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# SEROSURVEY FOR SELECTED PARASITIC AND BACTERIAL PATHOGENS IN DARWIN'S FOX (*LYCALOPEX FULVIPES*): NOT ONLY DOG DISEASES ARE A THREAT

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?1 Abstract: The Darwin's fox (*Lycalopex fulvipes*) is one of the most endangered carnivores worldwide, with the risk of disease spillover from domestic dogs being a major conservation threat. However, lack of epidemiologic information about generalist, non–dog-transmission-dependent protozoal and bacterial pathogens may be a barrier for disease prevention and management. To determine the exposure of some of these agents in Darwin's fox populations, 54 serum samples were collected from 47 Darwin's foxes in Southern Chile during 2013–18 and assessed for the presence of antibodies against *Brucella abortus*, *Brucella canis*, *Coxiella burnetii*, pathogenic *Leptospira* (serovars Grippotyphosa, Pomona, Canicola, Hardjo, and Copehageni), *Toxoplasma gondii*, and *Neospora caninum*. The highest seroprevalence was detected for *T. gondii* (78%), followed by pathogenic *Leptospira* (14%). All the studied *Leptospira* serovars were confirmed in at least one animal. Two foxes seroconverted to *Leptospira* and one to *T. gondii* during the study period. No seroconversions were observed for the other pathogens. No risk factors, either intrinsic (sex, age) or extrinsic (season, year, and degree of landscape anthropization), were associated with the probability of being exposed to *T. gondii*. Our results indicate that *T. gondii* exposure is widespread in the Darwin's fox population, including in areas with minimal anthropization, and that *T. gondii* and pathogenic *Leptospira* might be neglected threats to the species. Further studies identifying the causes of morbidity and mortality in Darwin's fox are needed to determine if these or other pathogens are having individual or population-wide effects in this species.

*Key words*: Conservation medicine, *Leptospira interrogans*, Sarcocystidae, South America, zoonosis.

## INTRODUCTION

Infectious diseases can jeopardize the survival of endangered species. The most obvious effect that pathogens can have for endangered populations is direct mortality in the form of epizootics (e.g., Roelke-Parker et al. 1996; Timm et al. 2009). Additionally, endangered populations often suffer demographic bottlenecks and associated losses of genetic diversity that may exacerbate the species' vulnerability to pathogens (Millán et al. 2009b). The majority of pathogens that have caused problems in endangered populations of canids are generalists with the ability to infect a wide range of species (Cleaveland et al. 2002) and persist in another reservoir population (Haydon et al. 2002). Consequently, they can spillover and cause one-off or repeated epizootics in threatened populations (Laurenson et al. 2004).

Darwin's fox (*Lycalopex fulvipes*; Martin 1837) is one of the most endangered carnivores in the world. Darwin's fox occurs in Chile across the temperate coastal forest in at least three distinct populations: two small, isolated mainland populations located in Nahuelbuta Range (37°36′S, 73°3′W) and Reserva Costera Valdiviana—Parque Oncol (40°7′S, 73°33′W), composed of about 230 mature individuals, and one bigger population on Chiloé Island (42°21′S, 74°2′W) composed of about 415 mature individuals (Silva-Rodríguez et al. 2016; Hidalgo-Hermoso et al. 2020).

Disease outbreaks are considered one of the greatest threats for Darwin's fox persistence (Silva-Rodríguez et al. 2016). Recently, Hidalgo-Hermoso et al. (2020) suggested that an outbreak of canine distemper virus (CDV), a generalist pathogen, might seriously decimate Darwin's fox populations. Similarly, other generalist pathogens represent a challenge for wildlife managers (Canessa et al. 2019; Portier et al. 2019) and may be key threats to the survival of free-ranging wild carnivores (Alexander et al. 2010). Our study aimed to determine the degree of exposure of Darwin's fox to some generalist pathogens, *Brucella abortus*, *Brucella canis*, *Coxiella burnetii*, pathogenic *Leptospira*, *Toxoplasma gondii*, and *Neospora caninum* (Greene 2012) that may be causing undetected morbidity or mortality.

## MATERIALS AND METHODS

### Study areas and sample collection

Darwin's foxes were captured across their known range in Southern Chile: Nahuelbuta area (37°45′S, 73°00′W; $n$=5); the Valdivian coastal range (40°07′S, 73°33′W; $n$=3); and Chiloé Island (42°21′S, 74°2′W; $n$=39) (Hidalgo-Hermoso et al. 2020; Moreira-Arce et al. 2021). Surveyed landscapes differ in vegetation structure and composition. Nahuelbuta area is a mosaic of agricultural lands, exotic forest plantation stands of Monterrey pine (*Pinus radiata*), *Eucalyptus* spp., and surrounding remnants of native forests of evergreen and *Nothofagus* tree species in different successional stages (Moreira-Arce et al. 2015a). The Valdivian coastal range represents a well-conserved landscape comprising several public and private protected areas dominated by Valdivian evergreen forest and continuous patches of alerce tree (*Fitzroya cupressoides*; Silva-Rodriguez et al. 2018). Finally, Chiloé Island presents ecosystems with different levels of disturbance. The central and north portions of the island comprise remnant fragments of Valdivian and North-Patagonian evergreen forests surrounded by an open matrix consisting mainly of grazed pastures, small agricultural fields, and shrublands (Moreira-Arce et al. 2021). In contrast, the western and southern sections of the island maintain large and well-conserved old-growth forest remnants dominated by the original species from Valdivian and North-Patagonian evergreen forests (Moreira-Arce et al. 2021).

From May 2013 to November 2018, we collected 54 blood samples from 47 Darwin's foxes: 30 males (25 adults, five juveniles) and 17 females (13 adults, four juveniles). Seven foxes in Chiloé and one in Nahuelbuta were each recaptured once. One individual was juvenile at the initial sampling and adult when recaptured; the others were adults in both capture events. The foxes were captured with a collapsible skunk trap, with one trap door, Tomahawk traps (Tomahawk Live Trap Company, Hazelhurst, Wisconsin, USA) baited with chicken or canned fish, activated in the evening and checked the next morning at dawn. Foxes were anesthetized using a combination of either 1 mg/kg xylazine plus 10 mg/kg ketamine or 0.04 mg/kg dexmedetomidine plus 5 mg/kg ketamine, then tagged subcutaneously with a chip (Felixcan, Albacete, Spain), for identification. Atipamezole (0.4 mg/kg) was used

to reverse the dexmedetomidine. Blood samples were obtained by venipuncture of the cephalic, saphenous, or jugular veins using an evacuated tube system (Vacutainer, Beckon, Dickson, and Company, Franklin Lakes, New Jersey, USA), and the serum was extracted by centrifugation and stored in a –20 C freezer until serologic analysis. Foxes were classified as juveniles (younger than 1 y) or adults (older than 1 y) based on tooth eruption (Harris 1978). All animal captures were approved by the Chilean Agriculture and Livestock Service of the Ministry of Agriculture (trapping permit nos. 2263/2010; 206/2012; 3155/2013; 3363/2015), while animal manipulations followed the Guidelines for the Capture, Handling, and Care of Mammals of the American Society of Mammalogists (Gannon and Sikes 2007).

### Serologic assays

*Toxoplasma gondii*: A multispecies competitive enzyme-linked immunosorbent assay (ELISA) test (ID Screen®, ID VET, Montpellier, France) was carried out to detect anti-*Toxoplasma* antibodies, as it had proven successful in previous studies in dogs (Ahmad et al. 2014) and wild carnivores (Reiterová et al. 2016; Ferreira et al. 2019).

For each plate, 10 µL of the negative and positive controls in 90 µL of thinner per well (1:10) was added to four wells. Dilutions of serum samples (1:10) were distributed among the rest of the wells and incubated at 25±5 C for 45±4 min. Then, every plate was washed three times with approximately 250 µL of washing solution. Next, 100 µL of a 1:10 secondary multispecies antibody dilution conjugated with horseradish peroxidase was added to each well. The microplates were incubated at 21±5 C for 30±3 min and then washed three times as before. Subsequently, 100 µL of development solution was put into each well and the plate was incubated in the dark at 21±5 C for 15±2 min. Finally, 100 µL of the reaction's stop solution was added to each well, and then wells were read in an ELISA plate reader (Bio-Rad®, Des Plaines, Illinois, USA) at a wavelength of 450 nm. Lastly, to determine the positive and negative sera, a serum positive percent (S/P%) via optical density (OD) was calculated as follows: S/P%=(OD sample–OD negative control)/(OD positive control–OD negative control)×100. Results >50% were considered positive; <40% were considered negative; and 40–50% were considered doubtful. Samples considered as doubtful were retested. A second doubtful result was considered as a negative sample.

*Brucella abortus* and *B. canis*: For diagnosis of exposure to *Brucella* species, we selected tests recommended for use in wildlife (World Organisation for Animal Health [OIE] 2018a) and previously used in other studies (Oliveira et al. 2012; Moya et al. 2019).

For *B. abortus* antibody detection, the Rose Bengal test (RBT) was used as a screening test and was carried out in the Infectious Diseases Laboratory of the University of Chile's Faculty of Veterinary and Animal Science, Santiago. This official test for the diagnosis of bovine brucellosis was carried out following the technical instructions provided by the Chilean Agricultural and Livestock Service (Servicio Agrícola y Ganadero; SAG), Santiago. Briefly, 30 µL of Rose Bengal antigen (Bengatest®, Parsippany, New Jersey, USA) and sample serum were mixed for 4 min on a glass slide until a homogeneous mixture was formed. The test was considered negative if there was no visible agglutination, and if a uniform pink color, translucent against the light, was seen. Any test that developed visible agglutination was taken as a positive result. Positive and negative bovine serums were used as controls. As a confirmatory test, a competitive ELISA was done by the SAG using a commercial kit (SVANOVIR® Brucella C-ELISA Antibody Test, SVANOVA, Uppsala, Sweden) in accordance with the manufacturer's instructions. The OD values for each of the controls provided in the kit and serum samples in the wells were read at 450 nm using a microplate photometer (Universal Microplate Reader, Bio-Tek Instruments, Inc., Burlington, Vermont, USA). The percent inhibition (PI) values were calculated according to the manufacturer's instructions. The results were expressed as negative for *Brucella* antibodies (PI<30%) or positive for *Brucella* antibodies (PI>30%).

For *B. canis*, serum samples were analyzed at the Microbiology Laboratory of the University of Chile's Faculty of Veterinary and Animal Sciences. Samples were screened for antibodies against *B. canis* using the rapid slide agglutination test with 2-mercaptoethanol (2ME-RSAT) with a *B. canis* antigen. For this, 25 µL of serum were placed in a serologic pipette and mixed manually for 2–3 s with an equal volume of 2-mercaptoethanol (0.2 M). Next, 50 µL of this treated serum were placed onto a glass slide and mixed with 50 µL of *B. canis* antigen until homogenized. In the case of a positive result, agglutination could be clearly seen after 2 min against the light. Positive and negative canine sera were used as controls. Because the 2ME-RSAT has a diagnostic specificity of only 74.3% (Salgado 2016), positive samples were confirmed through counterimmunoelectrophoresis with the rough lipopolysaccharide antigen of *B. ovis* in accordance with the standardized technique in the laboratory. Briefly, microscope slides were prepared with 1% agarose in barbital buffer, pH 8.6, and deposited in equidistant wells. When the agarose solidified,

the wells were loaded with the positive control serum, fox's serum, and antigen. Electrophoresis was performed at 220 volts with a current of 7.5 mA per microscope slide for 2 h, and then slides were incubated in 5% sodium citrate to remove nonspecific precipitates (Borie et al. 2002). This test has a diagnostic specificity of 96.82%; supporting its use as a confirmatory test (Salgado 2016).

*Leptospira interrogans*: Samples were analyzed using the microscopic agglutination test (MAT) in accordance with the OIE protocol (Moreno-Beas et al. 2015; OIE 2019) for the following *L. interrogans* serovars: Pomona, Grippotyphosa, Copenhageni, Hardjo, and Canicola. The serovars selected for analysis were cultivated in Ellinghausen–McCullough–Johnson–Harris liquid medium for *Leptospira* spp. at 29±1 C. Sera from sampled foxes was mixed with the serovars of the living antigen in question to determine the presence of antibodies by agglutination reactions. Screening was performed with an initial serum dilution of 1:50. After this, an equal volume of each antigen, equal to the volume of the diluted serum, was added to each well to make a final dilution of 1:100. The plates were incubated at 29±1 C for 2–4 h and were subsequently examined by dark-field microscopy. Samples showing 50% agglutination in comparison to a control cultivation were considered positive (OIE 2019).

*Neospora caninum*: Samples were analyzed by ELISA (CHEKIT* *Neospora caninum* Antibody Test Kit, IDEXX Laboratories, Bern, Switzerland) using 150 µL of Darwin's fox serum. The kit includes positive and negative control sera that were used as internal references for quality checks. The tests were run in accordance with the manufacturer's instructions (Meng et al. 2015; De La Torre et al. 2017).

*Coxiella burnetii*: samples were analyzed by ELISA (CHEKIT Q-Fever (*Coxiella burnetii*) Antibody Test Kit, IDEXX Laboratories) following the OIE recommendations (OIE 2018b). This test uses microtiter plates coated with *C. burnetii* antigen and monoclonal anti-ruminant IgG-peroxidase antibodies, and was run according to the manufacturer's instructions.

### Statistical analysis

All data analyses were performed using R software version 3.4. For detection rates estimation, the package *EpiR* was used (R Core Team 2017). Only one of the capture events was considered for the recaptured individuals. In such cases, a fox was considered positive if the laboratory result was positive in any of the events. The buffer area generated by Di Cataldo et al. (2020) around each fox capture site, based on the home range size described by Jiménez (2007), was used to extract the sample landscape data. For each capture site, we collected data on the presence or absence of houses, total number of houses, distance to the nearest house, land use, and vegetation cover (Hansen et al. 2013). The environments analyzed included native forests (broadleaf-evergreen forest, broadleaf-deciduous forest, shrub cover, and bush herbaceous cover), grasslands and scrublands, wetlands, water bodies, cultivated areas, urbanized areas, and mosaic areas (crops/forest). Risk factors were assessed only for *T. gondii* on Chiloé Island, due to inadequate sample sizes for the other pathogens and areas. To determine possible risk factors associated with exposure to *T. gondii* (binomial variable: exposed/not exposed), a set of intrinsic (age, sex, and their interaction) and extrinsic variables (season, year, and landscape anthropization factors: presence and number of houses, land use, and vegetation cover (Hansen et al. 2013) were evaluated by univariable generalized linear models. For the variables with no positive animals in any of the categories, the evaluation was carried out by Fisher's exact test. For the previously mentioned continuous factors, multivariable generalized linear models were used, and the best model was selected using the *dredge* function from the MuMIn package (Barton 2020).

## RESULTS

Although blood samples from 47 free-ranging Darwin's foxes were collected, due to insufficient serum volume from some individuals, the sample size for specific serologic tests varied between 26 and 46 individuals depending on the test (Table 1). Antibodies against *T. gondii* were confirmed in 36 out of 46 foxes (observed prevalence=78.26%, 95% confidence interval (CI)=63–89%; Table 1). No risk factors, either intrinsic or extrinsic, were significantly associated with *T. gondii* seropositivity (in all cases, $P>0.05$). Antibodies against *Leptospira* sp. were detected in six out of 42 individuals (14.29%, 95% CI=5.4–28.5%). All the studied serovars were confirmed in at least one fox (Table 1), with some foxes being seropositive to multiple serovars. All the seropositive foxes for *Leptospira* sp. were also seropositive for *T. gondii*. Two foxes that were sampled more than once seroconverted to *Leptospira* sp. and

TABLE 1. Seroprevalence of selected pathogens in free-ranging Darwin's foxes (*Lycalopex fulvipes*) from Chile, 2013–2018, by sex, age, and geographic area.[a]

| Pathogen | *n* | Prevalence (%) | 95% CI | Prevalence (%) by sex | | Prevalence (%) by age class | | Prevalence (%) by geographic zone | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Male, *n*=30 | Female, *n*=17 | Juvenile, *n*=9 | Adult, n=38 | Continental, *n*=8 | Chiloé Island, *n*=39 |
| *Brucella abortus* | 46 | 0 | 0.0–0.8 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Brucella canis* | 46 | 0 | 0.0–0.8 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Coxiella burnetti* | 26 | 0 | 0.0–0.8 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Leptospira interrogans* | 42 | 14.29 | 5.4–28.5 | 17.86 | 7.14 | 0 | 18.18 | 0 | 16.67 |
| Canicola | | 2.38 | | 3.57 | 7.14 | 0 | 3.03 | 0 | 2.78 |
| Copenhageni | | 2.38 | | 3.57 | 7.14 | 0 | 3.03 | 0 | 2.78 |
| Grippotyphosa | | 7.14 | | 10.71 | 0 | 0 | 9.09 | 0 | 8.33 |
| Hardjo | | 4.76 | | 7.14 | 0 | 0 | 6.06 | 0 | 5.56 |
| Pomona | | 7.14 | | 10.71 | 0 | 0 | 9.09 | 0 | 8.33 |
| *Neospora caninum* | 26 | 0 | 0.0–13.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Toxoplasma gondii* | 46 | 78.26 | 63–89 | 75.86 | 82.35 | 66.67 | 78.38 | 37.5 | 86.84 |

[a] *n* = number of tested animals; CI = confidence interval.

one to *T. gondii* during the study period. One animal tested positive to *B. abortus* (with the RBT) and one to *B. canis* (with the 2ME-RSAT), but neither was positive in the confirmatory tests (competitive ELISA for *B. abortus* and counterimmunoelectrophoresis for *B. canis*) and were therefore considered negative. All foxes were seronegative to *C. burnetii* and *N. caninum*.

## DISCUSSION

To date, similar to the situation of other wild canid species, efforts to reduce the impact that diseases have on Darwin's foxes have been focused on controlling pathogen transmission from domestic dogs. However, the high prevalence of exposure to generalist pathogens not transmitted by dogs, such as *T. gondii* and several serovars of *Leptospira interrogans* other than Canicola, found in these foxes highlights the importance of focusing prevention efforts beyond dogs. These pathogens could represent an overlooked threat, especially for fragmented fox populations that are estimated to be composed of less than 90 individuals, such as the Nahuelbuta population.

The prevalence of antibodies against *T. gondii* in Darwin's fox observed in this study is one of the highest reported in Chilean wildlife (Sepúlveda et al. 2011; Barros et al. 2018; Calvo-Mac et al. 2020) and is very high in comparison with other fox species worldwide (Dubey et al. 2021; Wei et al. 2021), indicating frequent exposure to the parasite and suggesting that *T. gondii* may be a threat to the Darwin's fox. Deaths by acute toxoplasmosis have been widely reported in several fox species (Dubey et al. 1990; Davidson et al. 1992; Dubey and Lin 1994; Kottwitz et al. 2004; Sørensen et al. 2005; Dubey and Pas 2008; Pas and Dubey 2008; Lindsay and Dubey 2020), highlighting the risk this pathogen poses to Darwin's fox. Recently, aberrant behavioral traits observed in red foxes (*Vulpes vulpes*), subsequently classified as Dopey Fox Syndrome (DFS), have been associated with this parasite (Milne et al. 2020). Moreover, *T. gondii* infection is often associated with clinical cases of CDV infection in fox species (Reed and Turek 1985; Davidson et al. 1992; Kelly and Sleeman 2003). However, Darwin's fox populations seem to be naïve to CDV (Hidalgo-Hermoso et al. 2020). Therefore, in the event of a CDV outbreak, immunosuppressive effects of CDV

infection may exacerbate pathologies caused by concomitant *T. gondii* infection. Unfortunately, there is a lack of information about the causes of morbidity and mortality in Darwin's fox to determine whether *T. gondii* is having individual or population effects in this species.

The lack of association between *T. gondii* and extrinsic or intrinsic factors suggests that this parasite may be present in all types of environments, including in the well-preserved forests of Southern Chiloé Island where domestic cats, the main reservoir, are absent. Whether the sympatric forest-dwelling guigna (*Leopardus guigna*), a South American felid that uses fragments in human-dominated landscapes, may be acting as disperser of the parasite in these areas deserves further investigation.

Seroprevalence to pathogenic leptospires in Darwin's fox was above the mean prevalence (10–12%) reported for worldwide *Canidae* species (Andersen-Ranberg et al. 2016) and in the range of those previously reported for other fox species in Chilean Patagonia (Moya et al. 2019). All the serovars detected in the current study have been previously reported in fox species in other countries (Cilia et al. 2020), but serovars Grippotyphosa and Hardjo have not previously been documented in a wild carnivore in Chile. Cattle are the most common reservoir for serovars Hardjo and Pomona (Adler and de la Peña Moctezuma 2010), while rodents are the main reservoirs for serovar Coppenhageni (Cilia et al. 2020) and can also be natural hosts for serovar Grippotyphosa. Dogs are natural hosts for the serovar Canicola (Lelu et al. 2015). Therefore, the Darwin's fox is coming into contact with serovars from different sources, either through rodents, their main prey (Moreira-Arce et al. 2015b), or water contaminated by domestic animals. Although leptospirosis is frequently considered subclinical in wild carnivores, pathologic case reports of leptospirosis (Kingscote 1986; Juan-Sallés et al. 2001; Bregoli et al. 2021) have been found, as well as necropsy findings of renal lesions associated with *Leptospira* in infected or exposed wild canid species (Millán et al. 2009a; Scialfa et al. 2013). These reports suggest that deaths by leptospirosis may be overlooked in wild canids and that their susceptibility could be similar to that of dogs, as they may develop infections that range from asymptomatic to severe (Ricardo et al. 2020). A clinical case of leptospirosis by serovar Canicola was recently diagnosed in an Andean fox (*Lycalopex culpeaus*) in Chile (Llanos-Soto et al. 2019), confirming the pathogenicity of some *Leptospira* serovars for South American foxes.

No evidence of exposure was confirmed for the other studied pathogens. This may be due to the low sample size, especially among the mainland populations, which could have impacted the ability to detect infections. Regarding *B. abortus*, previous studies in wild canids in Brazil and Argentina reported prevalences between 7% and 29% (Martino et al. 2004; Azevedo et al. 2010; Seles-Dorneles et al. 2014). The lack of exposure found in the Darwin's fox may be explained by the low prevalence, <1%, of *B. abortus* in cattle in Chile resulting from the governmental eradication program (SAG 2015). Our results confirm that the RBT can generate false positives and that using a confirmatory technique is highly recommended, especially with wildlife (Godfroid 2002). Antibodies against *B. canis* have been confirmed in the past in South American wild canids (Oliveira et al. 2012; Hayashi, 2013) and very recently in Andean foxes from Central Chile (Galarce et al. 2021). This study is the first reported *N. caninum* exposure in wildlife in Chile, although two captive Darwin's foxes were previously found seropositive (Patitucci et al. 2001). *Neospora caninum* is widely distributed in cattle in Southern Chile (Tuemmers et al. 2017; Moroni et al. 2018), so we recommend further surveillance of this parasite. Finally, our serosurvey confirms earlier studies indicating that *C. burnetii* is not a threat to the Darwin's fox (Cabello et al. 2013). Nevertheless, this bacterium has been found in Chilean wildlife, such as bats (Chiroptera; Müller et al. 2020).

In summary, *T. gondii* and different serovars of pathogenic leptospires are actively circulating in the environments where the last

Darwin's foxes persist, suggesting that diseases transmitted by dogs may not be the only threat to this species. Further studies on the causes of morbidity and mortality in Darwin's fox are needed to determine whether *T. gondii* and *Leptospira interrogans* are having individual or population effects on this species, particularly in Nahuelbuta and other small populations. Although these parasites and bacteria would not have the catastrophic effect of other agents such as CDV, the loss of a few individuals could deeply affect the persistence and/or recovery of fragmented and small populations such as the Nahuelbuta population.

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