



New insights on the infection of pathogenic *Leptospira* species in American mink (*Neovison vison*) in southern Chile

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Abstract

Leptospirosis is a zoonosis of global distribution, caused by the infection of pathogenic *Leptospira*, a group of bacteria capable of infecting both domestic and wild animals. Mink (*Neovison vison*) in southern Chile is recognized as a wild and synanthropic rodent predator (among various other prey), and *Leptospira* infection in them can be acquired through contact with the pathogen in the environment or by eating infected prey. Thus, the aim of this study was to provide more specifics regarding the source of the infection for the American mink under the conditions of Southern Chile. Minks were captured in the Los Ríos region, southern Chile, in an area with well-developed dairy farming. Two areas were selected for mink trapping, one with a high degree of dairy farming and a second with a low degree of dairy farming. Within them, 16 study sites were visited, and 45 American mink were trapped and euthanized to obtain kidney tissue and blood serum samples for bacteria isolation and determination of antibodies titers, respectively. Molecular characterization of the isolated strains was performed. Three minks from sites of high-dairy farming industry and only one from sites with low-degree dairy farming were detected as infected through molecular confirmation. This study shows evidence that confirms previous findings made in southern Chile, regarding mink as host of *Leptospira interrogans* serovar Hardjo-prajitno associated to cattle-farming areas. However, typing information (*Leptospira interrogans Copenhageni* and *Icterohaemorrhagiae*) suggests that the consumption of rodents may also be a potential source of infection.

Keywords *Leptospira* spp. · Cattle · Pathogen pollution · American mink

Introduction

Anthropogenic changes to the landscape increasingly threaten the survival of wild species, and recently, more records exist of the negative impacts of introduced alien species on endemic populations through the co-introduction of parasites or pathogens in a process termed “pathogen pollution” (Daszak et al.

2000; Cunningham et al. 2003; Medina-Vogel 2010). A good example of pathogen pollution in aquatic ecosystems is leptospirosis, one of the most important zoonotic bacterial diseases in animals and the most widely distributed in the world (Adler and de la Peña 2010). This infection is caused by spirochetes of the genus *Leptospira* and is characterized by fever, renal and hepatic insufficiency, lung diseases, and reproductive disorders (Guerra 2009; Adler and de la Peña 2010).

Water or moist soil contaminated with the urine of infected animals is known to be the main vehicle of inter- and intra-species transmission of pathogenic *Leptospira* (Faine 1994). The main transmission routes are through injured skin and via long periods of exposure to contaminated water or soil. The possibilities for transmission through direct contact within animal populations include sexual contact or artificial insemination (Faine 1994). However, predation of infected hosts (mostly rodents) could also be a transmission route (Reilly et al. 1970; Shophet and Marshall 1980; Cosson et al. 2014). Therefore, it

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has been suggested that the reduction of wildlife habitats favors spillback and spillover processes at the wildlife-livestock-human interface possibly leading to the emergence of leptospirosis in some geographic areas (Matthias et al. 2005; Matthias et al. 2008). Confirming the latter, Derne et al. (2011) observed that, in islands with higher biodiversity and lower human intervention, there were less cases of human leptospirosis. On the opposite, there are some geographic areas that promote the presence and the persistence of *Leptospira* in the ecosystem. Such is the case of the Tuscany area, as well as all of Central Italy, where the close contact between domestic and wild animals plus hunting activity and a wet environment makes pathogenic *Leptospira* spread favorable. An example of the latter is the situation of wild boar in the epidemiology of leptospirosis among wildlife in Central Italy (Cilia et al. 2020). Thus, the role of human intervention on ecosystems and its effect on the ecology of diseases is an ongoing discussion.

Considering the epidemiological aspects of this infection, wild animals with semi-aquatic habits would have a greater exposure to pathogenic *Leptospira* from a source that may be associated with domestic animals (Gaydos et al. 2007; Barros et al. 2014). Among others, the American mink (*Neovison vison*) meets this condition. American mink in Chile is considered an invasive species, and its current distribution is throughout a great part of the Patagonian territory with an apparent northern distribution now reaching the Malleco River approximately at 39° 60' South parallel. Its introduction to Argentina and Chile occurred in the 1930s in Patagonia because of the liberation of individuals from the fur industry (Rozzi and Sherriffs 2003). The first infectious link between this invader and pathogenic *Leptospira* was reported in a study of European mink (*Mustela lutreola*) and other small carnivores in southwestern France (Moinet et al. 2010), where they found high seroprevalence and pathogen detection using molecular tools. In Chile, molecular detection of pathogenic *Leptospira* was also reported from renal tissue of captured American minks (Barros et al. 2014), suggesting two sources of infection: rodents, as they are a regular item of the mink diet in the region (Medina 1997; Medina-Vogel et al. 2013), and livestock production due to the proximity to cattle herds. American mink could then be an important link in the ecology of *Leptospira*, mainly due to its wide distribution, semi-aquatic life, and diet (Medina 1997; Millán et al. 2009; Moinet et al. 2010; Sepulveda et al. 2014). Considering that *Leptospira* infection rate of cattle in southern Chile has been reported as high, estimating up to 75% of dairy herds infected in the study region, and that it has clear negative effects on the cattle's health (Salgado et al. 2014; Salgado et al. 2015), the present study aimed to provide more specifics regarding the source of the infection for the American mink under the conditions of Southern Chile.

Materials and methods

Study area

Between March and September 2017, a trapping campaign to catch American minks was performed in the Los Rios region, Chile, which extends approximately from the 39° 17' south up to the 40° 41' south parallel, and the 73° 43' west, up to the 71° 35' West. Through geoprocessing (geospatial information system, GIS), the location of cattle herds registered on the Chilean agriculture and livestock service database was associated with the area from where each mink was captured. This methodology allowed for the classification of five trapping sites as areas with a high degree of bovine herd concentration (mainly represented by a high density of dairy cattle operations), while the other 14 sites were assessed as having a low degree of dairy farming (Fig. 1), characterized for subsistence-farming activities with less human intervention and significantly smaller numbers of domestic animals, in comparison with the first described hunting area.

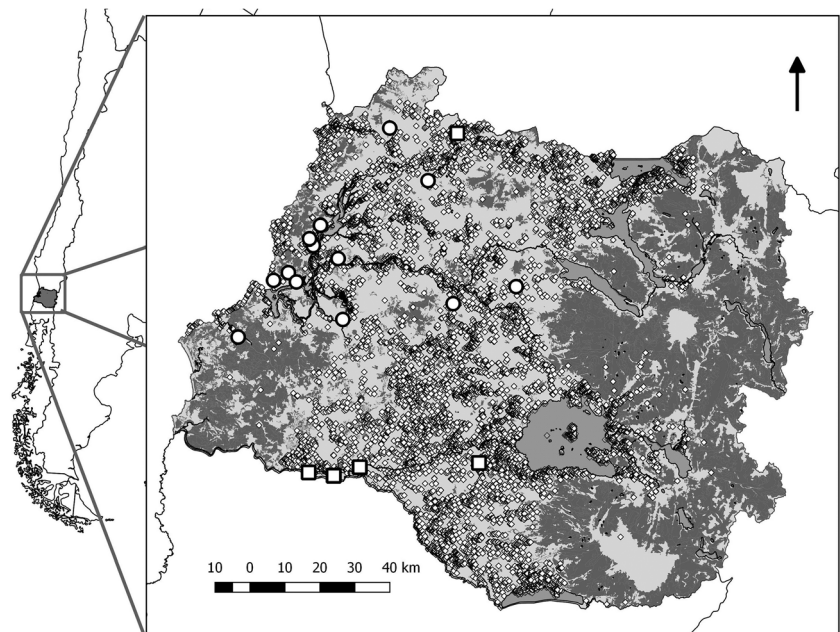
Trapping permits

The mink trapping was associated with a regional American Mink control initiative executed by the "Servicio Agrícola y Ganadero" (Agriculture and Livestock Service) which is undertaking a regional effort to control this invasive species which is considered as "detrimental to agriculture and biodiversity," therefore considered by Chilean law as a detrimental species that can be hunted on all the Chilean territory without quotas or hunting seasons (Chilean law N° 19.473, article 6 of the current regulation). The animals used on this research were culled under the regional mink control initiative, and the carcasses were delivered for research purposes; therefore, no special permits are required for research purposes according to the current regulation. Minks were trapped alive with modified Tomahawk traps with a single entrance.

Sampling

Each trapped animal was euthanized on site (to reduce stress) with an overdose of ketamine hydrochloride (100 mg per animal) via intra-cardiac injection following full sedation obtained by a previous intra-muscular injection of a combination of 100 mg of ketamine hydrochloride and 10 mg of xylazine hydrochloride (standard dose per individual). After this procedure, 5 ml of blood was obtained directly from the heart with a sterile blood collection tube. The trapped animals were then transported directly to the Instituto de Patología at the Universidad Austral de Chile (UACH) for necropsy, and during that procedure, the one kidney was extracted from each euthanized mink and deposited in a sterile 50-ml centrifuge tube. All samples were labeled with the animal's number,

Fig. 1 GIS that represents the mink capture area in the Los Ríos District. Mink capture areas associated with high degree of bovine herd concentrations (white squares) and low degree bovine herds (white circles). Bovine herds are represented with white rhombus. Native forests, exotic forest exploitations, and mixed forests are represented by dark gray polygons for spatial representation and graphical interpretation to explain the concentration of the bovine herds within the central valley of the district



date, and place of origin. Samples were kept at room temperature until they were transferred (less than 2 h) to the Infectious Diseases Lab, at the Instituto de Medicina Preventiva Veterinaria, UACH. In most cases, samples were immediately processed for the tissue culture protocol. A smaller number of samples (7) were refrigerated for a maximum of 2 days until processing. Blood samples were centrifuged upon arrival at the lab to extract the serum and then kept refrigerated until processed on the same day. Animals were handled strictly according to the recommendations in the university's guide on the use of animals for research (<https://www.uach.cl/organizacion/vicerrectoria-investigacion-desarrollo-y-creacion-artistica/utiles/subcomite-en-uso-de-animales-en-investigacion>); however, it is important to note that the mink trapping was associated with a regional American Mink control initiative.

Diagnostic approach

Sera were analyzed using MAT (microscopic agglutination test) for the presence of antibodies anti-six *Leptospira* serovars according to a published protocol (Salgado et al. 2014). Kidney tissue was processed according to Faine et al. (1999). Briefly, 200 μ l of the processed sample in four replicates was cultured in EMJH (Ellinghausen and McCullough and modified by Johnson and Harris) medium at 29 °C. The cultures were checked once a week for 13 weeks, and a positive result was reported with a typical Dinger ring growth. Thereafter, positive cultures were submitted for the DNA extraction-purification protocol using the High Pure PCR Template Preparation Kit (Roche), following the manufacturer instructions. DNA templates obtained from the above

protocol were analyzed in a qPCR system (Roche LightCycler 2.0), using a TaqMan probe and targeting the *LipL32*gen (Stoddard et al. 2009), which is specific only for pathogenic *Leptospira* species. The amplification mixture for each sample: 0.7- μ M primers, 0.15- μ M probe, 10- μ l Master Mix TaqMan Universal (Roche), and 5- μ l DNA template, in a total volume of 20 μ l. Samples were amplified with the following program: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation for 5 s at 95 °C and annealing/elongation for 30 s at 58 °C. The PCR protocol considered a negative and positive control to survey the proficiency of the reaction as well as negative and positive controls for the DNA extraction.

The “Multi Locus Sequence Typing” (MLST) method was used to molecularly characterize strains obtained from positive cultures. MLST was performed according to a protocol described by Thaipadungpanit et al. (2007), since it demonstrates the utility of this genotyping method for characterizing *Leptospira* isolates from a clinical perspective. Specifically, this protocol used the following housekeeping genes: *glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA*, and *caiB*. Briefly, PCR amplification was carried out using bacterial (*Leptospira*) DNA obtained from positive mink kidney cultures, where the initial denaturation step was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s; annealing/elongation was at 50 °C for 60 s for all genes; extension was at 72 °C for 50 s; and then the final extension was at 72 °C for 7 min. The PCR products were purified using the Geneaid™ “Gene/DNA Genetic Extraction” Kit, and sequencing was done in both directions with primers initially used for PCR amplification. Sequencing was performed using the sequencer kit “BigDye Terminator v. 3.1 Cycle Sequencing Kit (ABI)”, and the automated DNA

sequencer “ABI Prism 3130xl Genetic.” The sequences were analyzed using the Chrommas and BioEdit programs, and these were derived to the international database of free use (<https://pubmlst.org/leptospira/>) to obtain the allelic profile and assign the sequence type (ST).

Statistical analysis

Data was not normally distributed, so frequencies of positive culture results for each study area and sex were compared using Fisher exact test. The level of significance was $\alpha < 0.05$.

Results

Twenty-five (55.6%) minks were trapped in low-density dairy farming and 20 (44.4%) in the area of high-density dairy farming. Twenty-nine (64.4%) were males, and 16 (35.5%) were females (Table 1).

None of the hunted animals showed serological positive results to any *Leptospira* spp. serovars. Four (8.8%) out of 45 kidney samples clearly showed the characteristic Dinger ring indicative of *Leptospira* bacterial growth, and in all these, the presence of the *LipL32* gene was confirmed by qPCR.

Although a higher proportion of animals with positive culture was observed in high density of dairy farming (3) than the area of low-density dairy farming (1), this difference was not statistically significant ($P = 0.28$) (Table 2). In the first area, the typing information showed that the specific species were *Leptospira interrogans* serovar Hardjo-prajitno and *Leptospira interrogans Copenhageni* and *Icterohaemorrhagiae*. In the area of low-density farming activity, just one positive culture sample showed *Leptospira interrogans Copenhageni* and *Icterohaemorrhagiae*. Similarly, although more females were positive (3) than males (1), this difference was not significant ($P = 0.09$).

Table 1 Hunting general information, considering geographic area and individual information of the captured mink

Origin	Minks	Weight (grams)		
		Average	Max.	Min.
High herd density area	20	821.10	1327	471
Females	7	552.86	622	471
Males	13	965.54	1327	721
Low herd density area	25	840.32	1250	551
Females	9	609.89	713	551
Males	16	969.94	1250	630
Total	45	831.78	1327	471

Table 2 Proportion and comparison of individuals with culture-positive results between different categories

Categories	Total captured animals	Culture-positive animals	Rate of culture-positive animals
Area of major intervention	20	3	15%
Area of minor intervention	25	1	4%

$P = 0.28$

The MLST analysis of the four isolates revealed that three of them presented the ST-17 while one isolate belonged to the ST-20.

Discussion

The present study provided new information regarding the role of American mink in the epidemiology of *Leptospira* infection as sentinels of semiaquatic wildlife in Patagonia.

Only one of the two kidneys of each animal was subjected to bacterial culture, since to the authors' knowledge, there is no published evidence about increasing sensitivity of culture to growth pathogenic *Leptospira* after using both the kidneys.

The success of capture of male individuals was higher than females, probably since the former has a wider home environment, associated with more excursive behaviors, associated with territoriality and looking for mating, compared with females (Medina-Vogel et al. 2015). Although males move around more than females, the difference is not large enough to make any conclusion. Female home range is above the 60% of male home range. Furthermore, mink have intersexual territoriality in a relative linear habitat, so movements depend on neighbors and the season, as during reproduction, the territorial system breaks down. Also, the landscape characteristics are very important. So, we do not believe that we have data enough to make any conclusion about the difference between females and males.

The fact that none of the animals showed a serological response could be due to an early stage of the infectious process, especially in the four infected animals. According to the biology of this infection, the presence of antibodies cannot be detected before 15 to 20 days after the infection has occurred (Zuerner 2015). Another reason to explain the obtained results is the possibility that other serogroups that could be involved are missed in our MAT battery. The serologic (MAT) diagnostic battery used in this study consisted of 6 serogroups used routinely for different animal species (Salgado et al. 2014). It is important to highlight that there are more than 24 serogroups that cover more than 250 serovars. Finally, the lack of knowledge on the mink immune system response

against this infection should also be considered. For example, a dilution of 1:100 in MAT may overestimate the immune response and makes it insufficient for its detection by this serologic tool.

The lower detection rate of pathogenic *Leptospira*, in relation to what was reported in the study by Barros et al. (2014), can be explained by the fact that they used direct PCR for the diagnostic pathogen detection, which implies a higher diagnostic sensitivity, mainly due to a detection limit and contamination problems for culture. However, direct PCR could not confirm the presence of live *Leptospira* in urine specimen. Therefore, the interpretation of this finding is not conclusive or confirmatory of active infection. On the contrary, in the present study, one can infer that in those 4 animals with positive culture results, the infection was active since the pathogen could be isolated, and as such, there was the probability of dissemination by urine due to its viability in renal tissue.

The highest proportion of individuals with positive results to the bacteriological culture is reported in the macro area of greater human intervention. In this area, a large bovine dairy mass is concentrated specifically in the areas of Puerto Trumao and Cocule in the region of Los Ríos (see Fig. 1). The discussion based on this finding should alert us, despite the result of the statistical analysis, to the fact that, in this area, the reported infection rate for the dairy cattle population is considered high (Salgado et al. 2014). In addition, the trend of a higher proportion of positive results in this area would suggest that cattle could act as a source of pathogenic *Leptospira* infection for the region's mink population and for other semiaquatic mammals too, such as the endangered Southern river otter (*Lontra provocax*). Complementing these findings, the MLST technique has reported *Leptospira interrogans* serovar Hardjo-prajitno and *Leptospira interrogans* serovars Copenhageni and Icterohaemorrhagiae. The first serovar is directly associated with cattle, being responsible for severe clinical disease in this animal species (Ellis 1994). The abortion rate after *Leptospira borgpetersenii* Hardjobovis infection is 3 to 10%, whereas the rate increases up to 30% for *Leptospira interrogans* serovar Hardjo-type Hardjo-prajitno (Li Hardjo-prajitno) infection (Koizumi and Yasutomi 2012). In Chile, the virulence and severity of *Leptospira interrogans* serovar Hardjo-prajitno have been demonstrated in cattle from the Los Ríos region (Salgado et al. 2015). The results of the present study also allow us to consider that, although the rate of positive results was lower in the animals caught in lesser intervention area, the presence of the infection was also confirmed there.

Interestingly, three out of four isolates informed in this study were typed as *Leptospira interrogans* serovars Copenhageni and Icterohaemorrhagiae belonging to ST-17, which opens the interpretation about the potential source of infection, taking into account that strains with this genotype in

the PubMLST database have been isolated from rats, canines, porcines, and humans in countries of South America, Europe, and Asia (last accessed September 23, 2020). The latter suggests that predation of infected hosts (mostly rodents) could also be a transmission route (Reilly et al. 1970; Shophet and Marshall 1980; Cosson et al. 2014). Medina (1997) helps to support this discussion since minks practice predation of both synanthropic and wild rodents, which are recognized as reservoirs of pathogenic *Leptospira* species. Also, recently, ST-17 was the predominant genotype in dogs in a study conducted in Italy (Bertasio et al. 2020), and it was confirmed as the major cause of canine leptospirosis. Interestingly, the same MLST genotype was also informed in the PubMLST database in rats, mice, cats, and some farm animals. Free-roaming dogs possibly play an important role in the dissemination of pathogenic *Leptospira* to sympatric wildlife.

The demographic explosion of mink causes them to be in direct or indirect contact with different domestic and wild animals, and human populations suggest that the transmission of the pathogen is possible. They, therefore, constitute a risk to public health affecting the biodiversity of the native wildlife, being a probable focus of dissemination of this and other pathogens present in domestic animals towards wildlife and vice versa.

As far as we are aware, the STs described here correspond to the first Chilean genotypes deposited to the *Leptospira* MLST database, and it is hoped that this information will be a contribution for the organisms involved in health and environmental protection, so that they can be considered in future control plans for this species, as a tool to mitigate the negative effects that this alien species exerts in the region and in the country.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of animal rights The authors declare that the present study does not contain clinical studies or patient data. Animals were handled strictly according to the recommendations in the Universidad Austral de Chile's guide on the use of animals for research (<https://www.uach.cl/organizacion/vicerrectoria-investigacion-desarrollo-y-creacion-artistica/utiles/subcomite-en-uso-de-animales-en-investigacion>). Since American Mink in Chile is considered an invasive species, therefore, it can be hunted on all the Chilean territory without quotas or hunting seasons (Chilean law No. 19.473, article 6 of the current regulation).

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