Science of the Total Environment 703 (2020) 134900

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Antibiotic resistance genes as landscape anthropization indicators: Using a wild felid as sentinel in Chile

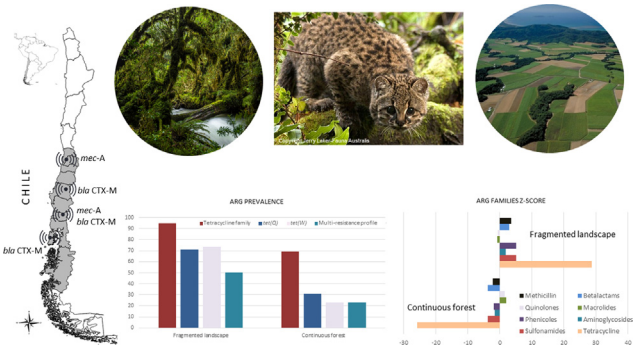
Irene Sacristán [a,*], Fernando Esperón [b], Francisca Acuña [c], Emilio Aguilar [c], Sebastián García [c], María José López [c], Aitor Cevidanes [a], Elena Neves [b], Javier Cabello [d,e], Ezequiel Hidalgo-Hermoso [f], Elie Poulin [g,h], Javier Millán [i], Constanza Napolitano [g,j,*]

[a] PhD Program in Conservation Medicine, Facultad de Ciencias de la Vida, Universidad Andres Bello, República 252, Santiago, Chile
[b] Grupo de Epidemiología y Sanidad Ambiental, Centro de Investigación en Sanidad Animal (INIA-CISA), Madrid, Spain
[c] Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Avda. Santa Rosa 11735, La Pintana, Santiago, Chile
[d] Facultad de Medicina Veterinaria, Universidad San Sebastián, sede de la Patagonia, Puerto Montt, Chile
[e] Centro de Conservación de la Biodiversidad Chiloé Silvestre, Ancud, Chile
[f] Departamento de Conservación e Investigación, Parque Zoológico Buin Zoo, Panamericana Sur Km 32, Buin, Chile
[g] Instituto de Ecología y Biodiversidad (IEB), Santiago, Chile
[h] Laboratorio de Ecología Molecular, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile
[i] Facultad de Ciencias de la Vida, Universidad Andres Bello, República 252, Santiago, Chile
[j] Departamento de Ciencias Biológicas y Biodiversidad, Universidad de Los Lagos, Osorno, Chile

HIGHLIGHTS

- Are antibiotic resistance genes indicators of landscape anthropization in wildlife?
- Higher prevalence of ARGs in guignas inhabiting fragmented landscapes.
- Presence of *mec*-A and bla_{CTX-M} in guignas inhabiting human-dominated landscapes.
- Wild carnivore species could act as sentinels of anthropogenic environmental ARGs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 July 2019
Received in revised form 24 September 2019
Accepted 7 October 2019
Available online 1 November 2019

Editor: Ewa Korzeniewska

Keywords:
Antimicrobial resistance
Leopardus guigna
Landscape anthropization

ABSTRACT

Antimicrobial resistance is a global emerging public health issue whose presence and impact in wildlife are widely unknown. Antimicrobial resistance genes (ARGs) are considered environmental contaminants, suitable to evaluate the degree of anthropic impact on wildlife and the environment. We used a wild felid, the guigna (*Leopardus guigna*), as a sentinel for the presence of ARGs in anthropized and pristine areas across their entire distribution range in Chile. We evaluated fecal samples from 51 wild guignas, collected between 2009 and 2018. Real-time PCR essays were employed to detect and quantify 22 selected ARGs in their fecal microbiome. All animals (100%) were positive for at least one ARG. The most prevalent ARG families were those that confer resistance to tetracycline (88.2%) and beta-lactamase (68.9%), with *tet*(Q) (60.8%), *tet*(W) (60.8%), and bla_{TEM} (66.7%) as the most prevalent ARGs. Multi-resistance profiles were observed in 43% of the guignas. Statistically significant differences were found between anthropized and pristine areas for *tet*(Q) ($p = 0.014$), *tet*(W) ($p = 0.0037$), tetracycline family ($p = 0.027$), multi-resistance profile prevalence ($p = 0.043$) and *tet*(W) quantification ($p = 0.004$). Two animals from anthropized

* Corresponding authors at: Instituto de Ecología y Biodiversidad (IEB), Santiago, Chile (C. Napolitano) and PhD Program in Conservation Medicine, Facultad de Ciencias de la Vida, Universidad Andres Bello, República 252, Santiago, Chile (I.Sacristán).
E-mail addresses: isacristan.vet@gmail.com (I. Sacristán), cnapolit@uchile.cl (C. Napolitano).

https://doi.org/10.1016/j.scitotenv.2019.134900

MecA
Bla$_{CTX-M}$
Public health

landscapes were positive for *mecA*, a gene associated with *Staphylococcus aureus* and other staphylococci resistant to methicillin, while three animals from anthropized areas were positive for *bla*$_{CTX-M}$, that encodes class A extended-spectrum beta-lactamase. Both genes have been identified in bacteria causing relevant nosocomial infections worldwide. This is the first study on ARGs in wild felids from Chile and the first detection of *mecA* in South American wild felids. We observed an association between the degree of landscape anthropization and ARG prevalence, confirming that ARGs are important indicators of wildlife exposure to human activity/presence, with a widespread distribution.

1. Introduction

Antimicrobial resistance is a worldwide phenomenon of public health concern (Marston et al., 2016; O'Neill, 2018). Worldwide dispersion of antimicrobial resistance has been attributed to the excessive and inappropriate use of antibiotics, as well as little development of new drugs due to reduced economic incentives and the complex regulatory requirements involved (Goossens, 2005; Gould and Bal, 2013; Ventola, 2015). Antibiotics are used worldwide not only in the treatment of human and livestock diseases but also as livestock growth promoters and improvers of feed efficiency (Bartlett et al., 2013; Sarmah et al., 2006), and in agriculture to treat bacterial infections in plants (Golkar et al., 2014), leading to considerable geographic dispersion and changes in environmental microbiota (Golkar et al., 2014; Ventola, 2015). For instance, approximately 90% of the antibiotics used in livestock are released to the environment through animal waste and widely dispersed through the use of slurry (Bartlett et al., 2013). Environmental pollution by antibiotics from urban, agricultural and livestock origin persists in soil and aquatic environments, leading to a selective pressure that contributes to the development and maintenance of antimicrobial resistance (Schwartz, 2012). Thus, the study of environmental antibiotic resistances and antibiotic resistance genes, including those found in wildlife, is paramount to better understand the anthropogenically-derived antimicrobial pollution in ecosystems (Swift et al., 2019).

The widespread human use of antibiotics may turn the presence of environmental antibiotic resistances into indicators of the degree of anthropogenic impact on ecosystems (Allen et al., 2010; Kozak et al., 2009; Radhouani et al., 2011). Several studies have reported that species inhabiting adjacent to human-populated or agricultural areas present a higher frequency of resistance gene-carrying bacteria than those inhabiting more remote areas (Cole et al., 2005; Furness et al., 2017; Kozak et al., 2009). Such findings suggest that wildlife species adapted to both anthropized and pristine areas could be used as indicators of the degree of anthropization of these ecosystems (Furness et al., 2017; Larsson et al., 2018; Sayah et al., 2005; Swift et al., 2019). However, a sentinel species should fulfill some requirements; occupy an elevated position in the trophic chain, and present territorial behavior, a long-life span and a varied diet. Carnivores are excellent sentinels, able to reflect their environment (Millán et al., 2014; Naccari et al., 2013). The guigna (*Leopardus guigna*) is a small felid species endemic to Chile and a restricted area of SW Argentina, closely associated with native forests and vegetation cover (Napolitano et al., 2015a, 2015b). Cataloged as Vulnerable by the IUCN (Napolitano et al., 2015b) with declining populations, its main threats are habitat loss and fragmentation, forcing guignas to adapt to human-dominated landscapes and inhabit forest fragments surrounded by a human matrix of agricultural and livestock activities. The use of a carnivore such as the guigna, able to inhabit both fragmented landscapes and pristine remote areas, as a sentinel of antimicrobial resistance genes, may enable the indirect evaluation of the degree of anthropogenic impact in different areas and its correlation with other health studies on this threatened species.

Likewise, understanding the role of wild species in the maintenance of antibiotic resistance would shed light on the dynamics of antibiotic resistance, its dispersion, medium transference and maintenance within bacterial populations.

The goal of the study was to evaluate the presence and load of antibiotic resistance genes (ARGs) in guignas from pristine and fragmented landscapes across their distribution range in Chile, in order to assess the degree of environmental anthropization as well as to elucidate the role of wildlife in antibiotic resistance gene dynamics in natural environments. We hypothesized that guignas inhabiting human-dominated landscapes would have a higher frequency and load of resistance gene-carrying bacteria than those inhabiting pristine areas.

2. Material and methods

2.1. Study area

The study area encompassed the whole distribution range of the guigna in Chile, corresponding to four biogeographic areas and nine administrative regions in central and southern Chile (33°S–46°S) (Napolitano et al., 2015b) (Fig. 1, Table S1).

Study sites included a gradient of different landscape types, ranging from continuous near pristine native forest with no human presence, to human-perturbed landscapes with fragments of remnant forest surrounded by a matrix of agriculture and livestock activities with high human densities. The central area of the country is the most populated, concentrating 79% of the total population of the country, while the southern area has lower population density (INE, 2017).

2.2. Sample collection

Fecal samples were collected between 2009 and 2018 from a total of 51 (Table S2). (23 live and 28 dead) free-ranging guignas. Direct fecal samples were collected from the rectum in live individuals captured using tomahawk-like live traps (n = 15) or upon admission into wildlife rescue and rehabilitation centers (WRRC; n = 8). Dead individuals, road-killed or euthanized at WRRC, were sampled during necropsies and fecal samples from the rectum were collected (n = 28). All samples were kept frozen at −20 °C until laboratory analysis.

Guigna trapping and fecal collection were conducted following previously published protocols (Napolitano et al., 2015a, 2015b), and handling and supervising guidelines within bioethical and animal welfare frameworks approved by the Chilean Agriculture and Livestock Service (SAG) (capture permits 814/13 2008, 109/9 2009, 1220/22 2010, 1708/26 2010, 7624/2015, 2288/2016, 2185/2017, 4072/2018). The anesthesia protocol was 5 mg/kg ketamine (Ketamine 100®, Chemie) and 0.05 mg/kg dexmedeto-

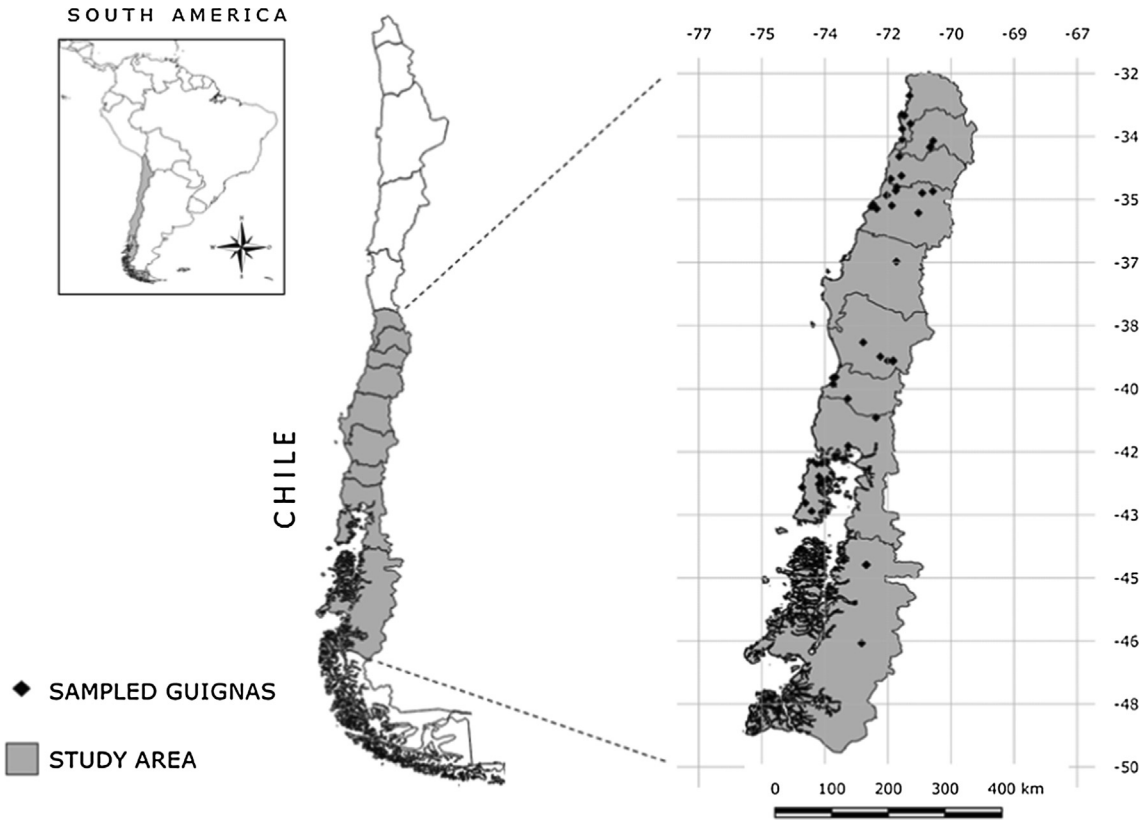


Fig. 1. Study area encompassing the entire distribution of guignas in Chile and each sampled guigna location.

midine (Dexdomitor® 0.5 mg, Ecuphar), adapted from methods described for other wild felids in South America (Pampas cat [*L. colocolo*] and Geoffroy's cat [*L. geoffroyi*] Beltrán et al., 2009). Sex, age range (estimated from dentition) and GPS location were recorded for each individual. A total of 23 females and 28 males, 40 adults and 11 juveniles were sampled.

2.3. Laboratory analysis

Total DNA extraction from fecal samples was performed by a pressure filtration method (QuickGene DNA Tissue Kit S, Fujifilm, Japan), following the manufacturer's instructions. The 16S rRNA gene was amplified in each sample by real time PCR (rtPCR) in 10-fold dilutions of extracted samples in order to detect the presence of bacterial genetic material, according to Jiang et al. (2013). A sample was considered as validated when a ten-fold dilution showed a cycle threshold (Ct) less than 25 (Esperón et al., 2018). Once validated, the samples were analyzed by a panel of 22 different ARGs by rtPCR. ARG selection was based on three main parameters: (1) genes with expected high frequency of detection (genes *aadA* [PCR protocol amplifies all the *aadA* variants], *cat, str* [PCR protocol amplifies both *str* genes, i.e., *strA* and *strB*], *sul* and *tet*); (2) use in animal production and/or human health (genes *erm*, bla_{TEM} and *qnr*); and (3) impact on public health (genes $bla_{CTX\text{-}M}$, *mcr-1* and *mecA*), as previously described (Chen et al., 2007; Cummings et al., 2011; Devarajan et al., 2016; Francois et al., 2003; Jiang et al., 2013; Marti and Balcázar, 2013; Nieto-Claudín et al., 2019; Wang et al., 2014, Table S2). All the ARGs were detected and quantified directly in the fecal microbiome by gel-based rtPCR using Eva Green (Biotools, B & M Labs, S.A., Madrid, Spain), except bla_{TEM} and *qnr*B, which were analyzed by conventional rtPCR using Sybr Green.

2.4. Data analysis

Relative quantification of positive samples was based on the cycle threshold (ct) of the 16S rRNA present in each sample and the specific ct for each gene. To normalize the study, ct was obtained from the fluorescence variation value ($(\Delta F/\Delta C) = 0.02$).

An estimate of the percentage of bacteria harboring ARGs (load percentage of each ARG) was calculated based on the formula % gene X = 10[2 + 0.33ct16S − ctgeneX], where ct is the cycle thresh-

Table 1
Prevalence of each family of antimicrobial resistance gene and prevalence of multi-resistance profiles in sampled guignas.

Family of antimicrobial resistant gene	Tetracycline	Sulfonamides	Aminoglycosides	Phenicoles	Macrolides	Quinolones	Betalactams	Methicillin	Colistin	Multi-resistance profiles
Prevalence (%)	88.2	15.69	19.61	3.92	33.33	15.69	68.63	3.92	0.0	43.14
Lower 95% CI	79.08	05.35	8.33	1.59	19.94	5.35	55.45	−1.59	0.0	29.07
Upper 95% CI	97.39	26.02	30.89	9.43	46.72	26.02	81.81	9.43	0.0	57.21
n	45	8	10	2	17	8	35	2	0	22

old (16S rRNA is for bacterial determination and ARG is for each gene), and 0.33 is the mean slope for all the genes tested. Results were expressed in $\log_{10}$ scale of the hypothetical percentage of bacteria presenting each gene, for the percentual load of ARGs. Our custom-made formula expresses the results in percentages and correlates highly with those previously published (Xie et al., 2016) ($R^2 = 0.997$; data not shown). The results range from −8 to +2; a value of −8 is given to a sample when considered negative. The z-score value (number of standard deviations a data point is from the mean value) of each ARG and ARG families was calculated and correlated with landscape variables.

Fecal microbiome samples resistant to three or more different classes of antimicrobials were classified as "multi-resistance profiles", following the classification proposed by Blanco-Peña et al. (2017). A k-means clustering method was applied to investigate the resistance patterns (GENESIS software v. 1.7.7, Graz University of Technology, Graz, Austria), based on the assignment of each sample to one cluster.

To identify and describe landscape features associated with guigna sampling locations, a circular buffer for each sample location was generated with the QuantumGIS 2.14® program, corresponding to the mean home range described for the species (males = 446 ha; females = 170 ha) (Dunstone et al., 2002; Sanderson et al., 2002; Schüttler et al., 2017). We described and quantified six landscape variables in each buffer area: (1) percentage of vegetation cover (Hansen et al., 2013); (2) presence of houses; (3) number of houses; (4) distance from capture location to the nearest house; (5) land use (fragmented landscape or continuous forest) and (6) local administrative region. GIS layers were obtained at the Ministerio de Bienes Nacionales. website (Ministerio de Bienes Nacionales, 2019). QGIS 2.14® software was used to extract landscape attributes.

Spatial and biological variables associated with ARGs, ARG family prevalence and quantification and the presence of multi-resistance profiles were assessed with crude and adjusted odds ratios (ORs) calculated by a logistic regression analysis with 95% confidence intervals and corresponding Kruskall Wallis and Mann-Whitney U tests. The Chi-square test was used to assess the relation between k-media clusters and spatial and biological variables, while Mann-Whitney U tests and the Spearman correlation test were used to establish the relation between z-score values and independent variables.

Goodness of fit models were evaluated by the Hosmer Lemeshow test, confidence intervals and an analysis of residuals. All statistical analyses were performed in R software (R Development Core Team, 2013) with a significance level of $p < 0.05$.

3. Results

3.1. Qualitative descriptive results

All sampled individuals (100%) were positive for at least one ARG. Gene families able to confer resistance to tetracyclines (88.2%) and beta-lactamase (68.6%) were the most prevalent (Table 1), significantly greater than the rest of the families of ARG ($K = 192.9$, $p < 0.05$). Multi-resistance profiles (positive for three or more different ARG families) were observed in 43% of sampled guignas (Table 1). *Tet*(Q) (60.8%), *tet*(W) (60.8%), and bla_{TEM} (66.7%) were the most commonly found ARGs (Table 2), significantly greater than the prevalence of the rest of ARGs ($K = 308.2$, $p < 0.05$).

K-means clustering showed most of the sampled individuals were included in Cluster 1 (high percentage load charge cluster) (Fig. 2).

Table 2
Prevalence of antimicrobial resistance genes in the sampled guignas.

Antimicrobial resistance gene	*tet(A)*	*tet(B)*	*tet(Y)*	*tet(K)*	*tet(M)*	*tet(Q)*	*tet(S)*	*tet(W)*	*sul1*	*sul2*	*str*	*aadA*	*catI*	*catI1*	*erm(B)*	*erm(F)*	*qnrS*	*qnrB*	bla_{TEM}	$bla_{CTX\text{-}M}$	*mecA*	*mcr-1*
Prevalence (%)	3.92	19.61	15.69	13.73	23.53	60.78	1.96	60.78	1.96	15.69	9.80	13.73	3.92	0.0	9.8	25.49	0.0	15.69	66.67	5.88	3.92	0.0
Lower 95% CI	−1.59	8.33	5.35	3.95	11.48	46.92	−1.98	46.92	−1.98	5.35	1.36	3.95	−1.59	0.0	1.36	13.11	0.0	5.36	53.28	−0.80	−1.59	0.0
Upper 95% CI	9.43	30.89	26.02	23.50	35.58	74.65	5.90	74.65	5.90	26.02	18.25	23.50	9.43	0.0	18.25	37.87	0.0	26.02	80.06	12.57	9.44	0.0
n	2	10	8	7	12	31	1	31	1	8	5	7	2	0	5	13	0	8	34	3	2	0

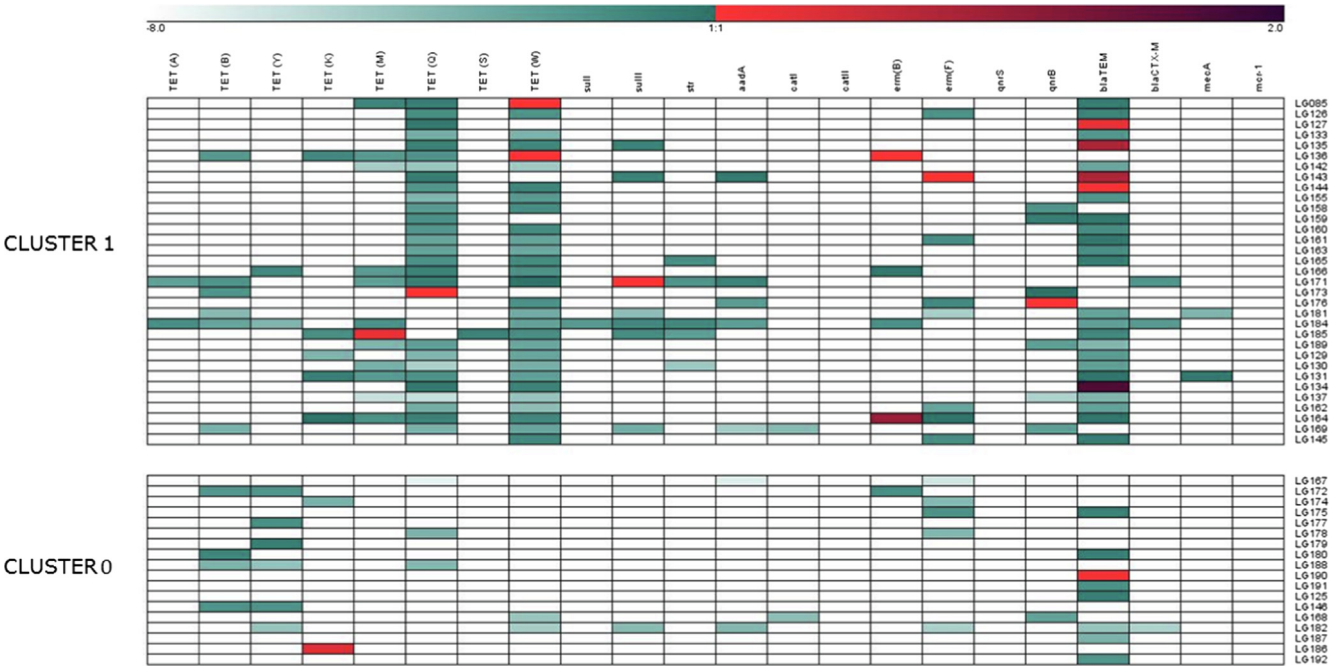


Fig. 2. Patterns of resistance obtained in guigna samples by k-means clustering of each antibiotic resistance gene. Cluster 1 includes samples with high relative percentage load charge and Cluster 0 shows samples with low relative percentage load charge. Relative percentage load charge is expressed in a color scale from a minimum of −8 to a maximum of +2.

3.2. Quantitative descriptive results

The highest mean ARG percentage load was observed for bla_{TEM} (-3.2), *tet*(Q) (-4.3) and *tet*(W) (-4.2) genes, showing significant differences in comparison to the rest of the ARGs analyzed ($K = 301.5$, $p < 0.05$) (Table 3).

3.3. Spatial and biological variables analysis

The only landscape use variable assessed that yielded significant results was landscape composition (fragmented landscape *vs*. continuous forest). Prevalence of *tet*(Q) ($p = 0.014$), *tet*(W) ($p = 0.003$), tetracycline family ($p = 0.02$) and multi-resistance profiles ($p = 0.04$) and *tet*(W) quantification ($U = 117.5$, $p = 0.004$) were significantly higher in guignas inhabiting fragmented landscapes than in those from continuous forests (Table 4, Table 5, Fig. 3). Significant differences associated with guigna sex, age or year of sampling were not observed.

Positive z-score values were observed mainly in individuals inhabiting fragmented landscapes, while negative values were found in guignas from continuous forests. The quinolone and macrolide families presented the opposite trend. Z-score values for the tetracycline family were significantly higher in guignas living in fragmented landscapes than in continuous forests ($U = 129.5$; $p = 0.01$) (Fig. 4).

Comparing animals from clusters 0 and 1 in relation to their type of landscape of origin, significantly more sampled individuals inhabiting fragmented landscapes belonged to cluster 1 (high load charge percentage) ($\chi^2 = 5.26$; $p = 0.02$). Two individuals from fragmented landscapes were positive for *mecA*, a gene associated with methicillin-resistant *Staphylococcus aureus*, and three others were positive for $bla_{CTX\text{-}M}$, an extended-spectrum beta-lactamase.

4. Discussion

Studies based on genetic analysis of antimicrobial resistance in wildlife are limited and most of them differ in the set of genes analyzed and in the diagnostic methodology used, based mostly on cultures (Blanco-Peña et al., 2017; Jiang et al., 2013; Marti et al., 2013). To the authors' knowledge, this is the first study to use direct (without culture) detection and quantification of ARGs by rtPCR in the fecal microbiome of wild felids worldwide. This method allowed a more complete analysis of the bacterial intestinal microbiome of guignas, given that approximately 80% of bacteria are not cultivable (Hamady and Knight, 2009). Unfortunately, due to its novelty, the comparison between our results and those from previous studies is limited.

Here we found that antibiotic resistance genes are very frequent in the fecal microbiome of guignas across the study area, since all individuals studied were positive for at least one ARG. This is consistent with other studies that reported a widespread presence of antibiotic resistance genes and phenotypes in bacteria from wildlife and wild settings (Allen et al., 2010; Cristóbal-Azkarate et al., 2014; Martinez, 2009). The most prevalent ARGs were those that potentially confer resistance to tetracycline and beta-lactam. Although studies of antibiotic resistance genes and antimicrobial resistances in wild felids are scarce, our genetic resistance profile is similar to the phenotypic resistance profile observed in four wild felid species (n = 7) of Mexico (Cristóbal-Azkarate et al., 2014), which described tetracycline and beta-lactam as the most prevalent antibiotic families detected via antibiotic susceptibility testing in cultured bacteria isolated from felid fecal samples. Likewise, tetracycline was also the most frequently observed resistance in *E. coli* isolates from wild small mammals in Canada (Kozak et al., 2009) and wild birds of Spain (Sacristán et al., 2014). Tetracyclines have been frequently used in veterinary medicine, being the first-line antimicrobials employed for disease prevention and growth promotion in livestock. Their widespread use has likely contributed to the high rates of resistance worldwide, and they are thus good models to compare different scenarios (Roberts, 1996).

To the authors' knowledge this is the first description of relevant public health-related ARGs (*mecA* and $bla_{CTX\text{-}M}$) in a wild felid in South America. The gene *mecA* has been mainly described in methicillin-resistant *Staphylococcus aureus* and also in other

Table 3
Relative percentage load of each antibiotic resistance gene expressed in logarithmic scale in the sampled guignas.

	tet(A)	*tet(B)*	*tet(Y)*	*tet(K)*	*tet(M)*	*tet(Q)*	*tet(S)*	*tet(W)*	*sul1*	*sul2*	*str*	*aadA*	*catI*	*catII*	*erm(B)*	*erm(F)*	*qnrS*	*qnrB*	*bla*$_{TEM}$	*bla*$_{CTX-M}$	*mecA*	*mcr-1*
Minimum	−7.0	−7.0	−7.0	−7.0	−7.0	−7.3	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0	−7.0
Maximum	−1.6	−1.1	−0.7	0.3	0.2	0.0	−1	0.0	−2.3	0.0	−1.3	−0.5	−4.1	−7.0	1.0	0.0	−7.0	0.0	1.7	−2.0	−0.5	−7.0
Mean	−6.8	−6.1	−6.3	−6.2	−6	−4.3	−6.9	−4.2	−6.9	−6.2	−6.5	−6.5	−6.9	−7.0	−6.4	−5.9	−7.0	−6.2	−3.2	−6.8	−6.8	−7.0
Std. Deviation	0.9	1.8	1.7	2.0	2.0	2.6	0.8	2.5	0.6	1.9	1.4	1.5	0.5	0.0	1.9	2.1	0.0	2.0	2.9	1.0	1.0	0.0
Std. Error	0.1	0.2	0.2	0.3	0.3	0.4	0.1	0.3	0.1	0.3	0.2	0.2	0.1	0.0	0.3	0.3	0.0	0.3	0.4	0.1	0.1	0.0

Table 4
Prevalence of antibiotic resistance genes of the tetracycline family and multi-resistant microbiome, prevalence and quantification of *tet(Q)* and *tet(W)* antibiotic resistance genes found in guignas inhabiting fragmented landscapes and continuous forests.

	Fragmented landscape $n = 38$ Prevalence (%); (95% CI)	Continuous forest $n = 13$ Prevalence (%); (95% CI)
Tetracycline family	94.7; (87.3–102.2)	69.2; (40.2–98.2)
tet(Q)	71.0; (55.9–86.1)	30.7; (01.7–59.8)
tet(W)	73.7; (59.0–88.3)	23.0; (-3.4–49.6)
Multi-resistance profile	50.0; (33.3–66.6)	23.0; (-3.4–49.6)
	Fragmented landscape $n = 38$ Mean relative percentage load (SD)	Continuous forest $n = 13$ Mean relative percentage load (SD)
tet(Q)	−3.8 (2.5)	−5.5 (2.4)
tet(W)	−3.6 (2.4)	−6.0 (2.0)

staphylococci (Chen et al., 2016). Of note, *Staphylococcus aureus* is one of the major causes of hospital and community-acquired infections, associated with high mortality rates (Lakhundi and Zhang, 2018) and increased hospital stays and health care costs (Antonanzas et al., 2015). The gene *bla*$_{CTX-M}$ has been related to extended-spectrum beta-lactamases (Devarajan et al., 2016). Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, currently classified as critical priority pathogens by the World Health Organization (WHO), have been increasingly reported in natural ecosystems and wildlife (Allen et al., 2010; Dolejska and Papagiannitsis, 2018; Fuentes-Castillo et al., 2019). In Chile, *bla*$_{CTX-M}$ presence in wildlife has been previously reported in wild birds of the order Strigiformes (Magellanic horned owl [*Bubo magellanicus*], and rufous-legged owl [*Strix rufipes*]) received at WRRC, rescued from different anthropogenically-impacted ecosystems (Fuentes-Castillo et al., 2019).

Our hypothesis regarding higher frequency of ARG in guignas from human-dominated areas was confirmed: animals from fragmented landscapes presented higher tetracycline prevalence and quantification, and higher prevalence of multi-resistance profiles in comparison to those inhabiting continuous pristine forests. Likewise, z-score and cluster analyses showed a relationship between ARG and fragmented landscapes; higher z-score values were observed for almost all ARG families in most individuals sampled in human-dominated landscapes, included in cluster 1 (high relative load percentage cluster). Previous reports have described higher ARG levels in species inhabiting human-dominated areas (Cristóbal-Azkarate et al., 2014; Kozak et al., 2009; Blanco-Peña et al., 2017; Cole et al., 2005; Hassell et al., 2017). In agreement with our results, antibiotic resistance in the fecal microbiota of different species of wild felids in Mexico was higher in animals inhabiting anthropized areas when compared to those from pristine areas (Cristóbal-Azkarate et al., 2014). Kozak et al. (2009), showed a relationship between anthropized areas and higher levels of antibiotic resistance in *E. coli* isolates from wild small mammals. A correlation between urbanization and the presence of high levels of ARGs has also been reported, mainly for wild birds (Blanco-Peña et al., 2017; Cole et al., 2005; Hassell et al., 2017).

The current literature associates the presence of antimicrobial-resistant bacteria in wildlife in contact with anthropogenic sources such as farms and human waste (Allen et al., 2010; Radhouani et al., 2011). Here we showed that, as previously described by other authors (Furness et al., 2017; Vittecoq et al., 2016), wildlife could act as sentinels of environmental ARGs in relation to the degree of anthropization of the landscape they inhabit. Likewise, wildlife may also become possible reservoirs of ARGs in the environment;

Table 5
Best model explaining multivariable relationships between predictor variables and ARGs, ARG families and multi-resistant microbiome prevalence using logistic regression analysis (n = 51).

		Regressioncoefficient	SE	Odds ratio	95% confidence interval	*P* value	Hosmer Lemeshow test
a) Tetracycline family	Fragmented landscape	2.0	0.9	8.0	1.3–64.7	0.02	*p*-value = 0.46
	Continuous forest	–	–	2.2	0.7–8.3		
a.1) *tet(Q)*	Fragmented landscape	1.7	0.7	5.5	1.5–24.1	0.01	*p*-value = 1.0
	Continuous forest	–	–	0.4	0.1–1.4		
a.2) *tet(W)*	Fragmented landscape	2.2	0.7	9.3	2.3–48.4	0.003	*p*-value = 1.0
	Continuous forest	–	–	0.3	0.06–1.0		
b) Multi-resistant microbiome	Fragmented landscape	1.7	0.8	5.4	1.2–31.7	0.04	*p*-value = 0.99
	Continuous forest	–	–	0.12	0.0–0.7		

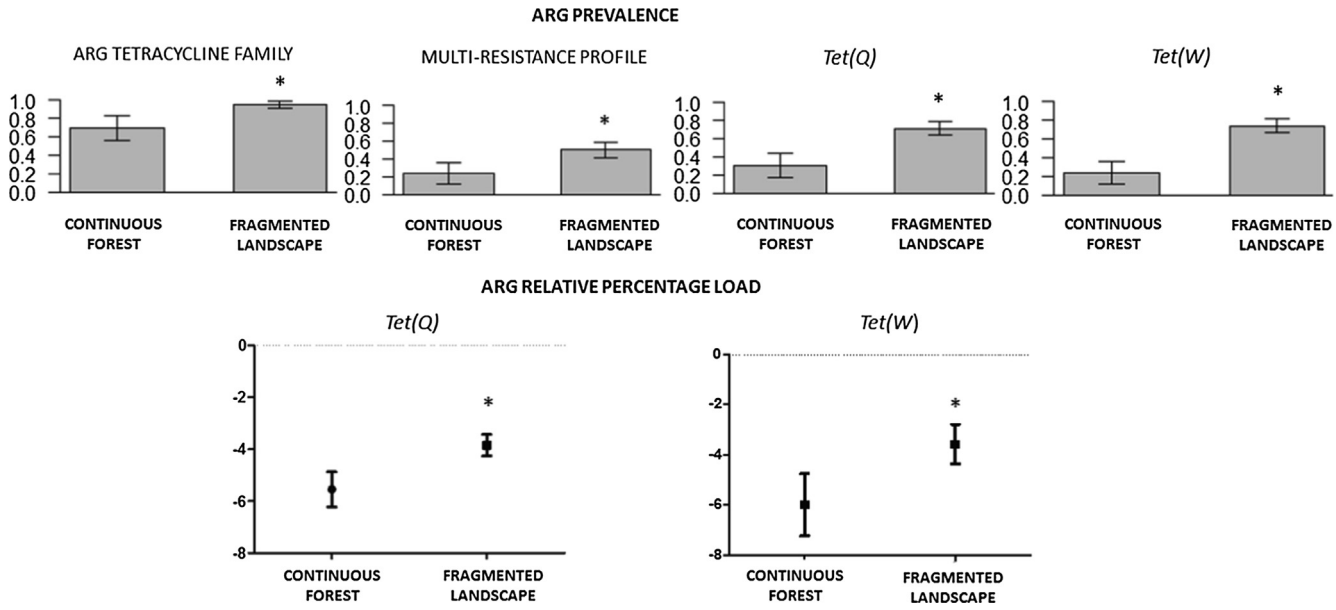


Fig. 3. Statistically significant differences between antibiotic resistance gene prevalence and relative percentage load between guignas inhabiting continuous forests and fragmented landscapes.

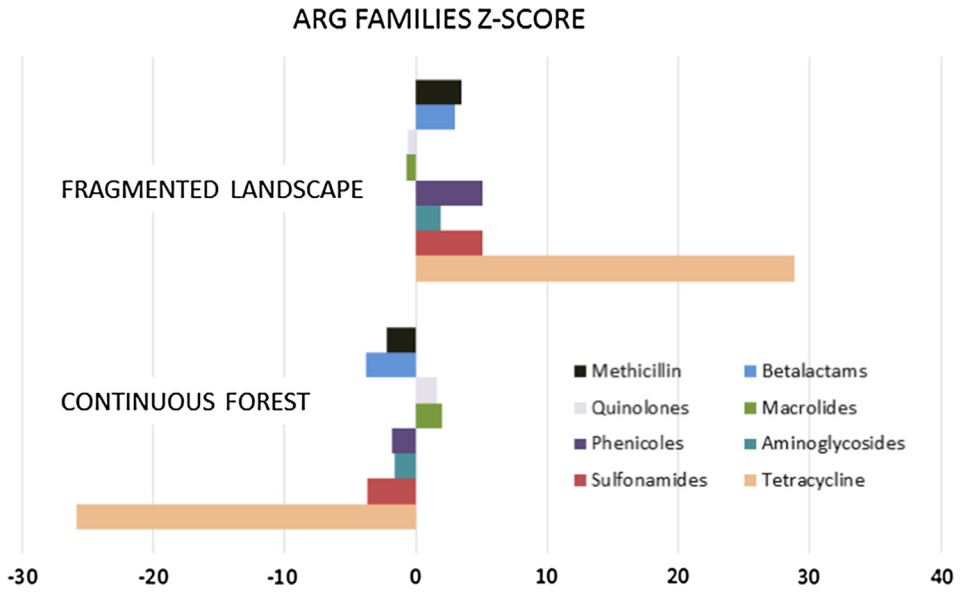


Fig. 4. Z-score values of antibiotic resistance gene families in guignas inhabiting fragmented landscape and continuous forest.

therefore, it is necessary to increase our knowledge of antibiotic resistance dynamics among wild species.

Human-caused modification of natural habitats is the main threat currently faced by guignas. According to our results, the study of ARGs is a valuable tool to identify and evaluate the degree of environmental contamination with ARG of the different types of landscapes used by guignas and provides new insights for the implementation of conservation strategies. Additionally, the presence of ARGs in the microbiome of threatened species is a potential danger for future disease control and conservation efforts.

5. Conclusion

This is the first study on ARGs in wild felids of Chile and the first detection of *mecA* in South American wild felids. We observed an association between the degree of landscape anthropization and ARG prevalence, confirming that ARGs are important indicators of wildlife exposure to human activities in Chile. Despite the sample size limitation, our results contribute to elucidate the antibiotic resistance dynamics in a wild-anthropized interface and can be used to implement conservation measures for threatened species such as the guigna. The results identify the human impact to guigna habitat and allows a better understanding of the anthropogenically-derived antimicrobial pollution in ecosystems. Further information on the ecological drivers of ARGs in wildlife, their transmission dynamics and range of conditions under which gene/bacteria exchange occurs are imperative to elaborate and implement solutions for this global public health crisis.

Acknowledgements

We gratefully acknowledge CONAF, especially Patricio Contreras, Patricia Barría, Andrea Bahamonde and Dennis Aldrigde; SAG, especially Diego Ramírez and Rodrigo Villalobos; the Ministry of the Environment, especially Sandra Díaz; Tantauco Park, especially Alan Bannister; and the Valdivian Coastal Reserve and Camila Dünner for logistic support. Special thanks to Diego Peñaloza, Nicolás Gálvez, Eduardo Silva, Maximiliano Sepúlveda, José Luis Brito, Daniel González, Nicole Sallaberry, Angelo Espinoza, Jorge Valenzuela, Nora Prehn, Camila Sepúlveda, Gerardo Morales, Andrea Roa, Gaby Svensson, Ricardo Pino, Frederick Toro, Mario Alvarado, Brayan Zambrano, Tomás Valdés and Manuel Valdés for their valuable support in sample collection. We acknowledge Irene Peña and Ana Carolina Ewbank for their support in laboratory work.

Funding

Our study was funded by CONICYT FONDECYT Iniciación, Chile 11150934 (CN), Morris Animal Foundation (MAF) Fellowship Training Award D15ZO-413 (CN), National Geographic Society Conservation Trust C309-15 (CN), Mohamed bin Zayed Species Conservation Fund 152510351 (CN), 2018 Endeavour Research Fellowship (Australian government) (CN), CONICYT PIA APOYO CCTE AFB170008 (CN, EP), the Wild Felid Association (IS), Fondo Interno UNAB Universidad Andres Bello, Chile DI-778-15/R (JM), Morris Animal Foundation D16Z-825 (JM), and CONICYT FONDECYT Regular 1161593 (JM, CN).

Author Contributions

Conceived and designed the study: IS, FE, CN. Performed field work, data collection and provided samples: IS, CN, FA, EA, SG, MJL, AC, JC, EHH. Performed lab work: IS, EN. Analyzed the data: IS, FE. Wrote the paper and prepared tables and figures: IS. Contributed in writing the paper: FE, CN, JM, EHH, EP. All authors discussed the results and contributed to editing the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134900.

References

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312.

Antonanzas, F., Lozano, C., Torres, C., 2015. Economic features of antibiotic resistance: the case of methicillin-resistant *Staphylococcus aureus*. Pharmacoeconomics 33, 285–325. https://doi.org/10.1007/s40273-014-0242-y.

Bartlett, J.G., Gilbert, D.N., Spellberg, B., 2013. Seven ways to preserve the miracle of antibiotics. Clin. Infect. Dis. 56, 1445–1450. https://doi.org/10.1093/cid/cit070.

Beltrán, L.F., Nallar, R., Villalba, M.L., Delgado, E., Berna, M., 2009. Inmovilización química, evaluación hematológica y coproparasitología de *Leopardus colocolo* en Khastor, Potosí, Bolivia. Revista de Investigaciones Veterinarias del Perú 20, 297–305.

Blanco-Peña, K., Esperón, F., Torres-Mejía, A.M., de la Torre, A., de la Cruz, E., Jiménez-Soto, M., 2017. Antimicrobial Resistance Genes in Pigeons from Public Parks in Costa Rica. Zoonoses Publ. Health 64, e23–e30. https://doi.org/10.1111/zph.12340.

Chen, J., Yu, Z., Michel Jr, F.C., Wittum, T., Morrison, M., 2007. Development and application of real-time PCR assays for quantification of *erm* genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl. Environ. Microbiol. 73, 4407–4416. https://doi.org/10.1128/AEM.02799-06.

Chen, M.M., Boardman, W.S., Brown, M.H., 2016. Methicillin resistance gene diversity in staphylococci isolated from captive and free-ranging wallabies. Infect. Ecol. Epidemiol. 6, 31507.

Cole, Dana, Drum, David J.V., Stallknecht, David E., White, David G., Lee, Margie D., Ayers, Sherry, Sobsey, Mark, Maurer, John J., 2005. Free-living Canada Geese and Antimicrobial Resistance. Emerg. Infect. Dis. 11 (6), 935–938. https://doi.org/10.3201/eid1106.040717.

Cristóbal-Azkarate, J., Dunn, J.C., Day, J.M.W., Amábile-Cuevas, C.F., 2014. Resistance to antibiotics of clinical relevance in the fecal microbiota of mexican wildlife. PLoS ONE 9,. https://doi.org/10.1371/journal.pone.0107719 e107719.

Cummings, D.E., Archer, K.F., Arriola, D.J., Baker, P.A., Faucett, K.G., Laroya, J.B., Pfeil, K.L., Ryan, C.R., Ryan, K.R., Zuill, D.E., 2011. Broad dissemination of plasmid-mediated quinolone resistance genes in sediments of two urban coastal wetlands. Environ. Sci. Technol. 45, 447–454. https://doi.org/10.1021/es1029206.

Devarajan, N., Laffite, A., Mulaji, C.K., Otamonga, J.-P., Mpiana, P.T., Mubedi, J.I., Prabakar, K., Ibelings, B.W., Poté, J., 2016. Occurrence of antibiotic resistance genes and bacterial markers in a tropical river receiving hospital and urban wastewaters. PLoS ONE 11, 1–14. https://doi.org/10.1371/journal.pone.0149211.

Dolejska, M., Papagiannitsis, C.C., 2018. Plasmid-mediated resistance is going wild. Plasmid, Antimicrob. Resistan. Mob. Genet. Elements 99, 99–111. https://doi.org/10.1016/j.plasmid.2018.09.010.

Dunstone, N., Durbin, L., Wyllie, I., Freer, R., Jamett, G.A., Mazzolli, M., Rose, S., 2002. Spatial organization, ranging behaviour and habitat use of the kodkod (*Oncifelis guigna*) in southern Chile. J. Zool. 257, 1–11. https://doi.org/10.1017/S0952836902000602.

Esperón, F., Sacristán, C., Carballo, M., de la Torre, A., 2018. Antimicrobial resistance genes in animal manure, manure-amended and non-anthropogenically impacted soils in Spain. Adv. Biosci. Biotechnol. 9, 469–480. https://doi.org/10.4236/abb.2018.99032.

Francois, P., Pittet, D., Bento, M., Pepey, B., Vaudaux, P., Lew, D., Schrenzel, J., 2003. Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay. J. Clin. Microbiol. 41, 254–260. https://doi.org/10.1128/JCM.41.1.254-260.2003.

Fuentes-Castillo, D., Farfán-López, M., Esposito, F., Moura, Q., Fernandes, M.R., Lopes, R., Cardoso, B., Muñoz, M.E., Cerdeira, L., Najle, I., Muñoz, P.M., Catão-Dias, J.L., González-Acuña, D., Lincopan, N., 2019. Wild owls colonized by international clones of extended-spectrum β-lactamase (CTX-M)-producing *Escherichia coli* and *Salmonella infantis* in the Southern Cone of America. Sci. Total Environ. 674, 554–562. https://doi.org/10.1016/j.scitotenv.2019.04.149.

Furness, L.E., Campbell, A., Zhang, L., Gaze, W.H., McDonald, R.A., 2017. Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. Environ. Res. 154, 28–34. https://doi.org/10.1016/j.envres.2016.12.014.

Golkar, Z., Bagasra, O., Pace, D.G., 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J. Infect. Dev. Countries 8, 129–136. https://doi.org/10.3855/jidc.3573.

Goossens, H., 2005. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study 365, 9. https://doi.org/10.1016/S0140-6736(05)17907-0.

Gould, I.M., Bal, A.M., 2013. New antibiotic agents in the pipeline and how they can help overcome microbial resistance. Virulence 4, 185–191. https://doi.org/10.4161/viru.22507.

Hamady, M., Knight, R., 2009. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. 19, 1141–1152. https://doi.org/10.1101/gr.085464.108.

Hansen, M.C., Potapov, P.V., Moore, R., Hancher, M., Turubanova, S.A.A., Tyukavina, A., Thau, D., Stehman, S.V., Goetz, S.J., Loveland, T.R., 2013. High-resolution global maps of 21st-century forest cover change. Science 342, 850–853. https://doi.org/10.1126/science.1244693.

Hassell, J.M., Begon, M., Ward, M.J., Fèvre, E.M., 2017. Urbanization and disease emergence: Dynamics at the wildlife–livestock–human interface. Trends Ecol. Evol. 32, 55–67. https://doi.org/10.1016/j.tree.2016.09.012.

Instituto Nacional de Estadística (INE), 2017. Cited September, 2019. URL http://www.ine.cl/estadisticas/medioambiente/informes-anuales, n.d.

Jiang, L., Hu, X., Xu, T., Zhang, H., Sheng, D., Yin, D., 2013. Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. Sci. Total Environ. 458–460, 267–272. https://doi.org/10.1016/j.scitotenv.2013.04.038.

Kozak, G.K., Boerlin, P., Janecko, N., Reid-Smith, R.J., Jardine, C., 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Appl. Environ. Microbiol. 75, 559–566. https://doi.org/10.1128/AEM.01821-08.

Lakhundi, S., Zhang, K., 2018. Methicillin-resistant *Staphylococcus aureus*: molecular characterization, evolution, and epidemiology. Clin. Microbiol. Rev. 31, e00020–e118. https://doi.org/10.1128/CMR.00020-18.

Larsson, D.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., de Roda Husman, A. M., Fagerstedt, P., Fick, J., Flach, C.-F., Gaze, W.H., Kuroda, M., 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138. https://doi.org/10.1016/j.envint.2018.04.041.

Marston, H.D., Dixon, D.M., Knisely, J.M., Palmore, T.N., Fauci, A.S., 2016. Antimicrobial Resistance. JAMA 316, 1193–1204. https://doi.org/10.1001/jama.2016.11764.

Marti, E., Balcázar, L., 2013. Real-Time PCR assays for quantification of *qnr* genes in environmental water samples and chicken feces. J. Appl. Environ. Microbiol. 75, 1743–1745. https://doi.org/10.1128/AEM.03409-12.

Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS ONE 8,. https://doi.org/10.1371/journal.pone.0078906 e78906.

Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ. Pollut. 157, 2893–2902. https://doi.org/10.1016/j.envpol.2009.05.051.

Millán, J., García, E.J., Oleaga, Á., López-Bao, J.V., Llaneza, L., Palacios, V., Candela, M. G., Cevidanes, A., Rodríguez, A., León-Vizcaíno, L., 2014. Using a top predator as a sentinel for environmental contamination with pathogenic bacteria: the Iberian wolf and leptospires. Memórias do Instituto Oswaldo Cruz 109, 1041–1044. https://doi.org/10.1590/0074-0276140258.

Ministerio de Bienes Nacionales website. Ministerio de Bienes Nacionales, 2019. (http://www.ide.cl/descarga/capas/advanced-search/92.html). n.d. Accessed September 18, 2019.

Naccari, C., Giangrosso, G., Macaluso, A., Billone, E., Cicero, A., D'Ascenzi, C., Ferrantelli, V., 2013. Red foxes (*Vulpes vulpes*) bioindicator of lead and copper pollution in Sicily (Italy). Ecotoxicol. Environ. Saf. 90, 41–45. https://doi.org/10.1016/j.ecoenv.2012.12.011.

Napolitano, C., Díaz, D., Sanderson, J., Johnson, W.E., Ritland, K., Ritland, C.E., Poulin, E., 2015a. Reduced genetic diversity and increased dispersal in guigna (*Leopardus guigna*) in chilean fragmented landscapes. J. Hered. 106, 522–536. https://doi.org/10.1093/jhered/esv025.

Napolitano, C., Gálvez, N., Bennett, M., Acosta-Jamett, G., Sanderson, J., 2015b. Leopardus guigna. IUCN Red List Threatened Spec. https://doi.org/10.2305/IUCN.UK.2015-2.RLTS.T15311A50657245.en.

Nieto-Claudín, A., Esperón, F., Blake, S., Deem, S.L., 2019. Antimicrobial resistance genes present in the faecal microbiota of free-living Galapagos tortoises (*Chelonoidis porteri*). Zoonoses Publ. Health 00, 1–9. https://doi.org/10.1111/zph.12639.

O'Neill, J., 2018. Tackling drug-resistant infections globally: Final report and recommendations. 2016. HM Government and Welcome Trust: UK.

Radhouani, H., Igrejas, G., Carvalho, C., Pinto, L., Gonçalves, A., Lopez, M., Sargo, R., Cardoso, L., Martinho, A., Rego, V., Rodrigues, R., Torres, C., Poeta, P., 2011. Clonal Lineages, Antibiotic Resistance and Virulence Factors in Vancomycin-Resistant Enterococci Isolated from Fecal Samples of Red Foxes (*Vulpes vulpes*). J. Wildl. Dis. 47, 769–773. https://doi.org/10.7589/0090-3558-47.3.769.

R Development Core Team 3.0.1., 2013. A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Viena, Austria.

Roberts, M.C., 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19, 1–24. https://doi.org/10.1111/j.1574-6976.1996.tb00251.x.

Sacristán, C., Esperón, F., Herrera-León, S., Iglesias, I., Neves, E., Nogal, V., Muñoz, M. J., de la Torre, A., 2014. Virulence genes, antibiotic resistance and integrons in *Escherichia coli* strains isolated from synanthropic birds from Spain. Avian Pathol. 43, 172–175. https://doi.org/10.1080/03079457.2014.897683.

Sanderson, J., Sunquist, M.E., Iriarte, A.W., 2002. Natural history and landscape-use of guignas (*Oncifelis guigna*) on Isla Grande de Chiloé, Chile. J. Mammal. 83, 608–613. https://doi.org/10.1644/1545-1542(2002) 083<0608: NHALUO>2.0.CO;2.

Sarmah, A.K., Meyer, M.T., Boxall, A.B., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026.

Sayah, R.S., Kaneene, J.B., Johnson, Y., Miller, R., 2005. Patterns of Antimicrobial Resistance Observed in Escherichia coli Isolates Obtained from Domestic- and Wild-Animal Fecal Samples, Human Septage, and Surface Water. Appl. Environ. Microbiol. 71, 1394–1404. https://doi.org/10.1128/AEM.71.3.1394-1404.2005.

Schüttler, E., Klenke, R., Galuppo, S., Castro, R.A., Bonacic, C., Laker, J., Henle, K., 2017. Habitat use and sensitivity to fragmentation in America's smallest wildcat. Mamm. Biol. 86, 1–8. https://doi.org/10.1016/j.mambio.2016.11.013.

Schwartz, T., 2012. Strategies to assess and minimize the biological risk of antibiotic resistance in the environment. In: Kenn P, Montforts M, editors. Antimicrobial resistance in the environment. John Wiley & Sons 251-64. Hoboken, NJ, USA.

Swift, B.M.C., Bennett, M., Waller, K., Dodd, C., Murray, A., Gomes, R.L., Humphreys, B., Hobman, J.L., Jones, M.A., Whitlock, S.E., Mitchell, L.J., Lennon, R.J., Arnold, K.E., 2019. Anthropogenic environmental drivers of antimicrobial resistance in wildlife. Sci. Total Environ. 649, 12–20. https://doi.org/10.1016/j.scitotenv.2018.08.180.

Ventola, C.L., 2015. The antibiotic resistance crisis. Pharm. Therapeut. 40, 277–283. PMID: 25859123.

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., Arnal, A., Aberkane, S., Jean-Pierre, H., Gauthier-Clerc, M., Thomas, F., Renaud, F., 2016. Antimicrobial resistance in wildlife. J. Appl. Ecol. 53, 519–529. https://doi.org/10.1111/1365-2664.12596.

Wang, F.-H., Qiao, M., Su, J.-Q., Chen, Z., Zhou, X., Zhu, Y.-G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Environ. Sci. Technol. 48, 9079–9085. https://doi.org/10.1021/es502615e.

Xie, W.-Y., Yang, X.-P., Li, Q., Wu, L.-H., Shen, Q.-R., Zhao, F.-J., 2016. Changes in antibiotic concentrations and antibiotic resistome during commercial composting of animal manures. Environ. Pollut. 219, 182–190. https://doi.org/10.1016/j.envpol.2016.10.044.