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Original Contribution

Xenopus laevis and Emerging Amphibian Pathogens in Chile

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Abstract: Amphibians face an extinction crisis with no precedence. Two emerging infectious diseases, ranaviral disease caused by viruses within the genus *Ranavirus* and chytridiomycosis due to *Batrachochytrium dendrobatidis* (Bd), have been linked with amphibian mass mortalities and population declines in many regions of the globe. The African clawed frog (*Xenopus laevis*) has been indicated as a vector for the spread of these pathogens. Since the 1970s, this species has been invasive in central Chile. We collected *X. laevis* and dead native amphibians in Chile between 2011 and 2013. We conducted post-mortem examinations and molecular tests for *Ranavirus* and Bd. Eight of 187 individuals (4.3 %) tested positive for *Ranavirus*: seven *X. laevis* and a giant Chilean frog (*Calyptocephallela gayi*). All positive cases were from the original area of *X. laevis* invasion. Bd was found to be more prevalent (14.4 %) and widespread than *Ranavirus,* and all *X. laevis* Bd-positive animals presented low to moderate levels of infection. Sequencing of a partial *Ranavirus* gene revealed 100 % sequence identity with *Frog Virus 3*. This is the first report of *Ranavirus* in Chile, and these preliminary results are consistent with a role for *X. laevis* as an infection reservoir for both *Ranavirus* and Bd.

Keywords: *Ranavirus*, *Batrachochytrium dendrobatidis*, *Xenopus laevis*, Reservoir, Emerging infectious diseases, Chile

Introduction and Purpose

Amphibians are considered the most imperilled class of vertebrates (Stuart et al. 2004). In recent years, evidence for the critical involvement of emerging infectious diseases in the decline of amphibian populations has grown and become more convincing, especially in the case of chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Bd; Berger et al. 1998). Lethal outbreaks caused by *Ranavirus* have been reported in many parts of the world (Cunningham et al. 1996; Jancovich et al. 1997; Green et al. 2002; Greer et al. 2005; Fox et al. 2006; Muths et al. 2006; Balseiro et al., 2010; Une et al. 2009; Kik et al. 2011; Stöhr et al. 2013), long-term population declines confirmed for the common frog (*Rana temporaria*) in the United Kingdom (Teacher et al. 2010) and severe amphibian community level impacts described in Spain (Price et al. 2014). Chytridiomycosis has been implicated in the extinction of several amphibian species from Australia, Costa Rica and

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Chile (Daszak et al. 1999; Pounds et al. 2006; Schloegel et al. 2006; Soto-Azat et al. 2013a, b).

Ranaviruses cause systemic haemorrhagic disease in amphibians, fish and reptiles (Hyatt et al. 2000; Miller et al. 2011). The pathogen infects multiple amphibian hosts, including tadpoles and adults, and may persist in aquatic and terrestrial environments through amphibian, fish or reptile reservoirs (Hyatt et al. 2000). The chytrid fungus Bd is a highly pathogenic and a virulent pathogen, which appears to be capable of infecting the entire class *Amphibia* (Berger et al. 1998; Gower et al. 2013; Olson and Ronnenberg 2014). In susceptible adult amphibians, Bd colonizes the skin, disrupting the integrity of the epidermis, with subsequent electrolyte depletion and osmotic imbalance leading to death (Voyles et al. 2009). Tadpoles and resistant species or populations may act as reservoirs of infection (Berger et al. 1998; Daszak et al. 1999; Schloegel et al. 2006). The type species of *Ranavirus*, *Frog Virus 3* (FV3) and a hypervirulent genotype of *Bd*, termed the global pandemic lineage (BdGPL), are known to be globally widespread, while other species of *Ranavirus* and other lineages of Bd appear to be more restricted in distribution (Farrer et al. 2013; Duffus et al. 2015). Although poorly studied in South America, evidence of *Ranavirus* has been obtained from free-ranging amphibians in Venezuela, Argentina and Peru (Zupanovic et al. 1998; Fox et al. 2006; Warne et al. 2016), and from farmed North American bullfrogs (*Lithobates catesbeianus*) from Uruguay and Brazil (Galli et al. 2006; Mazzoni et al. 2009). Better studied, Bd appears to be widely distributed in South America (Mazzoni et al. 2003; Hanselmann et al. 2004; Pounds et al. 2006; Schloegel et al. 2010, 2012; Solís et al. 2010, 2015; Bourke et al. 2011; Soto-Azat et al. 2013a; Olson and Ronnenberg 2014; James et al. 2015; Warne et al. 2016).

Chilean batrachofauna consist of 63 anuran species, characterized by a high degree of endemism (72 %, Soto-Azat et al. 2015). Since its introduction in the 1970s, the African clawed frog (*Xenopus laevis*) has become established throughout much of central Chile (Lobos and Jaksic 2005) and, recently, Bd infection has been described in this species in Chile (Solís et al. 2010). Whilst *X. laevis* is generally resistant to developing ranaviruses or chytridiomycosis, it is tolerant to infection with both causative pathogens (Robert et al. 2007; Ramsey et al. 2010). This species is thus theoretically capable of disseminating both *Ranavirus* and Bd to new geographical areas and amphibian populations, where it might also serve as a reservoir of infection (Hanselmann et al. 2004; Fisher and Garner 2007; Schloegel et al. 2010; Greenspan et al. 2012). In this study, we investigated the *Ranavirus* and Bd carrier status of *X. laevis* in Chile and looked for evidence of infection in sympatric native species.

Materials and Methods

Study Area

Amphibians were collected from seven sites in central Chile from 2011 to 2013 (see Fig. 1), all within or near the invasive range of *X. laevis*. These included natural environments as well as those transformed through agriculture. Only adult frogs were collected, and each site was visited once during the amphibian breeding season (November to March). We also responded to reports of mortality events by visiting sites as soon as possible to collect fresh carcasses.

Sampling

Our opportunistic sampling consisted of animals that had died in the wild as well as euthanized animals (in this case only *X. laevis*). Amphibian carcasses found recently dead were collected following mortality events. Carcass numbers ranged from single animals to 79. We also received the internal organs of eight individuals harvested for human consumption from a commercial giant Chilean frog (*Calyptocephallela gayi*) aquaculture facility. In addition, *X. laevis* was live captured using funnel traps set up at the margin of water bodies. This species is considered harmful under the Chilean Wildlife Act (Law N° 19473), and can be captured all year round without limits on the number and use of captured individuals. Traps were baited with chicken heart and checked twice daily. Each of the captured *X. laevis* was euthanized individually via immersion in a buffered solution of 10 g/l tricaine methanesulfonate (Dolical 80 %, Centrovet), which has been demonstrated to be safe for Bd studies based on molecular detection (Webb et al. 2005). Immediately after collection of dead amphibians or euthanasia of *X. laevis*, each individual was skin swabbed for Bd detection following Hyatt et al. (2007), examined for gross lesions and dissected following standard necropsy procedures to obtain liver, kidney and spleen for molecular tests for *Ranavirus*. New sterile disposable scalpels were used to avoid cross-contamination. Tissues were collected separately in 2 ml sterile Eppendorf tubes containing 95 % sterile ethanol. Each individual was handled using a new pair of disposable gloves. Furthermore, in

order to minimize any contamination of samples or the spread of pathogens within or between study sites by researchers, equipment or materials, a strict field sampling and disinfection protocol was followed, with reference to Phillot et al. (2010).

PCR Assay for Bd

Tips of skin swabs were each added to 1.5 ml Eppendorf tubes containing 60 ml of PrepMan Ultra (Applied Biosystems) and between 30 to 40 mg of Zirconium/silica beads of 0.5 mm diameter (Biospec Products). For each sample, DNA was extracted following the protocol of Boyle et al. (2004). Extracted DNA was diluted (1:10) in double-distilled water and analyzed using a quantitative real-time PCR Taqman assay (qPCR) with primers specific for the ITS-1/5.8S ribosomal DNA region of Bd. In addition, bovine serum albumin (BSA) was included in the Taqman mastermix to minimize PCR inhibition (Garland et al. 2010). Each assay was run in 25 µl PCR reactions, and thermocycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 50 cycles. For each sample, diagnostic assays were performed in duplicate, and standards of known zoospore concentration were included within each PCR plate, as were negative controls. In order to quantify the Bd genome equivalents (GE) in each well, we multiplied the qPCR result by 120, as described by Hudson et al. (2016). A result was considered positive when (1) amplification (i.e. a clearly sigmoid curve) occurred in both replicate PCR assays, (2) values higher than 0.1 GE were obtained from both replicated reactions and (3) a sample's mean GE value was greater than its standard deviation.

PCR Assay, DNA Sequencing and DNA Sequence Analysis for *Ranavirus*

Small pieces (0.01–0.05 g) of sampled visceral organs of the same animal were pooled and analyzed. Samples were homogenized together in tubes containing 250 µl of lysis buffer and then incubated at 56°C overnight. DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. MCP-F and MCP-R primers were used to amplify a 530 base pair fragment of the *Ranavirus* major capsid protein (MCP) in 25 µl PCR reactions, following the protocol of Mao et al. (1997), modified by Greer et al. (2005). Thermocycling conditions were 5 min at 94°C, 30 s at 94°C, 30 s at 55°C and 30 s at 60°C, cycled 35 times, followed by an extension of 2 min at 72°C. All PCR assays were run in duplicate with a positive (previously obtained FV3 DNA) and a negative (water) control tested alongside the unknown samples. PCR products were stained with Sybr Safe (Invitrogen) and visualized following electrophoresis on 2 % agarose gels. Samples were considered positive when bands matched the size of the positive control bands. The PCR products of positive samples were submitted (Beckman Coulter Genomics, UK) for Sanger sequencing of both

Table 1. Summary of *Ranavirus* (Rv) and *Batrachochytrium dendrobatidis* (Bd) Prevalence by Site Between 2011 and 2013 in Central Chile.

Site	Location	Habitat type	Species	n	Rv+	Prevalence of infection (±95 %CI)	Bd+	Prevalence of infection (±95 %CI)
Rinconada	33°29′40″S; 70°49′47″W	Lagoon	XL	24	4	0.167 ± 0.160	10	0.417 ± 0.213
El Peral	33°30′18″S; 71°36′13″W	Lagoon	XL, PT	81 (79 XL, 2 PT)	0	0	1	0.01 ± 0.02
Talagante	33°41′05″S; 70°54′28″W	Agriculture channels	XL, CG	41 (40 XL, 1 CG)	4	0.098 ± 0.095	6	0.146 ± 0.113
San Guillermo	33°50′56″S; 71°47′11″W	Pond	XL	8	0	0	2	0.250 ± 0.387
Talca	35°26′45″S; 71°42′10″W	Pond	XL	24	0	0	0	0
Longaví	35°55′57″S; 71°34′57″W	Frog farm	CG	8	0	0	8	1.000
Nahuelbuta	37°49′46″S; 73°09′49″W	River	TB	1	0	0	0	0

Results of specific PCR assays from 187 amphibians of mixed species.
XL—*Xenopus laevis*, PT—*Pleurodema thaul*, CG—*Calyptocephalella gayi*, TB—*Telmatobufo bullocki*.

Table 2. Summary of *Ranavirus* (Rv) and *Batrachochytrium dendrobatidis* (Bd) Prevalence by Host Species Between 2011 and 2013 in Central Chile.

Species	n	Rv+	Prevalence of infection (±95 %CI)	Bd+	Prevalence of infection (±95 %CI)	co-infections	Prevalence of co-infections (±95 %CI)
Calyptocephalella gayi	9	1[a]	0.111 ± 0.256	8[b]	0.889 ± 0.256	0	0
Pleurodema thaul	2	0	0	1[a]	0.500 ± 6.353	0	0
Telmatobufo bullocki	1	0	0	0		0	0
Xenopus laevis	175	7[c]	0.040 ± 0.029	18[c]	0.103 ± 0.046	3[c]	0.017 ± 0.020
TOTAL	187	8	0.043 ± 0.029	27	0.144 ± 0.051	3	0.016 ± 0.018

[a]Dead in the wild.
[b]Tissue obtained from aquaculture.
[c]Euthanized after live capture.

DNA strands. Sequences generated from the reverse primer were reverse-complemented prior to alignment of all sequences using MEGA6 (Tamura et al. 2013). Sequences were trimmed to remove low-quality base calls and checked by eye for consistency between complementary DNA strands. We then compared our processed sequences to other publicly available *Ranavirus* sequences in the National Center for Biotechnology Information (NCBI) nucleotide database using BLAST.

Results

A total of 187 individuals of four amphibian species were investigated for evidence of *Ranavirus* and Bd infection. Characteristics and results of molecular tests for each study site are shown in Table 1. Of the amphibians examined, 96 *X. laevis* were captured with the use of baited funnel traps, 79 *X. laevis* and four individuals of native species were collected dead from mortality events and the tissues of eight *C. gayi* were obtained from an aquaculture facility. Overall, 4.3 % and 14.4 % of animals tested were positive for *Ranavirus* and Bd, respectively. All *Ranavirus*-positive amphibians (7 *X. laevis*, 1 *C. gayi*) were from two sites within the Metropolitan Region near to Santiago, the original site of *X. laevis* introduction in Chile (Fig. 1). Bd was found to be more widespread amongst sites and species, with all but one site with *X. laevis* being positive (Fig. 1).

All *Ranavirus*-positive *X. laevis* were apparently healthy individuals; they were live captured and did not present any lesions consistent with ranaviruses. In contrast, the other *Ranavirus*-positive animal, a 2.2 kg female *C. gayi* (22.4 cm snout-vent-length, and estimated to be more than 15 years old based on size), was found dead by a member of the public and then collected for investigation. The animal had been stored frozen until the post-mortem examination, where it presented with abundant clear serosanguinous subcutaneous fluid. Within the intracoelomic cavity, a large amount of a dark serosanguinous fluid was found. Internal organs were moderately oedematous. The internal surface of the left lung was extensively haemorrhagic. No other macroscopic changes were noticeable. Histopathological analyses were not informative as autolysis of organs was advanced. None of the other amphibians studied from mortality events, comprising two four-eyed toads (*Pleurodema thaul*), a Bullock's toad (*Telmatobufo bullocki*) and 79 *X. laevis*, gave *Ranavirus*-positive results. Most (24 of 27) Bd-positive cases were categorized as low to moderate intensity infections by qPCR (30-9816 GE), including all Bd-positive *X. laevis*. Three *C. gayi*, however, had severe intensities of infection ($<$25,368 GE) indicative of disease. However, no signs or lesions attributable to chytridiomycosis were observed in any of the surveyed animals. All *C. gayi* from one site (Longaví) were infected with Bd (and negative to *Ranavirus*). The individual of *C. gayi* found dead in Talagante, co-habiting with *X. laevis*, resulted positive for *Ranavirus*, but negative for Bd. Three animals with co-infections were detected, all of which were *X. laevis*: two from Rinconada and one from Talagante (Fig. 1; Table 1).

Nucleotide sequences of *Ranavirus* PCR products were obtained from four positive frogs (3 *X. laevis*, 1 *C. gayi*). All sequences were 100 % identical to each other and to FV3 (NCBI ref. AY548484) and had 99 % similarity with *Rana grylio Iridovirus* (JQ654586), *Rana catesbeiana virus* (AB474588) and *Common midwife toad Ranavirus*

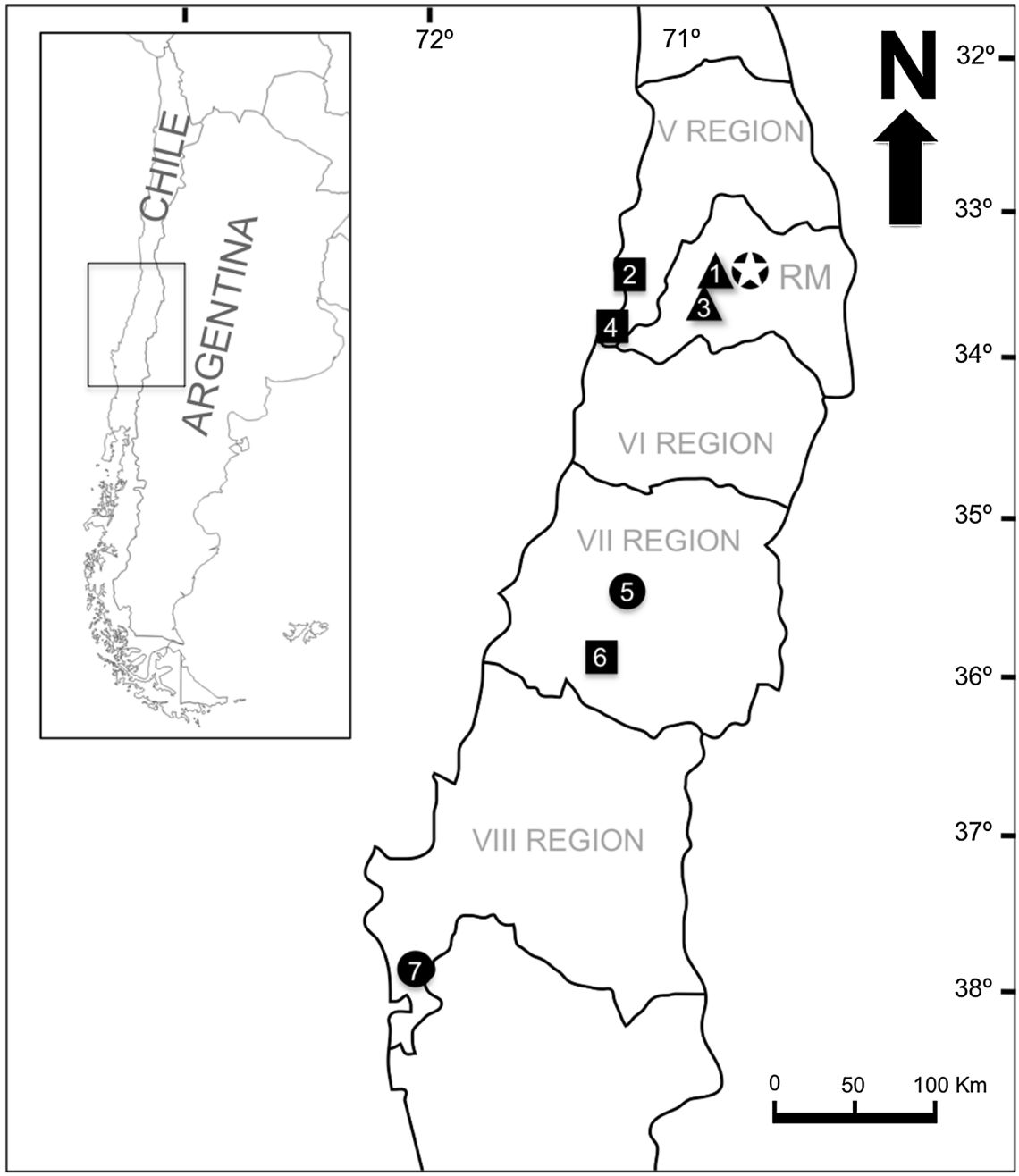

Figure 1. Map of central Chile showing locations of sites from which amphibians were sampled for *Ranavirus* and *B. dendrobatidis* (Bd) infection using PCR. The *star* indicates Santiago, the capital city of Chile. Each *square* indicates a site with Bd-positive animals. Each *triangle* indicates a site with Bd-positive and Ranavirus positive animals. Each *circle* indicates a site where neither pathogen was detected. No site was positive for Ranavirus only. *1* Rinconada, *2* El Peral, *3* Talagante, *4* San Guillermo, *5* Talca, *6* Longaví, *7* Nahuelbuta.

(KP056312); 98 % similarity with *Bohle Iridovirus* (AY187046) and 95 % similarity with *Ambystoma tigrinum virus* (KR075877). The sequences showed no significant similarity to any non-*Ranavirus* sequences in the NCBI nucleotide database, confirming the specificity of the PCR assay.

Discussion

We found evidence of infection of the emerging amphibian pathogens, *Ranavirus* and Bd, in the invasive *X. laevis* and in native species in central Chile. Although clinical chytridiomycosis was not detected, a *Ranavirus*-positive

individual of *C. gayi* which had been found dead had internal lesions consistent with ranaviruses. Unfortunately, the condition of the tissues (frozen/thawed and autolysed) precluded histopathological examination; thus, this presumptive cause of death could not be confirmed. Of the animals tested, 4.3 % were positive for *Ranavirus*. Our sequence analyses of the MCP region of *Ranavirus* are a robust confirmation of our initial PCR findings, and follow OIE recommendations (OIE 2015) to support imperfect molecular methods with corroborative evidence, especially when assessing *Ranavirus* occurrence in a previously unstudied region. In addition, these sequence data serve as initial genetic characterisation of the *Ranavirus* found in central Chile, which appears to be closely related to the type *Ranavirus*, FV3. Our findings further extend the patchy, global distribution of this virus type (Duffus et al. 2015). Isolation and whole genome sequencing of local isolates, as well as the development of primers targeting hypervariable DNA regions of *Ranavirus* to distinguish between different strains, will undoubtedly help to further characterize ranaviruses in Chile and may provide information on their evolutionary history and source (endemic *vs* introduced) through comparative phylogeny (Holopainen et al. 2009; Jancovich et al. 2015).

To the best of our knowledge, this is the first evidence of *Ranavirus* in Chile. We detected *Ranavirus* infection at only two of our study sites, but sample sizes were generally small, limiting our ability to detect the pathogen if at a low infection prevalence. This is seen in the large confidence intervals obtained for those sites and species underrepresented (Tables 1, 2). It is possible, therefore, that *Ranavirus* infection is more widespread than our findings suggest. Increasing the number and range of study sites, the numbers of animals sampled per site and the number of species sampled may improve detection and extend the current known distribution of *Ranavirus* in Chile.

In contrast to our *Ranavirus* results, at least one Bd-positive animal was detected from five of our seven study sites. All sites with *X. laevis* presence except one resulted positive for Bd. In the *C. gayi* aquaculture facility (area still not invaded by *X. laevis*, but expected to occur within the next years), all studied animals (eight) resulted positive for Bd. This pathogen has been reported from *X. laevis* in central Chile (Solís et al. 2010) and from a range of native species across a latitudinal extension of ~3000 km (Bourke et al. 2011; Soto-Azat et al. 2013a; Solís 2015). In the current study, we found the prevalence of Bd infection to range from zero to 41.7 % in the *X. laevis* populations sampled, with all individuals showing low to moderate levels of infection, suggestive of a Bd reservoir function of this species when co-habiting with other susceptible amphibian species. Also, we found all eight of the farmed *C. gayi* tested to be Bd-positive, even with molecular evidence supporting the occurrence of chytridiomycosis, indicating endemicity of infection on the frog farm in question and possibly in other amphibian aquaculture in Chile, as has been reported for frog aquaculture elsewhere in South America (Mazzoni et al. 2003; Schloegel et al. 2012).

Bd was detected in one of two dead *P. thaul* collected from the El Peral lagoon in April 2012. In contrast, no evidence of Bd was obtained from the 79 dead *X. laevis* collected from a mass mortality event at the same site in 2013. On 27 May 2013, ~2000 *X. laevis* left El Peral lagoon coincident with a period of heavy rain. On the following day, many hundreds of these frogs were found dead in the surrounding area, but only fresh carcasses or moribund (euthanized) animals were sampled. This mass movement of *X. laevis* appears to be associated with the colonization of new environments that may occur during heavy rainfall (Tinsley et al. 1996). *X. laevis*, originally from Africa, was introduced to Chile in the 1970s, with the initial site of introduction being the international airport near Santiago. Solís et al. (2010) and Soto-Azat et al. (2013a) speculated that Bd might have been co-introduced to Chile with *X. laevis*. Non-native host introductions have been identified as a predictor of Bd occurrence at the global level (Liu et al. 2013), and urban development has been positively correlated with the presence of both Bd (Murray et al. 2011; Rhor et al. 2011) and *Ranavirus* (North et al. 2015; St-Amour et al. 2008). In Chile, the highest occurrence of Bd has been found in the central region between Santiago and Concepción, an area containing >70 % of the country's human population (Soto-Azat et al. 2013a; James et al. 2015). In addition, Robert et al. (2007) found that a significant fraction of *X. laevis* adults raised in captivity in different places in the United States carried covert FV3 infections, which may have contributed to the spread of *Ranavirus* in the United States. An apparent restricted distribution of *Ranavirus*, associated with the occurrence of invasive *X. laevis* in central Chile, compared to the widespread distribution of Bd in the country, maybe the result of different introductions processes or mechanisms of spread (for instance, better habitat suitability for Bd). However, whether either Bd or *Ranavirus* is a recent introduction to Chile via *Xenopus* requires further inves-

tigation, including comparative pathogen genomics. In effect, this study and preliminary Bd genotype data provide evidence on the occurrence of FV3 and BdGPL in the country. Efforts to isolate endemic strains of both pathogens (if any) have not been successful so far, all this giving support to a role of *X. laevis* in pathogen introduction, maintenance and spread.

Amphibian species show differential susceptibility to Bd and *Ranavirus* depending of life-stage (Fisher et al. 2009; Miller et al. 2011; De Jesús Andino et al. 2012). Since samples obtained in this study were opportunistic, and included only four species (one potentially a competent reservoir), and considering that sites were visited once and only adult amphibians were studied, our results may underestimate the extent of occurrence of these pathogens in Chile, a reason to extend future studies to include sampling of tadpoles, as well as samples of additional sites and species. This study also is a good example of using wildlife mortalities and invasive species as a convenient source of information to study wildlife diseases of conservation concern (Sleeman et al. 2012).

Conclusion

Emerging infectious diseases have been increasingly recognized as a threat to biodiversity, especially as wildlife populations become more fragmented and are increasingly living in sub-optimal environments (Smith et al. 2009). For example, Soto-Azat et al. (2013a) showed that Bd is likely driving precipitous declines of Darwin's frogs (*Rhinoderma* spp.) in Chile. Currently, 47 % of Chilean amphibian species are threatened with extinction, and 37 % have undergone population declines (Soto-Azat et al. 2015). Among these, the once abundant *C. gayi* is currently listed as Vulnerable by the IUCN, and its populations have markedly declined over the last two decades, due to overexploitation for food and agricultural development (Veloso et al. 2010). In addition, chytridiomycosis (Soto-Azat et al. 2013a) and *Ranavirus* (this report) have been identified as potential additional threats to this endemic species (Soto-Azat et al. 2015). Whether *Ranavirus* and/or Bd are negatively impacting this and other native amphibians in Chile should be further investigated. All *Ranavirus*-positive cases were restricted to the invasive distribution of *X. laevis,* and all Bd- positive *X. laevis* showed low to moderate levels of infection. Our results are consistent with a reservoir role of *X. laevis* for *Ranavirus* and Bd in Chile; however, additional field and laboratory analyses are required to verify this.

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Compliance with Ethical Standards

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

Ethical Approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Collection of wild amphibians was approved under the permits of the Chilean Agriculture and Livestock Service (N°7993/2010, 300/2012, 5666/2013) and the Universidad Andres Bello Bioethical Committee (1939/2012 and 19/2013), and followed the Bioethic Guidelines of the Comisión Nacional de Investigación Científica y Tecnologica de Chile (CONICYT 2009).

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