticum* (Kreiter and Semenas, 1997; Casalins et al., 2015), whereas *D. latum* has been cited as parasitizing humans (Semenas, 2006). Dogs, and also cats, acquire the infection when they eat raw fish and discarded viscera (Leštinová et al., 2016). Considering the low host specificity of the species that infect terrestrial mammals, it is not surprising that humans act as accidental definitive hosts (Waeschenbach et al., 2017).

As far as we are aware, findings of *Diphyllobothrium* eggs in South American go back at least from 1951 in Chile (Neghme and Bertín, 1951a; 1951b). In Argentina, the first report of a species of *Diphyllobothrium* was the description of *Diphyllobothrium grañaia* from dogs (Bacigalupo, 1948). This species is recognized by Schmidt (1986, see page 94 mentioned as *D. granaia*) but is currently not included in the Cestode database (Gibson et al., 2005) or in updated publications on Diphyllobothriidea (Kuchta and Scholz, 2017). Successful experimental infection of 4 dogs with plerocercoids of rainbow trout from Nahuel Huapi Lake (Patagonia, Argentina) were later reported and assigned to *D. latum* (Bacigalupo and D'Alessandro Bacigalupo, 1952).

In Argentinean Patagonia, the first record of *Diphyllobothrium* eggs in canine feces was that of Chubut Province (Zunino et al., 2000). Posteriorly, eggs in feces were found in Neuquén Province (Soriano et al., 2010) and in Bariloche, Río Negro Province (Semenas et al., 2014; Flores et al., 2017) (Table 1). In Northeastern Argentina, eggs in canine feces have also been found in the provinces of Santa Fé, Corrientes and Misiones (Milano and Oscherov, 2005; Ruiz et al., 2011; Rivero et al., 2015), including ecoregions with subtropical and temperate climates, which lie outside the natural epidemiological environment for these cestode species (Table 1). Regarding infection in wild carnivores, in our country adult worms assigned to *Diphyllobothrium* (cited as *Bothriocephalus*) were found in two species of felines in Buenos Aires Zoo: *Herpailurus yagouaroundi* (eyra), and *Panthera onca* (yaguareté) (Parodi and Widakowich, 1917) and also in *Puma concolor* (puma), from La Plata Zoo in Buenos Aires Province (Mc Donagh, 1930). More recently, in the Northeastern provinces of Argentina, *Diphyllobothrium* adults and eggs were found in carnivores kept in captivity: *Procyon cancrivorus* (crab eating racoon), *Lycalopex gymnocercus* (Pampa fox), and *Chrysocyon brachyurus* (aguará guazú) (Martínez et al., 2000).

All the previous records of *Diphyllobothrium* in domestic dogs and wild carnivores in our country represent isolated occurrence data, and there are no epidemiological studies that analyze social, environmental or economic factors involved in the transmission and persistence of this zoonosis, or its public health relevance. The aim of this work is to describe the epidemiology of Diphyllobothriosis in urban dogs from Patagonia, determining which species of *Diphyllobothrium* are involved, and obtaining information about the detection and treatment of this disease in domestic dogs. Data on the search for this parasite species in wild carnivores are also included.

2. Materials and methods

2.1. Study area

The survey was conducted in three Andean Patagonian National Parks: Lanín (LNP, Neuquén Province), Nahuel Huapi (NHNP, Río Negro and Neuquén Provinces), and Los Alerces (LANP, Chubut Province). A large hydrographic network characterizes these protected areas, with numerous glacial lakes surrounded by deciduous and perennial forests in the West, and steppe in the East. Nahuel Huapi Lake (NHNP) is the biggest lake in the area, and two cities are located on its coasts: Villa La Angostura in the North and Bariloche in the South (Fig. 1). The epidemiological study of urban dogs was carried out in the city of Bariloche (41°8′S; 71°27′W), which is located in the Andean foothills, and is characterized by a temperate climate with a rainy and snowy winter. The city covers a large area within a National Park, and has a rugged topography, surrounded by lakes and rivers. Bariloche has a population of 112,887 (INDEC, 2010) and is one of the most important tourist destinations in the country. The society has a very heterogeneous composition with many neighborhoods having low incomes and many Unsatisfied Basic Needs (UBN%) (Territorial Management Plan, 2011), that translate into a city with marked spatial differences regarding socioeconomic aspects (Table 3).

2.2. Epidemiology in urban dogs

2.2.1. Sampling and processing of feces

To analyze the epidemiology of infection in dogs, we performed a descriptive, observational, and transversal study. Feces were collected in thirteen neighborhoods (inhabited by more than 20,000 people in total) (Fig. 1, Table 3) with different UBN % (Territorial Management Plan, 2011). This index reflects the socio-economic condition of the neighborhood, and includes educational level, housing deficiencies, and water and health services, among other characteristics. During autumn-winter (April to August) of 2016, we collected from 6 to 20 samples of fresh dog feces per neighborhood (118 samples in total) from streets, parks, squares, and peridomiciliary areas.

The collected samples were inactivated at −70 °C for at least 7 days prior to processing. Each stool was analyzed using two egg concentration methods: sedimentation (modified Telemann) and flotation (Sheather) (Thienpont et al., 1979). Subsequently, two cover glasses for each method per sample were observed under optical microscope at 100× by three different trained professional (Roth, D., Flores, V., and Viozzi, G.) For the Sheater method, each cover-glass was left on the surface of the homogenate for 20 min. A sample was considered positive when at least one of the methods yielded positive results.

Table 1
Records of *Diphyllobothrium* spp. in naturally infected feces and dogs from South America.

Bibliographical source	Species	Origin	N	Percentage of infection	City	Country
Neghme and Bertín, 1951 a,b	*Diphyllobothrium* sp.	W	38	18%	Villarrica	Chile
Bacigalupo and D'Alessandro Bacigalupo, 1952	*D. grañaia*	W	2	2/2	Buenos Aires	Argentina
Torres et al., 1983	*D. pacificum*	W	1	1/1	Valdivia	Chile
Torres et al., 1989	*Diphyllobothrium* sp.	F	105	5.8%	Riñihue	Chile
Torres et al., 1991	*Diphyllobothrium* sp.	F	166	9.1–18.8%	Villarrica	Chile
Zunino et al., 2000	*Diphyllobothrium* sp.	F	30	3.3%	Puelo	Argentina
Cabrera et al., 2001	*D. pacificum*	W	1	1/1	Chincha Alta	Perú
Milano and Oscherov, 2005	Diphyllobothriidae	F	362	2.8%	Corrientes	Argentina
Soriano et al., 2010	*Diphyllobothrium* sp.	F	236	0.10%	Neuquen rural	Argentina
Mocetti et al., 2011	*Diphyllobothrium* sp.	F	131	0.8%	Lima	Perú
Ruiz et al., 2011	*Diphyllobothrium* sp.	W	1	1/1	Santa Fe	Argentina
Costa Santos et al., 2012	Diphyllobothriidae	F	45	2.2%	Serra do Cipó	Brazil
Semenas et al., 2014	*Diphyllobothrium* sp.	F	27	0–33%	Bariloche	Argentina
Rivero et al., 2015	*Diphyllobothrium* sp.	F	405	0.5%	Misiones	Argentina
Flores et al., 2017	*Diphyllobothrium* sp.	F	118	16.9%	Bariloche	Argentina
Present work	*D. latum*	F	118	0–66%	Bariloche	Argentina

W: worms; F: feces

2.2.2. Estimation of free-roaming dogs

The population of free-roaming dogs in each neighborhood was estimated by counting dogs in the street and public spaces, along 10 blocks (aprox. 1000 m) per neighborhood in the morning hours (9–12 h) in one single day. We considered free-roaming dogs to be animals wandering around without any type of control or restriction, whether or not they have an owner (Molina et al., 2006).

2.2.3. Veterinary surveys

Surveys were conducted at all veterinary clinics registered in the Veterinary Medical College of Bariloche. Veterinarians were asked about Diphyllobothriosis diagnoses, with the following questions: (1) Do you perform routine copro-parasitological tests? (2) Have you ever diagnosed Diphyllobothriosis in dogs? (3) On what basis was the diagnosis of Diphyllobothriosis made? (4) What neighborhood did the positive dogs come from? (5) Was there a recurrence of the disease? (6) Did the owner carry out any activity related to fishing or fish processing?

2.3. Morphological and morphometric identification of eggs

Two types of eggs were obtained; those from dog feces and those from adult worms collected during the study from naturally infected dogs. Eggs were measured (length and width) using a graduated eyepiece; mean and range were calculated, with measurements expressed in micrometers. From 1 to 16 eggs *per* positive neighborhood and *per* method were measured, plus 10 eggs for each adult worm obtained.

2.4. Molecular identification

We aimed to extract DNA from both eggs and adult worms. In the case of eggs, twelve positive samples were obtained from infected feces. Then we used two different methods in order to concentrate the eggs (four with the Teleman method and eight from Sheater method, which was prepared without formaldehyde). Samples were then centrifuged and the pellet washed twice with distilled water and transferred to a new tube. Pellets were immersed in liquid nitrogen and pulverized using stainless steel balls in a Mixer Mill (Retsch). Following this, one set of six samples was processed following the manufacturer's recommendations for the DNeasy kit Blood & Tissue (Qiagen). The other set of six samples were processed using Chelex 5% for DNA extraction *sensu* Walsh et al. (1991). Briefly, the methods involve boiling suspensions of eggs in a 5% suspension of Chelex, followed by amplification by PCR of an aliquot of the supernatant.

In the case of adult worms (from El Pilar I and San Cayetanito neighborhoods) a piece of about 3 mm was sectioned and ethanol was allowed to evaporate at room temperature. Both samples, as with the eggs, were immersed in liquid nitrogen and pulverized with a MixerMill, and DNA was then extracted with the DNeasy Blood & Tissue kit (Qiagen).

PCR reaction followed a multiplex PCR protocol (Wicht et al., 2010), which allowed simultaneous identification of both *D. latum* and *D. dendriticum,* using universal primers (MulRevCom, located in positions 1492 to 1512) and two specific forward primers: MulLat3 (position 1055 to 1077) for *D. latum* and MulDen4 (position 1174 to 1200) for *D. dendriticum*. PCR products differ in size and are clearly differentiable (437 bp for *D. latum* and 318 bp for *D. dendriticum*). Each reaction contains 0.3 μM of each single primer and 0.5 μM of the common primer, with one unit of Taq (Life Technologies) when performing multiplex. The reactions were run in a thermal cycler (Applied Biosystems) with a cycle of 94 °C for 5 min followed by 25 cycles at 94 °C for 30 s, 60 °C for 1 min 30 s, 72 °C for 1 min 30 s and a final extension cycle at 72 °C for 10 min. Reactions were run in 1% agarose gels, and stained with SyberSafe (Life Technologies). We included negative and positive controls for each species alone, and for a mixture of both (i. e. one tube with the set of primers for *D. latum*, another one with the set of primers for *D. dendriticum* and a third one in multiplex with both primer pairs). In the case of positive controls we run one tube for each species separately and another one with both species together, using the multiplex reaction. Samples with bands compatible with *Diphyllobothrium* spp. were *exo*-sap purified and sent to Korea's Macrogen Company for sequencing, using the universal primer MulRevCom.

2.5. Wild carnivores

Wild animal carcasses were collected over a period of 22 years (from 1996 to 2018) in Lanín, Nahuel Huapi, and Los Alerces National Parks. The thirty-six wild carnivores analyzed, including 8 species of the Families Mustelidae, Felidae, and Canidae, were road killed or found dead (Table 2). All specimens were kept at −20 °C until the necropsy was performed. The digestive tubes were removed and examined under stereoscopic microscope.

2.6. Data analysis

The percentage of positive feces was calculated for each neighborhood. Non-parametric Spearman Rho correlation tests were performed to analyze the correlation between the percentage of positive feces in relation to number of free-roaming dogs, UBN %, and distance to the nearest lake or river. The distance was measured as a straight line from the center of the neighborhood area to the nearest river or lake, on Google Earth maps. Differences in the length and width of eggs

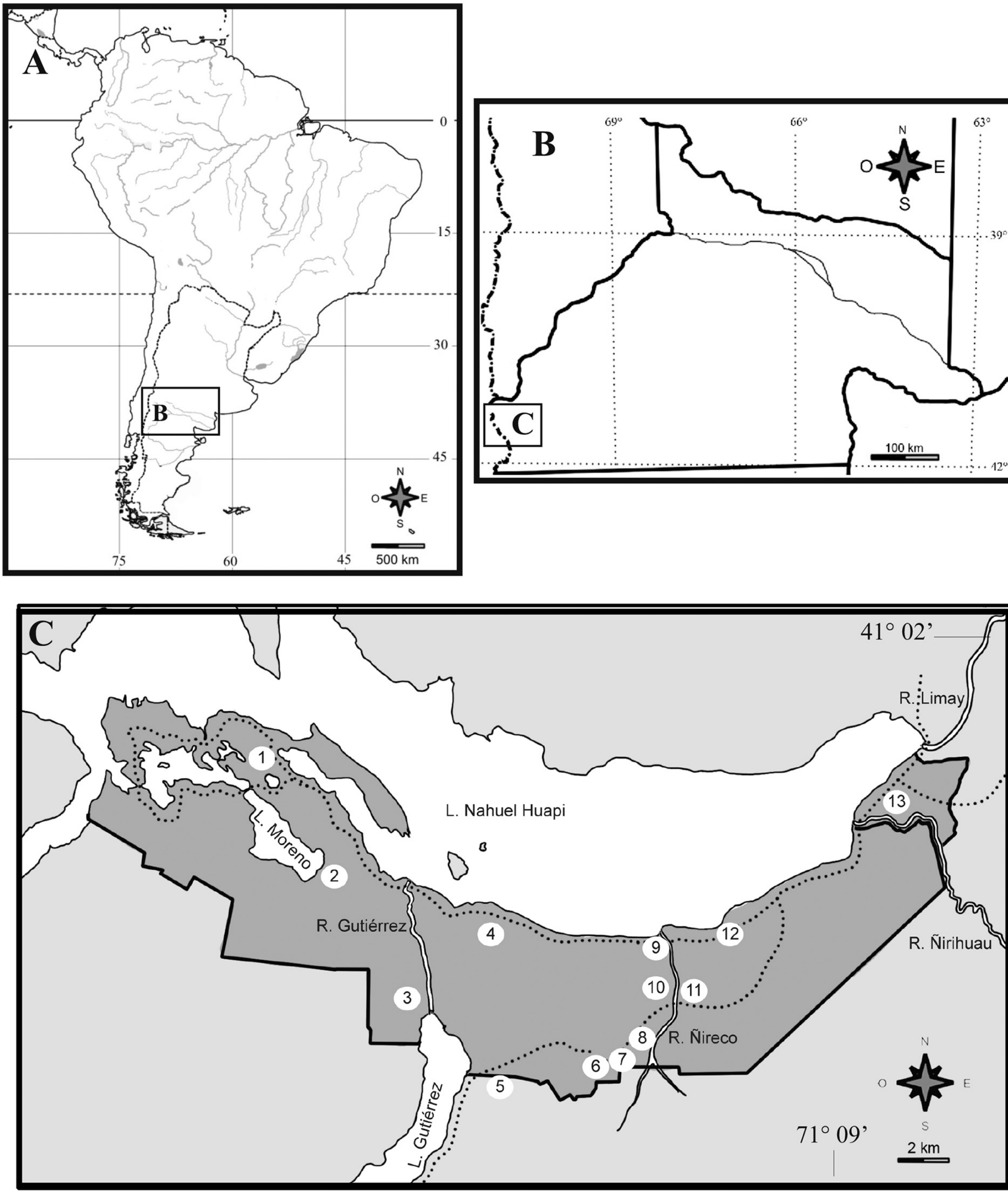


Fig. 1. Geographic locations: A. Argentina; B. Bariloche city; C. neighborhoods.

obtained by the different concentration methods and those obtained from proglottids were analyzed by one-way ANOVA tests. Post-hoc Tukey HSD test was performed to detect differences between egg group pairs. Significance levels were set at 0.05.

3. Results

3.1. Epidemiology in urban dogs

Of a total of 118 feces 20 (16.9%) were positive for *Diphyllobothrium*, and were found in 9 of 13 neighborhoods (69.2%). In these neighborhoods the percentage of infected feces ranged between 10% (Los Coihues) and 66% (Vivero), and the number of free-roaming dogs/100 m varied from 0.5 in Pilar I to 7.2 in Vivero (Table 3). The percentage infection in canine feces correlated positively with the number of free-roaming dogs/100 m ($N = 13$, $R = 0.754$, $p = 0.0028$) and with UBN % (N = 13, $R = 0.661$; $p = 0.0233$), but showed no correlation with distance to nearest water body (N = 13, $R = -0.204$, $p = 0.5033$).

Twenty-two surveys were conducted at veterinary clinics where the following information was recovered: four (18.2%) veterinarians were unaware that Diphyllobothriosis is present in Patagonia; eight (36.4%) professionals perform routine copro-parasitological analysis. Of the four veterinarians (18.2%) who have diagnosed the disease in dogs, two

Table 2
Data of specimens of families Felidae, Canidae, and Mustelidae sampled in Lanin, Nahuel Huapi, and Los Alerces National Parks.

Host	Vernacular name	Family	Date	Sex	Locality (National Park and Province)	Coordinates
Neovison vison (I)	American mink	Mustelidae	Mar-96	nr	Cerro Tronador Route (NHNP, RN)	41°15′S-71°44′W
			Mar-00	M	Hua Hum River (LNP, N)	40°07′S-71°39′W
			Mar-00	M	Hua Hum River (LNP, N)	40°07′S-71°39′W
			Sep-10	M	Fortin Chacabuco (NHNP, N)	40°50′S-71°05′W
			aug-10	M	Pond Larga, Victoria Island (NHNP, RN)	40°53′S-71°32′W
			aug-10	M	Pond Larga, Victoria Island (NHNP), RN	40°53′S-71°32′W
			May-12	F	Lake Nahuel Huapi, Bonita Beach (NHNP, RN)	41°07′S-71°23′W
			Nov-15	nr	Salmonicultura KM10 (NHNP, RN)	41°06′S-71°25′W
			apr-16	nr	Lake Moreno Pisciculture (NHNP, RN)	41°05′S-71°30′W
			apr-17	M	Waterfall Ñivinco (LNP, N)	40°28′S-71°31′W
Galictis cuja (A)	South american wolverine	Mustelidae	aug-05	F	Las Chacras (NHNP, RN)	41°05′S-71°11′W
			Mar-08	M	Rincon Chico (NHNP, N)	40°59′S-71°06′W
			Feb-14	nr	R40, km 1992 (NHNP, RN)	41°26′S-71°29′W
			?	F	Esquel (LANP, Ch)	42°59′S-71°29′W
			Mar-18	M	National Park Nahuel Huapi	?
Lyncodon patagonicus (A)	Patagonian weasel	Mustelidae	Oct-10	M	R40N-R231 Crossroad (NHNP, N)	41°02′S-71°11′W
Lontra provocax (A)	Southern river otter	Mustelidae	apr-10	M	Pichi Traful (NHNP, RN)	40°29′S-71°35′W
Puma concolor (A)	Puma	Felidae	Sep-10	F	Lake Falkner (NHNP, N)	40°26′S-71°32′W
Leopardus geoffroyi (A)	Geoffroy's cat	Felidae	aug-05	M	Las Chacras (NHNP, RN)	41°06′S-71°12′W
			Oct-09	M	Mascardi Village (NHNP, RN)	41°20′S-71°30′W
			Oct-11	M	Villegas Ranch (NHNP, RN)	41°31′S-71°27′W
			aug-12	M	Bariloche Airport (NHNP, RN)	41°08′S-71°10′W
			Sep-12	F	Lake Mascardi (NHNP, RN)	41°19′S-71°29′W
			May-13	M	Villegas River (NHNP, RN)	41°35′S-71°29′W
			May-13	F	Lakes Mascardi-Gutiérrez Watershed (NNHP, RN)	41°15′S-71°28′W
			Mar-18	M	National Park Nahuel Huapi	???
Leopardus colo colo (A)	Pampa's cat	Felidae	Sep-14	F	R40 N-R231 Crossroad (NHNP, RN)	41°02′S-71°11′W
Lycalopex culpaeus (A)	Andean fox	Canidae	aug-12	nr	Anfiteatro Route 237 Dpt. Los Lagos (NHNP, N)	40°59′S-70°05′W
			jan-14	F	Route 40 North Dpt. Los Lagos (NHNP, N)	40°48′S-71°35′W
			Oct-15	M	Quintriqueo (NHNP, N)	40°57′S-71°19′W
			Feb-16	M	Confluencia Traful (NHNP, N)	40°43′S-71°05′W
			jan-18	F	Secc. Limay, Route 2 (NHNP, N)	41°03′S-71°08′W
			Mar-18	M	Las Chacras (NHNP, RN)	41°06′S-71°12′W
			ap-18	F	Catedral Mountain (NHNP, RN)	41°10′S-71°26′W
			May-18	H	Cerro Frey Mountain Path (NHNP, RN)	41°10′S-71°26′W
			May-18	M	Route 82 (NHNP, RN)	41°07′S-71°24′W

References: (LANP) Los Alerces National Park; (LNP) Lanin National Park; (NHNP) Nahuel Huapi National Park; (Ch) Chubut province; (N) Neuquen province. (RN) Rio Negro province; nr not recorded.
(I) introduced species; (A) autochtonous species.
? Data not recorded.

Table 3
Characteristics of the neighborhoods, sampling data of feces and percentage of infection with Diphyllobothrium latum.

Neighborhoods	Estimated population	%NBI		Feces		Distance from the nearest waterbody (Km)	Number of roaming dogs/100 m
		Min	Max	Sample size	% of infection		
Trampa Ñireco	500	18	34	6	0	0.12	1.2
INTA	250	34	50	6	33	0.05	2
San Francisco I	600	34	50	10	40	0.15	1.4
San Cayetanito	400	50	100	6	16	0.05	1.2
Pilar l	255	34	50	6	0	2.5	0.5
Don Bosco	600	18	34	6	16	0.37	0.75
Jamaica	800	34	49	6	33	1.4	2.1
Vivero	1000	50	100	6	66	0.21	7.2
La Cascada	4000	0	7	6	0	0.62	1
Los Cohiues	2500	7	18	10	10	0.3	0.9
Dina Huapi	3500	0	7	10	0	0.39	1
Nahuel Hue	4000	50	100	20	25	0.7	4
Nuestras Malvinas	4000	50	100	20	20	0.9	4

detected it by identification of proglottids dismissed with feces (two dogs), and the other two by identification of *Diphyllobothrium* sp. eggs in canine stools (two dogs). Three of the four dogs diagnosed had gastrointestinal signs; the one without specific signs was diagnosed by a copro-parasitological analysis. The owners of these four animals lived in areas near lakes or rivers, and the dogs had free access to streets. It is interesting to mention that in three of the four dogs diagnosed, the owner had no connection with fishing or fish processing. The deworming treatment chosen in all cases was oral administration of Praziquantel and was effective in three cases. No further information was obtained about the remaining case. The three dewormed dogs had no recurrence of the disease.

3.2. Morphological and morphometric identification of eggs

A total of 89 eggs retrieved from dog feces, were measured; 30

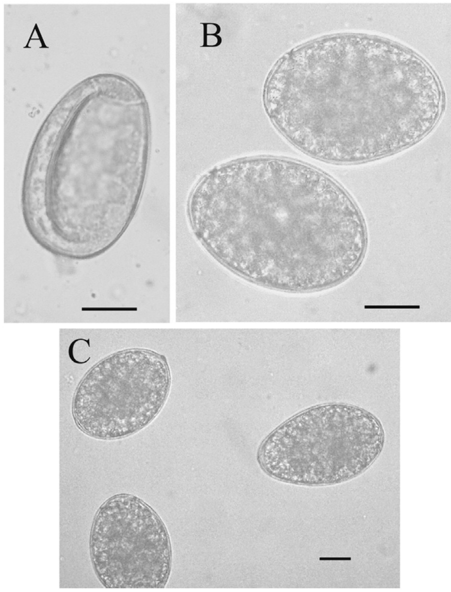


Fig. 2. Microphotographs of *Diphyllobothrium latum* eggs from dogs. **A.** from stools processed by Sheater flotation method, **B.** from stools processed by Telemann sedimentation method, **C.** from adult worms. Scale: 20 μm.

obtained by Sheater and 59 obtained by Telemann methods. Length varied between 58 and 73 μm and width between 40 and 53 μm for those recovered by the Sheater method; these eggs were generally collapsed (Fig. 2 A, Table 4). The length of eggs recovered by the Telemann method varied between 58 and 70 μm, and the width between 40 and 55 μm (Table 4). Four adult worms were obtained from dogs of three neighborhoods (San Cayetanito, Pilar I, and Virgen Misionera), and one from a private veterinary clinic. Forty eggs from adult worms were measured. The length of these eggs varied between 58 and 73 μm and the width between 43 and 55 μm, but the minimum and mean values for egg length from the adult worm used for PCR analysis, are lower than the values for pooled eggs from all adult worms (Fig. 3, Table 5). The results of the one-way ANOVA indicate that there are significant differences in length (F = 5.636; fd = 2; $p = 0.00453$) and in width (F = 7.943; fd = 2; $p = 0.0005$) of the eggs measured (Sheater, Telemann and from proglottids). The Tukey HSD *posteriori* test showed that significant differences in length were found only between the eggs obtained from the two concentration methods used, while in terms of width, the values obtained for proglottids differed from those recovered from feces (Fig. 3).

3.3. Molecular identification of adults and eggs

We were not able to amplify by PCR the eggs isolated by flotation, which were obtained from positive feces samples (independently of the isolation technique or the concentration method). In the case of the adults, the multiplex PCR positively amplified DNA, resulting in bands of about 390 bp compatible with *D. latum*. The specificity of these primers was confirmed by the successful amplification of DNA from positive controls. In addition, the absence of amplification at the negative control indicates low possibility of cross contamination due to contaminated reagents. We performed a Blast search with the two adult sequences and compared them with the sequences in pre-existing databases of the NCBI. Both new amplifications were identical (Accession numbers: MH414914 and MH414915) showing 100% similarity with all the sequences of *D. latum* published in GeneBank, which were obtained from different hosts and sites (i. e. "*Homo sapiens*" from Switzerland and Canada, *Perca fluviatilis* from Switzerland and Italy (Como Lake), and fish from Estonia: Lake Peipsi) and with different cover values that ranged from 88 to 100% of the query sequence. Accession numbers: KY552871.1; AP017663.1; GU997614.1; GU997613.1; GU997612.1; AM712906.2; AM778554.2; AB269325.1; DQ985706.1; DQ768197.1; GU997615.1; KU341712.1; KU341699.1; KU341705.1; KU341702.1; KU341707.1; KU341711.1; KU341701.1; KU341716.1; KU341700.1; KU341698.1.

3.4. Wild carnivores

The thirty-six specimens of wild carnivores analyzed (10 *Neovison vison*, 1 *Lontra provocax*, 5 *Galictis cuja*, 1 *Lyncodon patagonicus*, 1 *Puma concolor,* 8 *Leopardus geoffroyi,* 1 *Leopardus colo colo*, 9 *Lycalopex culpaeus*) proved negative for *Diphyllobothrium* species (Table 2).

4. Discussion

The Diphyllobotrhiosis produced by *D. latum* is an endemic disease of the Andean Patagonia, where it was introduced by the immigration of infected Europeans during the first half of the last century, and maintained in the environment by the introduced salmonids (Semenas, 2006). In this region, two species co-exist, *D. latum* and *D. dendriticum* so, it is difficult to diagnose the parasites or their eggs at specific level by morphometric analysis of eggs or adults. Although *Diphyllobothrium* sp. eggs were previously detected in canine feces collected in neighborhoods of Bariloche, the infections was not determined at a specific level, nor were epidemiological studies performed in order to determine the environmental factors promoting the occurrence. Also, no data on the diagnosis of the disease made by local veterinarians were available, neither published records on the occurrence of the Diphyllobothriosis in wild carnivores from Northern Patagonia. This work contributed to understand the occurrence of the disease in urban dogs, and the possible circulation of the parasite in the city and surrounding lakes.

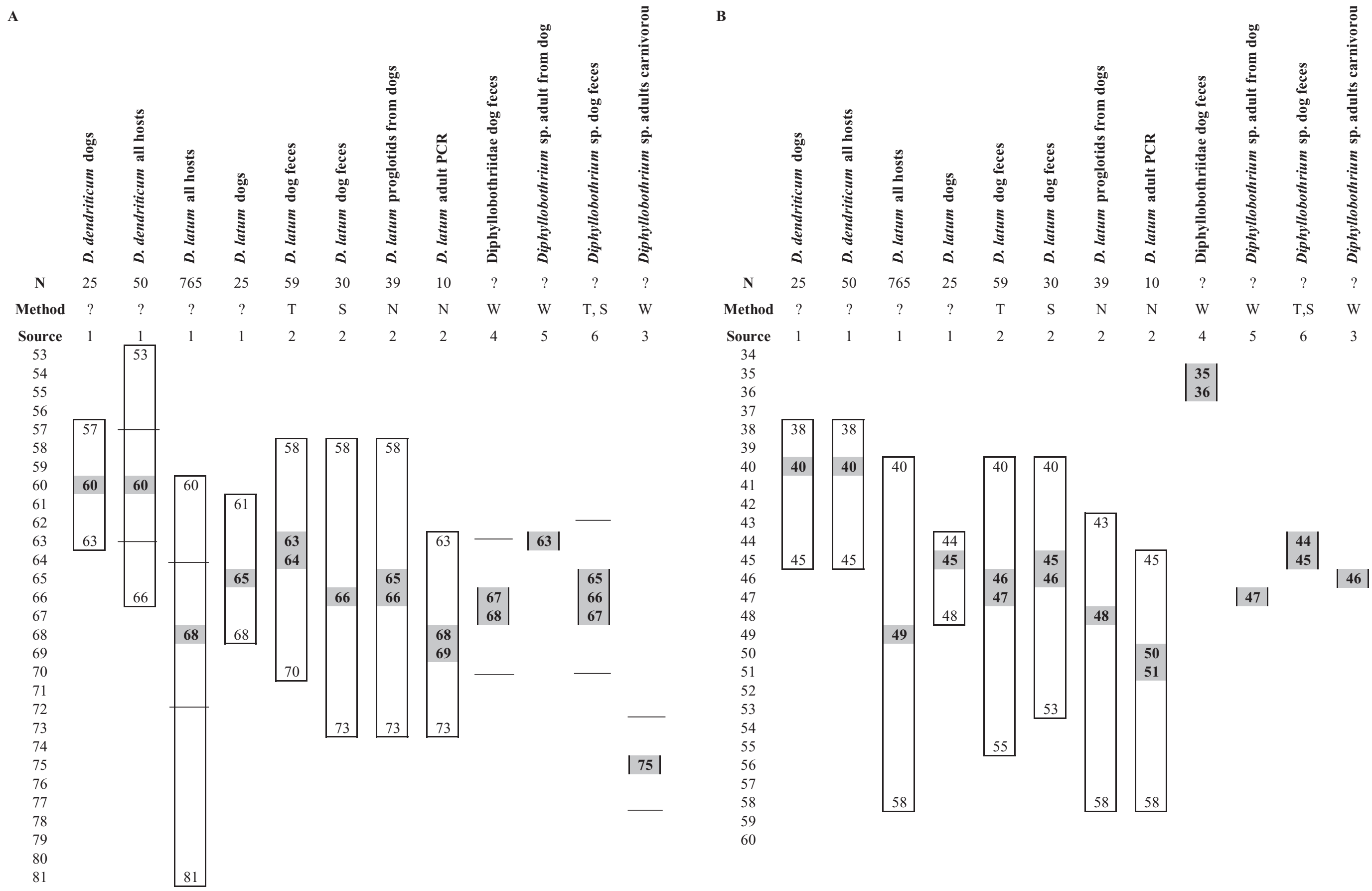
4.1. Epidemiology of Diphyllobothriosis in urban dogs

Occurrence of Diphyllobothriosis in urban dogs from Bariloche is widespread throughout the city, and is directly related to the number of free-roaming dogs, and the socioeconomic status of the neighborhoods. Besides, the percentage of infection is not related with distance to the

Table 4
Measurements of *Diphyllobothrium latum* eggs from feces of different neighborhoods obtained by Sheater and Telemann methods.

Methods	Sheater			Telemann		
Neighborhoods	N	Mean length (range) μm	Mean width (range) μm	N	Mean length (range) μm	Mean width (range) μm
Inta	8	65.0 (63–68)	45.0 (45–45)	10	65.1 (62–70)	52.1 (50–55)
San Francisco	11	66.5 (58–73)	44.8 (40–53)	16	62.1 (59–70)	44.4 (42–48)
San Cayetanito	0	–	–	8	62.4 (60–65)	44.7 (41–48)
Don Bosco	3	67.5 (65–70)	47.5 (45–50)	1	58.8	44.8
Jamaica	0	–	–	14	64.8 (58–70)	46.3 (43–48)
Vivero	8	66.9 (60–70)	45.0 (42–50)	10	66 (60–70)	44.8 (40–50)

– eggs were not obtained.



1: Léstinová et al., 2016; 2: Our study; 3: Martínez et al., 2000; 4: Milano and Oscherov, 2005; 5: Ruiz et al., 2011; 6: Rivero et al., 2015

N: natural from adult worms; S: Sheater; T: Telemann; W: Willis

Fig. 3. Comparisons of length (A) and width (B) of *D. latum* eggs measured in this study with those of other studies of Diphyllobothriidae from Argentina and other parts of the world. Rectangles represent the range of the measure in μm; the shaded numbers in the rectangle represent the average; horizontal bars represent standard deviation.

Table 5
Measurements of eggs from Diphyllobothrium latum obtained from proglotids of dogs.

Neighborhoods	N	Mean length (range) μm	Mean width (range) μm
San Cayetanito[a]	10	68.0 (63–73)	50.8 (45–55)
Pilar	10	61.8 (58–65)	47.8 (43–50)
Virgen Misionera	10	65.8 (58–70)	47.5 (45–50)
Veterinary Clinic	10	66.3 (63–73)	46.5 (43–50)

[a] Specimen used for extraction and sequencing of DNA.

nearest water body. Despite being an endemic zoonotic disease, it is striking that only 20% of veterinarians diagnosed it, either due to lack of knowledge or not performing routine copro-parasitological analysis of dog feces. The percentage of infected feces in the present study is higher than those recorded in other cities from South America (range: 0.8 − 18.8%; see Table 1).

It is known that the gross domestic product (GDP) *per capita* is inversely linked to the percentage of stray or semi-domesticated dogs (Otranto et al., 2017). The association between poverty and helminthiasis has already been recorded in a previous study of helminth infections in dog feces in 5 neighborhoods of Bariloche (Semenas et al., 2014). The distribution of Diphyllobothriosis in the different neighborhoods and their relation with UBN % is indicative of practices such as the feeding of dogs with raw fish viscera or their inadequate disposal. The subsistence fish poaching is a common practice in the winter in low-income neighborhoods (San Cayetanito, Vivero, Nahuel Hue, and Nuestras Malvinas). During this season, two situations contribute to this practice: 1) the return of salmonids from Nahuel Huapi Lake to the spawning sites in the tributary Ñireco River and 2) the increase in unemployment since the building industry comes to an almost complete halt in winter. In this context, fish poaching represents a source of food for people in these neighborhoods. Informal talks with neighbors confirmed this practice. This scenario of poverty facilitates Diphyllobothriosis transmission, as was also observed for other zoonotic diseases like echinococcosis (Flores et al., 2017). In a study conducted on dog owners' practices in low income neighborhoods from Bariloche, it was recorded that 96.4% of inhabitants know that dogs can transmit diseases but none know which diseases are involved and/or how to prevent them (Garibotti et al., 2017).

Diphyllobothriosis have particularly high values of infection in dogs from Bariloche, and it is clear that social and economic factors have a greater influence than environmental factors on the distribution of this zoonosis. Furthermore, the lack of monitoring of this zoonosis by veterinary professionals in the city is evident.

4.2. Identification of the causative agent of the Diphyllobothriosis in urban dogs

The causal agent of Diphyllobothriosis in urban dogs of Bariloche is *D. latum* according to the results of the morphometric analysis of eggs and the molecular analysis of adult worms. The mean length and width of eggs found in dog feces and those obtained from proglottids are similar to those of *D. latum sensu* Leštinová et al. (2016), but the ranges of both measurements, obtained by either method, are larger than those given by these authors for *D. latum* in dogs, and also larger than those of *D. dendriticum*. Measurements of the eggs obtained from the worm from the San Cayetanito neighborhood can be considered as a reference for *D. latum* in Patagonia, since that worm was determined molecularly to species level. On the other hand, the amplicons from both strains of the adult worms obtained using the multiplex reaction MulRevCom, MulLat3 and MulDen4 show size compatibility with *D. latum,* which was later confirmed by sequencing the fragment and comparing it with existing databases. To our knowledge, this is the first molecular determination of the specific identity of *D. latum* from dogs in South America.

One of the necessary conditions in epidemiological studies is assessment of the validity of the data processing. In this way, the combination of sedimentation and flotation methods enables us to detect positive samples with maximum likelihood (Becker et al., 2016). However, the chosen concentration method affects the size and shape of the eggs, as was observed in our study, particularly in those obtained by the Sheater method, which were generally collapsed. Therefore, whenever comparisons or identifications are made which depend on the measurements and shape of the eggs, it is necessary to take into account the method of fixation and/or concentration of eggs used. Although the molecular methods provide solutions and allow answering questions about accurate identification of species, sometimes problems arise such as the impossibility found in our study to extract DNA from the eggs. Our inability to amplify the egg samples (regardless of the concentration method used) could indicate that the eggshell was not properly disrupted, as we used only samples that were identify as positive by microscope examination. One method that succeeded in amplification accomplishes this by Sonication (two to three times for 10 s each at medium intensity) (Scholz et al., 2009), while we used liquid nitrogen and a MixerMill instead. This method may not be appropriate for the disruption of eggshells and release of the DNA content.

Molecular and morphometric results indicate that dogs in the Andean Patagonian region are infected with *D. latum.* Special care must be taken with regard to the method of fixation and concentration used to obtain the eggs, and more variable regions (e. g. ITS) could help resolve question about intraspecific variability and improve our understanding of morphological variation in eggs.

4.3. Wild carnivores

Although in our survey we analyzed wild, native and introduced, carnivores of the families Felidae, Mustelidae, and Canidae, we did not find Diphyllobothriidea specimens parasitizing them. The analysis of the data on wild carnivores had limitations considering the fact that samples were not part of a systematized survey, and the specimens were collected dead in different national parks and by different people in a period of two decades. However, the processing of the samples was carried out by the same professionals, and other cestodes like unidentified cyclophyllidean were found in L. *geoffroyi*, *L. culpaeus*, and L. *patagonicus* (unpublished data). Besides, other parasite registered in the same samples of wild carnivores is the nematode *Toxocara cati* in the felines *P. concolor* and, *L. geoffroyi* (Vega et al., 2018).

In Chilean Patagonia, the native mustelid L. *provocax* is the only species that has been registered as a host for *Diphyllobothrium* sp., with 3.5% (N = 84) of infected feces (Proboste, 2011). In the USA, *Lontra canadensis* was also reported as a host, with 11.2% (N = 409) of infected feces (Crait et al., 2015), and in Poland 1.9% (N = 106) of *Lutra lutra* feces were infected (Górski et al., 2010). The low values of infection registered in mustelids in other parts of the world, and the absence of infection we found in wild carnivores indicates that a higher number of specimens should be examined in Argentinean Patagonia in order to understand the role of mustelids and other carnivorous species in the transmission of *Diphyllobothrium* spp. Proximity to cities may be considered an important factor affecting levels of infection. In Andean Patagonia, although high values of prevalence (64–76%) and mean intensities (5–34.2) of *Diphyllobothrium* spp. plerocercoids were found in salmonids in lakes near the cities (Revenga and Semenas, 1991; Ortubay et al., 1994; Revenga et al., 1995), this pattern of infection is not reflected in the occurrence in wild carnivores. It is likely that human settlements prevent these wild animals gaining access to infected fish or their discarded viscera.

In South America, *D. latum* was cited in the Northern tiger cat (*Leopardus tigrinus*) and margay (*Leopardus weidii*) (Travassos, 1965), and *Diphyllobothrium* sp. were recorded in wild carnivores like *Conepatus chinga* (Molina's hog-nosed skunk), *Cerdocyon thous* (crab-eating fox), *Procyon cancrivorus*, *Panthera onca*, *Lycalopex gymnocercus* and *Philander opossum* (gray four-eyed opossum) (Carneiro et al., 1972; Rego and Schäffer, 1992; Santos et al., 2004; Vieira et al., 2008). Also, in the Northeastern region of Argentina adult worms of *Diphyllobothrium* sp. have been found in wild carnivores (Martínez et al., 2000), but the eggs had a narrower opercular end, and the mean length of these eggs (75 μm ± 2.1) is longer than that of *D. latum* for all hosts (68 μm ± 3.5) *sensu* Leštinová et al. (2016) (Fig. 3).

In Argentina, published studies provide little information on the number of measured eggs or their morphometric characteristics; neither do the figures provide enough information to determine the taxon involved. Though no infection was found in the wild, we have to consider that the number of carnivores examined was low (36), so that Patagonian wild animals should not yet be dismissed as hosts. Based on the cases of Diphyllobothriidea in wild carnivores recorded in Northeastern Argentina and Southern Brazil, we can infer that more than one species is circulating in the area, and that they are not necessarily the same as those found in Patagonia.

5. Conclusion

Infection by *D. latum* in urban dogs is widely distributed throughout the neighborhoods of Bariloche city in Northwestern Patagonia. The socioeconomic characteristics in low-income neighborhoods, such as a large number of free-roaming dogs and the lack of deworming (see Garibotti et al., 2017) contribute to the presence of the disease. The occurrence of Diphyllobothriosis in dogs is underestimated among local veterinarians due to the lack of routine copro-parasitological analysis.

Conflict of interest statement

None.

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Ethical statement

This study did not affect human or animal rights and did not harm the environment, animals and future generations. The researcher knows and carried out the safeguards foreseen in all the ethical, legal and legal requirements established in the national bioethics norms (ANMAT5330/97 Disposition) and international (Nuremberg Code, Helsinski Declaration and its modifications, Universal Declaration on the Human Genome and Rights Human rights approved by the general conference of UNESCO of November 11, 1997).

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