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Newly Discovered Sites and Potential Threats for the Critically Endangered Frog, *Alsodes pehuenche*, in Southern South America

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Abstract.—**The Pehuenche Spiny-chest Frog (*Alsodes pehuenche*) is a nocturnal aquatic frog that inhabits small streams and wetlands in the Central Andean Cordillera near the border between Argentina and Chile. The International Union for Conservation of Nature (IUCN) classified it as Critically Endangered (CR) due to its small extent of occurrence (EOO) and area of occupancy (AOO), as well as the continuing decline in the number of mature individuals and habitat quality due to road paving, livestock activities, and nonnative predatory fish. From March 2017 to February 2020, we conducted 11 d of research during five field trips to search for tadpoles and adults on the Argentine side of the border. We used information from local communities and satellite imagery to identify potential habitats. We found *A. pehuenche* individuals in 12 of the 26 sampled streams, which we grouped into three populations. These new populations extend the geographic range 6 km to the southeast and 19 km to the northeast. This increased the EOO fivefold, from 95 km^2 to 497.9 km^2. Nevertheless, the AOO remains small at 4.84 km^2. We found individuals positive for Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) in both new and previously known populations. We discovered salmonid fish in streams with and without frogs, and at one location we observed a Brown Trout (*Salmo trutta*) preying on tadpoles. The newly discovered populations face the same threats as those identified for previously known populations, except for road paving, which suggests that rapid responses are necessary to ensure the survival of the species.**

Key Words.—amphibian threats; Andean high-altitude wetland; Argentina; chytrid fungus; endemic frog; new records; Ranita del Pehuenche; Spiny-chest Frog.

Resumen.—**La ranita del Pehuenche *Alsodes pehuenche* es un anfibio acuático de hábitos nocturnos que habita pequeños arroyos y mallines en la Cordillera Central, próximos al límite entre Argentina y Chile. La Unión Internacional para la Conservación de la Naturaleza (UICN) clasificó a la especie como En Peligro Crítico debido a su restringida extensión de ocurrencia y área de ocupación, así como a la disminución continua en la calidad del hábitat y número de individuos maduros debido a la pavimentación de una ruta, presencia de ganado y presencia de peces exóticos. En esta contribución reportamos el descubrimiento de tres nuevas poblaciones en territorio argentino, extendiendo el rango geográfico 6 km al sudeste y 19 km al noreste de las poblaciones conocidas. Encontramos individuos positivos para el hongo quitridio tanto en las nuevas poblaciones como en las previamente conocidas. También registramos truchas en arroyos habitados y no habitados por la especie y en una ocasión observamos una trucha marrón *Salmo trutta* depredando sobre larvas de *A. pehuenche.* Las nuevas poblaciones encontradas enfrentan las mismas amenazas que las poblaciones previamente conocidas, excepto aquellas relacionadas a las obras de pavimentación, lo que implica una necesidad urgente de realizar acciones que aseguren la supervivencia de la especie.**

Palabras clave.—amenazas; anfibio endémico; Argentina; hongo quitridio; nuevos registros; Ranita del Pehuenche; Sapo de pecho espinoso; vegas de altura.

Introduction

The Pehuenche Spiny-chest Frog (*Alsodes pehuenche*; also called *Ranita del Pehuenche* or Pehuenche's Frog) is a medium-sized nocturnal frog (the mean snout-vent length in adults is 54.51 ± 4.07 [standard deviation] mm; Corbalán et al. 2014) that inhabits small streams and wetlands in the Central Andean Cordillera, on both sides of the border between Argentina and Chile (Fig. 1). The Argentine Herpetological Association categorized this species as Endangered (*En Peligro*), the maximum threat level for amphibians in Argentina (Vaira et al. 2012). In 2013, the International Union for Conservation of Nature (IUCN) listed it as Critically Endangered based on the small, estimated extent of occurrence (EOO) of 9 km^2 and an estimated area of occupancy (AOO) of 5 km^2 (IUCN 2013). At the time, researchers had only identified this frog at a single location, where they tracked a continuous decline in the quality of its stream habitat and in the number of mature individuals (Vaira et al. 2012). The principal threats were the modification of watercourses, as streams dried up where the frogs had lived (Corbalán et al. 2010). The paving of International Road 145 led to high mortality of tadpoles and adults (Corbalán et al. 2010).

Subsequently, Correa et al. (2018) reported new occurrences, primarily in Chile. Prado et al. (2019) also reported the presence of the species in other streams that are near previously known populations in Argentina. Until our study, the only place in Argentina where researchers had documented the species was in snowmelt streams near Paso Pehuenche (Mendoza Province). In Chile, the species also occurs in streams near Paso Pehuenche, tributary streams of the Maule Lake, and the drainage basin of Cajón Lo Aguirre (Lo Aguirre Grande and Lo Aguirre Chico, Maule Region; Corbalán et al. 2010; Correa et al. 2013, 2018). All reported sites (yellow points in Fig. 1) are located near International Road 145 and within a latitudinal band at -36°S, between -70°24' and -70°36', and between 2,215 and 2,600 m elevation. Even though the populations discovered by Correa et al. (2018) increased the EOO to 95 km^2, a 2018 reevaluation still placed the species in the category of Critically Endangered (IUCN 2019). In addition to previously identified threats to the species, others have been reported over the last decade: Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis* [*Bd*]; Ghirardi et al. 2014), the presence of livestock, and predation by non-native salmonid fish such as Rainbow Trout, (*Oncorhynchus mikyss*; IUCN 2019; Kacoliris et al. 2022; Zarco et al. 2021). Climate change is also apparently affecting the population at Paso Pehuenche because reduced winter snowfall means less water in streams where the frogs live. Some streams have dried up completely (Kacoliris et al. 2022).

Our goal was to locate new *A. pehuenche* populations to provide a more complete knowledge of the distribution of the species. Based on information provided by locals and a geospatial analysis of potential habitats, we explored areas much farther from International Road 145 in Argentina. We present new records that increase the EOO of *A. pehuenche* and provide a more realistic

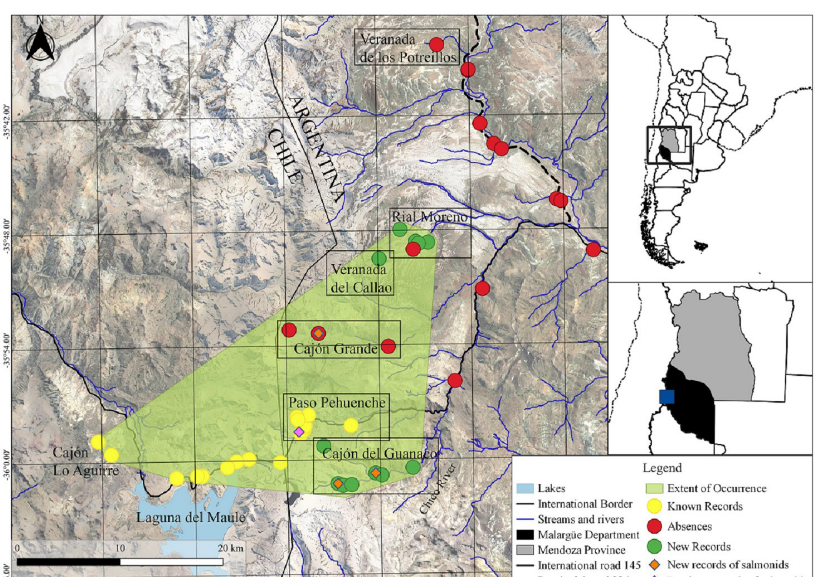

Figure 1. Current distribution of the Pehuenche Spiny-chest Frog (*Alsodes pehuenche*). Known records and sampled locations in this study are shown. New Records refer to sampled sites where the species was found, and Absences refer to sampled sites where the species was not found. The estimated Extent of Occurrence (EOO) and the presence of salmonid fish are indicated. Names and rectangles on map refer to geographic areas. Polygons in upper right map represent provinces. Blue square represents enlarged map.

estimate of AOO. We also report the prevalence of *Bd* in both known and newly discovered populations. We note the presence of salmonid fish in the streams we visited. We believe this information will be useful for re-designing conservation strategies.

Materials and Methods

The study area was located in the central Andes of Argentina (Malargüe Department, Mendoza Province), between -35°38'14.25" and -36°01'33" and between -70°23'48.73" and -70°04'2.51" (Fig. 1). The mean minimum temperature for the coldest month (August) is -6.2° C, whereas the mean maximum temperature for the warmest month (January) is 21.5° C (Ortíz Maldonado 2001). Precipitation in the area is influenced by the position of the westerlies wind belt and the South Pacific semi-permanent anticyclone, with heavy winter snowfalls (Trombotto Liaudat et al. 2014) and annual precipitation of 400–600 mm (Rivera et al. 2018). Snowmelt during the warm season feeds the wetlands (known locally as *mallines* or *vegas*). In these habitats, *A. pehuenche* tadpoles and adults inhabit narrow streams (Corbalán et al. 2008, 2010).

The main source of income for local people is from herding, with mixed herds of goats and cattle. Herders practice transhumance, a cultural practice that involves moving livestock seasonally, from the lowlands during the winter (*invernada*) to the highlands in the summer (*veranada*; Lacoste 2018). The summer sites are high-altitude wetlands (> 2,000 m elevation) where livestock have food and water. Herders stay in a temporary settlement called a rial. We learned of most of the sites we sampled from talking to herders and tour guides. We showed them photographs of the anuran species inhabiting the area, such as *A. pehuenche*, Large Four-eyed Frog (*Pleurodema bufoninum*), and Warty Toad (*Rhinella spinulosa*). We asked if they knew of *A. pehuenche* and where they had seen it. We also used Google Earth Pro (Google LLC, Mountain View, California, USA) to visually identify potential habitats for the species based on the presence of mountain wetlands and streams that can be identified from satellite images taken at an altitude of 10–25 km.

Field methods.—Based on the information above, we planned five trips to the field. We conducted the first trip in March 2017 by driving along Provincial Road 226 and International Road 145 (1 d, two researchers). We made three additional field trips in January 2019: (1) on foot to Cajón del Guanaco following the Guanaco stream to the confluence with the Chico River (3 d, two researchers); (2) on horseback following the Cajón Grande (2 d, three researchers); and (3) on horseback towards the Rial Moreno (and surrounding areas) and the Veranada del Callao (3 d, three researchers). The last trip (2 d, two researchers) was also made on horseback to the wetland Veranada de Potrerillos in February 2020 (Fig. 1). We made all horseback trips with a local guide.

On the trips, we visually inspected all streams and ponds for frogs and salmonids primarily during the daytime. We georeferenced streams that were separated by at least 30 m. In Cajón del Guanaco, the wetland is continuous, so the georeferenced points indicate where the search effort was most intense. We focused on permanent streams because *A. pehuenche* is strictly dependent on water availability (Corbalán et al. 2014). Adults take cover in small holes or tunnels along the edges of streams or stones covered by water during the day (Cei 1980). Tadpoles occupy backwater sections of the streams in densities of 30–80 individuals/m^2 (Corbalán et al. 2008). Their length can be up to 62 mm near metamorphosis (Corbalán et al. 2008). The larval period of *A. pehuenche* is prolonged, involving at least four winters as tadpoles (Corbalán et al. 2014). Because cohorts at different larval stages coexist at the same microsites, it is relatively easy to find tadpoles during the day when the site is inhabited by the species (Corbalán et al. 2008, 2010, 2014). Therefore, we first searched for tadpoles along the watercourse. When we found tadpoles or when herders told us they had previously seen frogs at the site (such as in Veranada del Callao), we searched for adults by putting our hands into holes and tunnels along the edge of the stream. When possible, we searched for adult frogs at night, when individuals are active (submerged or semi-submerged in the water; Correa et al. 2013).

Genetic analyses.—To verify that the frogs were *A. pehuenche*, we sequenced *Cyt b* from 10 individuals from five sites along Cajón del Guanaco and five in the area surrounding Rial Moreno. We followed the sampling procedure in Correa et al. (2018). For adults, we obtained buccal mucosa using 516CS01 flocked swabs (Copan, Murrieta, California, USA), which we immediately dried with silica gel. We selected tadpoles with a snout-vent length > 25 mm. We anesthetized tadpoles with 0.2 ml of topical benzocaine 10% and then excised a small piece of the end of the tail fin (3 × 3 mm, approximately) and preserved it in ethanol (96%). Finally, we submerged tadpoles in boxes with fresh stream water until they recovered mobility and then released them.

We extracted whole cellular DNA from tissues following the salting out technique (Miller et al. 1988). Using the Polymerase Chain Reaction (PCR), we amplified a fragment of the mitochondrial cytochrome *b* gene using the primers CytbDen3-LForward AAYATYTCCRYATGATGRAAYTTYGG and CytbDen1-H Reverse GCRAANAGRAAGTAT-

CATTCNGGYTTRAT (Santos and Cannatella 2011). The thermal profile of the reaction was an initial denaturation at 95º C for 2 min, followed by 30 cycles of amplification (95º C for 30 s, 50º C for 45 s, and 72º C for 30 s), with a final extension at 72º C for 2 min. We purified PCR products and sequenced them in both directions in an ABI3730XL automatic sequencer (Macrogen Inc., Seoul, South Korea). We deposited the sequences in GenBank under the accession numbers OP661156–OP661165.

Estimated area of occupancy.—Once we confirmed the identity of *A. pehuenche*, we recalculated the total AOO of the species, including all records for Chile and Argentina from previous research and this paper. The AOO is an area within the EOO that is actually occupied by a taxon, excluding vagrancy (IUCN 2012). This reflects the fact that a taxon often does not occur throughout its EOO, which may contain unsuitable or unoccupied habitats. We reviewed Sentinel-2 satellite images with a 10 × 10 m resolution (Copernicus Sentinel-2 mission, European Space Agency, Paris, France). We used a mosaic image with the best quality pixels from spring-summer months, November 2021-March 2022. We conducted imagery processing with Google Earth Engine (GEE; Google LLC1600 Amphitheatre Parkway, Mountain View, California, USA). To estimate the extent of high-altitude wetlands, we used the Enhanced Vegetation Index (EVI):

$$EVI = 2.5 \times (NIR - RED) / (NIR + 6 \times RED - 7.5 \times BLUE + 1)$$

where 2.5 and 1 are correction values, NIR is the shortwave infrared band, and RED and BLUE are the visible blue and red bands (Phiri et al. 2020). We filtered the resulting layer with a threshold value of 0.35 and converted it from raster to vector format. Then, we measured the AOO by intersecting a 50-m buffer of all records of *A. pehuenche*, a 50-m buffer of the permanent streams (Instituto Geográfico Nacional de Argentina and Google Earth Pro), and the high-altitude wetlands layer. Subsequently, we calculated the EOO area as the shortest continuous boundary that encompasses all known, inferred, or projected sites of the present occurrence of a taxon excluding cases of vagrancy (IUCN 2012). Then, this area is represented by the Minimum Convex Polygon (Jenrich and Turner 1969) that includes the estimated AOO of all populations.

Disease determination.—We sampled 37 adult frogs for *Bd* (six from Veranada del Callao, one from Rial Moreno, and 30 from the known population near Paso Pehuenche, Argentina). First, we checked for clinical signs of disease (erythema on the ventral surface, abnormal posture such as splayed limbs, slow righting reflex, abnormal skin shedding or ulceration, or tetanic spasms upon handling (Berger et al. 2009; Berger and Skerratt 2012). Next, we gently but firmly swabbed the body, especially on the ventral surface, hind limbs, and interdigital membrane, with a 155C plain swab (Copan, Murrieta, California, USA; Livo 2004; Hyatt et al. 2007). We stored the swabs in numbered plastic cryogenic vials with ethanol (96 %) and kept them refrigerated until DNA extraction. We used Qiagen's DNeasy kit and followed the Animal Tissues protocol and the modifications proposed by Kosch and Summers (2013). We used real-time PCR to determine the presence and quantification of *Bd* load using a StepOnePlus thermal cycler (Applied Biosystems, Foster City, California, USA) following the standard protocol (Boyle et al. 2004). We ran duplicates for all the samples. The thermal profiling of the reaction was 95° C for 20 s, 50 cycles at 90° C for 1 s, and finally, 60° C for 20 s. We used StepOne v2.3 software (Applied Biosystems) to estimate *Bd* DNA loads in zoospores equivalents (ZE) from the amplification curves (Boyle et al. 2004). We considered an individual *Bd* positive when at least one sample resulted in a DNA amplification. After sampling, we released all individuals at the exact capture site.

Results

We found *A. pehuenche* individuals at 12 of the 26 georeferenced points investigated: seven points along the Cajón del Guanaco, one point at Veranada del Callo, and four points at and around Rial Moreno (Figs. 1 and 2). We only found adults (no tadpoles) in the diurnal sampling at Veranada del Callao (2,825 m elevation). We found adults when we actively searched potential hiding places. One researcher captured six individuals within an hour. At and around Rial Moreno (2,616 m elevation), we performed a sampling at night. At the northern point of the distribution, three researchers found 26 adults, four juveniles, and tadpoles within an hour. Along Cajón del Guanaco, we found *A. pehuenche* tadpoles and adults as well as salmonids in two streams. We also observed a Brown Trout (*Salmo trutta*) preying on an *A. pehuenche* tadpole. We found another stream with salmonids in Cajón Grande. Here, we did not observe any frogs (Fig. 1), nor in the streams and wetlands near roads that we sampled in 2017, nor in the wetland Veranada de Potrerillos.

We compared *Cyt b* sequences from individuals sampled at Cajón del Guanaco and Rial Moreno to those reported by Blotto et al. (2013), which are available in GenBank. This comparison confirmed that the frogs we sampled are *A. pehuenche*. Our samples were identical (100%) to the topotypic specimens of the species (GenBank accession JX203962–64).

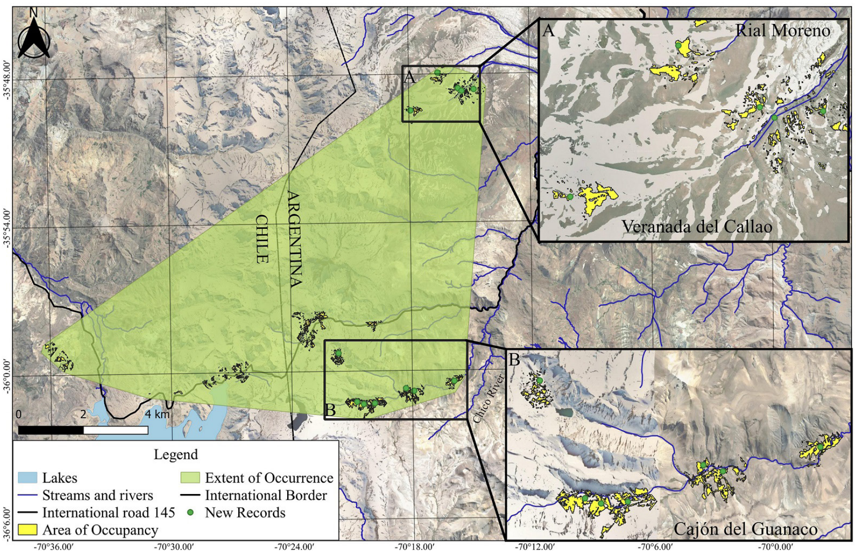

Figure 2. Extent of Occurrence (EOO) and Area of Occupancy (AOO) of Pehuenche Spiny-chest Frog (*Alsodes pehuenche*).

The new populations extend the distribution 6 km to the southeast and 19 km to the northeast of the previously documented population in Argentina, which increases the EOO to 497.9 km^2. These new sites also move the lower elevation limit of the frog down to 2,150 m, with an upper elevation limit of 2,825 m. We calculated the AOO as 4.84 km^2 (Fig. 2).

We detected *Bd* in six of the 37 sampled individuals, i.e., a prevalence of 16%. The individuals who tested positive were from Veranada del Callao (3/6, 50%) and Paso Pehuenche (3/30, 10%). In all cases, the load of *Bd* was lower than one zoospore equivalent. There were no clinical signs of chytridiomycosis at the time of sampling (Fig. 3).

Discussion

The streams inhabited by *A. pehuenche* are surrounded by wetlands (Corbalán et al. 2008). Some of these areas are connected by water, forming a continuous landscape where individuals can come into contact. In other cases, streams are distant from one another (> 10 km) or they originate at different points and flow away from each other, preventing contact among frogs. Taking this into account, we identified three new unconnected populations of *A. pehuenche*: (1) along Cajón del Guanaco; (2) at Veranada del Callao; and (3) at and near Rial Moreno.

The information provided in this study is valuable for conservation because the new populations increase the EOO fivefold, from 95 km^2 (IUCN 2019) to 497.9 km^2. Despite this substantial increase, the extent of wetlands within the EOO is limited, meaning that the available habitat for the species remains small. We estimated AOO at only 4.84 km^2. This is similar to the initial estimate (5 km^2; IUCN 2013) and smaller than the most recent: 32 km^2 (IUCN 2019). We feel our estimate is more accurate because we used precise imagery with 10 × 10 m cells, whereas the previous estimate was based on lower-resolution images with 4 × 4 km cells. Larger cell size may be appropriate for some taxa but could overestimate areas for taxa that live in specific microhabitats, such as these frogs. Although we

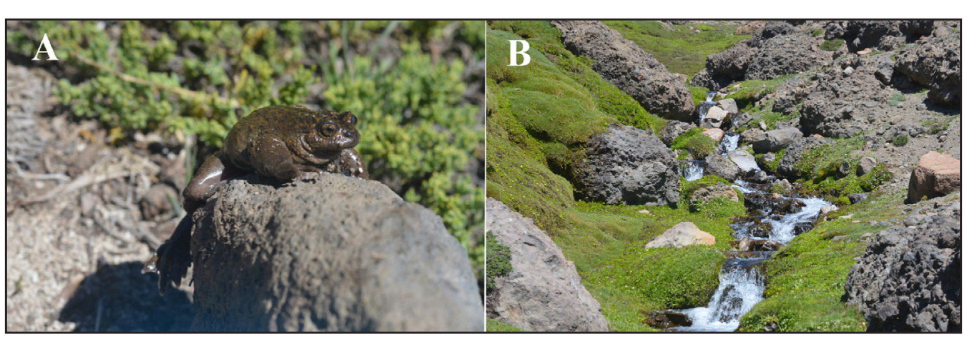

Figure 3. (A) Adult male Pehuenche Spiny-chest Frog (*Alsodes pehuenche*) sampled for *Bd* at Veranada del Callao (*Bd*-positive). (B) Habitat of *A. pehuenche* at Veranada del Callao. (Photographed by Valeria Corbalán).

Figure 4. Livestock as potential threat for the Pehuenche Spiny-chest Frog (*Alsodes pehuenche*). (A) Breeding pond at Paso Pehuenche, Argentina, in 2008. (B) The same pond after recent impact by livestock. Since 2016 no frogs have been found at this place. (Photographed by Valeria Corbalán).

did not measure habitat variables, the new sites we found were very similar to previously documented habitats. That is, new occurrences do not necessarily mean that there is a wider range of environmental conditions that the species can withstand.

The information provided by tour guides and herders was essential for locating new populations. Guides notice tadpoles when they take their horses to drink at streams, and herders identify adults when they channel streams for watering the cattle. Tour guides and herders were both able to distinguish temporary and permanent streams, which helped us focus our sampling efforts. Given the logistical effort involved in sampling remote sites, we recommend dialogue with local people to find new populations. The involvement of local inhabitants is also necessary when implementing conservation strategies, especially if their activities, i.e., herding livestock and the introduction of salmonids for fly fishing, could affect conservation efforts. Conservation actions involving local herders could include, for example, the development of watering stations outside of natural water courses and wetlands to keep cattle out of the area occupied by frogs.

Significantly, the new populations are not threatened by road paving, which is the main threat for the only other population in Argentina, near Paso Pehuenche. The species is also studied by Chilean researchers, who do not report negative effects on *A. pehuenche* due to paving or nonnative salmonids. On the other hand, the Paso Pehuenche population has repeatedly and abruptly declined since paving began (Corbalán et al. 2010; IUCN 2019). This is because widening the road changed water courses, in addition to the construction of a curb and roadside drains that caused the deaths of many individuals due to falling and dehydration (IUCN 2019). Therefore, the discovery of new populations far from International Road 145 enhances chances for survival of this species. Around Rial Moreno, we found 26 individuals/h, similar to the rate reported in Chile (Correa et al. 2013).

We detected three potential threats and should be taken into account for the management of the species: nonnative salmonids, the fungus *Bd*, and livestock. A recent study carried out by Kacoliris et al. (2022) revealed that invasive nonnative species and diseases are the main causes of declines in amphibian populations in Argentina and Chile, affecting 66% of known populations. *Oncorhynchus mikyss* severely affects the occupancy of the native El Rincon Stream Frog (*Pleurodema somuncurense*) and the Common Toad (*Rhinella arenarum*; Velasco et al. 2018). Arroyo Pehuenche has seen a recent illegal introduction of *O. mikyss*, perhaps because fly fishing is increasingly popular there, according to a recent study by Zarco et al. (2021). These authors note the presence of *O. mikyss* in a tributary of Arroyo Pehuenche that is also inhabited by *A. pehuenche* near Paso Pehuenche, as well as frog predation. Hence this nonnative fish is a real threat to the endangered frogs. We also observed predation by *S. trutta* on a tadpole in Cajón del Guanaco. The absence of frogs in Cajón Grande, where salmonids were recorded, may be because these fish have prevented the establishment of frog populations, which happens with other amphibian species (Velasco et al. 2018). If *ex-situ* frog breeding is used as a future conservation strategy, salmonids should be completely removed before frogs are released. Moreover, the introduction of salmonids should be banned.

Bd-related mortality events were reported in Argentina for the Patagonia Frog (*Atelognathus patagonicus*), the Atacama Water Frog (*Telmatobius atacamensis*), and Pisano's Frog (*T. pisanoi*; Fox et al. 2006; Barrionuevo and Mangione 2006; Kacoliris et al. 2022). Fortunately, the absence of clinical symptoms and a low load of *Bd* infection, which is far below the threshold of 10,000 ZE that is seen in mass mortalities (Vredenburg et al. 2010), suggest a low susceptibility of *A. pehuenche* to this fungus. Our data are important, however, because climate fluctuations may cross thresholds for certain pathogens, and trigger outbreaks (Pounds et al. 2006;

Ma et al. 2019; Semenza and Paz 2021). Some diseases are expected to become more lethal or spread more readily with global warming (Epstein 2001; Harvell et al. 2002; Rodó et al. 2002). Future conservation initiatives should frequently monitor the prevalence of *Bd* and assess the role of livestock movements as a potential dispersal agent of this fungus.

Small-holder grazing, ranching, or farming is the second most common threat associated with declines of amphibians in Argentina and Chile (Kacoliris et al. 2022). The area around Paso Pehuenche is impacted by domestic cows and goats, which walk through swamps and small ponds (Corbalán et al. 2010; Kacoliris et al. 2022). In fact, surveys conducted in January 2016 showed that frogs were no longer living in previously occupied ponds, probably due to trampling and defecation by livestock (IUCN 2019; Fig. 4). The newly discovered populations, especially at Cajón del Guanaco, are also exposed to trampling by cows. This situation could motivate future studies to evaluate the magnitude of impacts on *A. pehuenche.*

The evidence presented here indicates that despite the discovery of new populations, the AOO remains small. Moreover, the available habitat is highly fragmented, and the extent and quality of the habitat near Paso Pehuenche population continues to decline. Over the last decade, efforts to reverse the damage, such as restoring original water courses or creating wildlife crossings have failed because no support has been provided. If nothing is done in a short time, it is probable that this frog population will continue to decline. In addition, the presence of salmonids, *Bd*, and livestock near most (if not all) Argentine populations of *A. pehuenche* may threaten the survival of the species. Until the reversal of these threats is guaranteed, we suggest that the species continue to be categorized as Critically Endangered, as defined by the IUCN.

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