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Back Home: Strategies for the Reintroduction of *Chelonoidis chilensis* Into the Wild

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Abstract.—**The Argentinian Tortoise (*Chelonoidis chilensis*) is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora and classified as Vulnerable by the International Union for the Conservation of Nature; nevertheless, they are illegally traded as pets in Argentina. Many tortoise owners who kept them as pets gave their tortoises to *Protectora Rosario*, which sent them to a Wildlife Rescue Center in Santa Fe, Argentina. Our goals were to clinically and behaviorally evaluate these tortoises to analyze the feasibility of their release; determine the genetic population of origin of each specimen by identifying its mitochondrial haplotype; and generate educational actions to prevent the commercialization of this species. The genetic analysis identified six haplotypes, four corresponded to the Dry Chaco region, one with the Monte region, and two were new haplotypes for the species. Clinical and behavioral evaluations after a quarantine process showed that the tortoises were suitable to be reintroduced into the wild because they were disease-free, they showed a correct usage of their microhabitat, reacted to environmental stimuli, and had independence from humans for their food. Monitoring showed that the reintroduction of the tortoises was successful: we relocated 23 of the 28 released tortoises during the 6 mo after their release and none showed signs of disease or damage. We suggest that the evaluation methods and genetic analysis we applied in this study can be used as a baseline for the establishment of standardized protocols to apply in reintroduction programs for other tortoises.**

Key Words.—clinical and ethological diagnosis; genetic-molecular analysis; geographic origin; mascotism; release; Testudinidae; wildlife trafficking

Introduction

The Argentine Tortoise (*Chelonoidis chilensis*; Fig. 1) is distributed from the Chaco region in Bolivia, Paraguay, and Argentina to the north of Patagonia. Its distribution is limited mainly by the annual average temperature, the thermal amplitude, the maximum temperature, and summer precipitation (Richard 1999). Significant declines in tortoise populations in Argentina have been observed in recent decades. Currently, estimated densities are < 1 tortoise/ha in Salta, and 3/ha in Córdoba. These data contrast with the density estimates of 30 tortoise/ha that were found in Córdoba during the 1960s (Prado et al. 2012). Illegal trade is a major cause of decline in turtle populations (Prado et al. 2012).

Figure 1. An Argentinian Tortoise (*Chelonoidis chilensis*) from Villa Minetti, Santa Fe, Argentina. (Photographed by Pablo Siroski).

As with other species from other parts of the world, *Chelonoidis chilensis* has become a much sought-after pet due to its adaptability to captive conditions, gentile nature, and ability to live in small spaces (Richard 1999), and approximately 10,000 tortoises are sold per year (https://sib.gob.ar/portada). Access to this species has been greatly facilitated by the destruction and fragmentation of the Chaco Forest in central and northern Argentina. This is largely due to the expansion of the agricultural-livestock frontier, which has dramatically decreased the natural habitat of these chelonians (Prado et al. 2012). The species is protected by the National Fauna Law No. 22421. There also is a law (S-0754/17) that declares this species as a natural monument (in the terms of Article 8 of Law No. 22351), and prohibits the commercialization, hunting, or intentional capture throughout its distribution (Prado et al. 2012). Legislation in the Santa Fe Province (Law No. 4830) also prohibits its commercialization, possession, and transfer throughout national and provincial territories. *Chelonoidis chilensis* has been considered threatened since 2012 according to the Argentine Herpetological Association and has been categorized as Vulnerable by the Secretaría de Ambiente y Desarrollo Sustentable since 2013. This classification is based on the fact that this species has an extensive and relatively continuous distribution, and their populations are protected in various areas within provincial and national territories (https://sib.gob.ar/portada). Internationally, *C. chilensis* has been included in Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; https://cites.org/sites/default/files/document/1997.pdf) since 1997, which allows the regulated trade of its products, and it is classified as Vulnerable on the Red List of the International Union for the Conservation of Nature (IUCN; Prado et al. 2012). Despite the fact that national and international organizations could allow it under regulated programs, currently in Argentina, there are no management programs for this species. Therefore, all the tortoises that are currently marketed in Argentina come from illegal trafficking.

An additional issue that contributes to the declines of *C. chilensis* is the low survival of nests and juveniles in the wild (Richard 1999). Clutches are especially vulnerable due to the long incubation period they require, which ranges from 13–16 mo. During this long incubation time, embryos can die from several different causes, including water stress, fungal attacks, and predators (Waller et al. 1989). Collectively, all of factors cause low abundance in most of the localities that constitute its habitat, apparent absence of populations in many of the previously studied localities (Sánchez et al. 2014) and narrowing and loss of quality of its habitat, mainly in the Dry Chaco region (Biasatti 2016; Gatica et al. 2021).

Furthermore, the taxonomic classification of *C. chilensis* is still unclear. Gray (1870) proposed that in the southern region of its distribution in South America there was only one species of tortoise, *Chelonoidis chilensis*. Later, Freiberg (1973) determined that there were large phenotypic differences between tortoises belonging to populations in Chaco and Patagonia, in Argentina, which led him to consider them as different species. He also added other species within the Chaco region: *C. petersi* and *C. donosobarrosi*, neither of which was given a specific common name different from *C. chilensis*. Subsequently, Fernández (1988) proposed that there were only two species in the Chaco region, *C. petersi*, with two morphological varieties (one with a light-colored carapace and the other with a dark brown carapace), and in the Monte region, *C. chilensis* (synonym of *C. donosobarrosi*). More recent molecular studies suggest that tortoises in Argentina correspond to a single species (Fritz et al. 2012; Sánchez 2012; Sánchez et al. 2017). The study performed by Sanchez (2012) is a comprehensive analysis of the distribution of mitochondrial haplotypes (cytochrome b gene) in samples of tortoises from most of their range in Argentina, where the author concluded that the genetic variability observed corresponds to a single species: *C. chilensis* (Gray 1870), which presents great phenotypic variability. These data corroborated the hypothesis proposed by Fritz et al. (2012), which was based on the study of a smaller number of samples. In addition, the mitochondrial DNA analysis established two haplogroups in *C. chilensis*, one corresponding to the tortoises of the Monte region (plains and plateaus), and the others to the Dry Chaco region (Fig. 2).

Currently, there is an increasing tendency to reintroduce rescued animals recovered from illegal wildlife trafficking. There are recommendations provided by national and international organizations

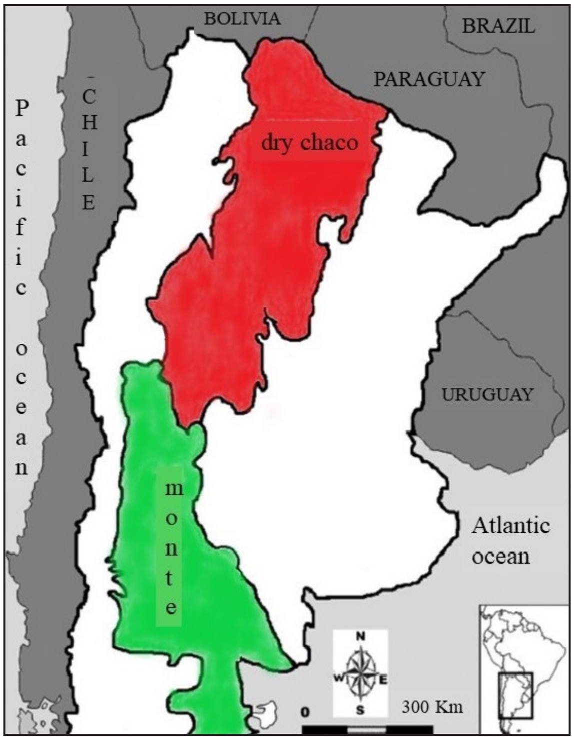

Figure 2. Haplotype distribution of Argentinian Tortoises (*Chelonoidis chilensis*) in Argentina: Dry Chaco region (red), Monte region (green). (Modified from Sánchez et al. 2014).

that detail guidelines for planning actions to reintroduce wild animals into natural habitats. For example, the IUCN (2020) established that the assessment of the feasibility of the reintroduction of the animals should cover the entire spectrum of relevant biological and non-biological factors, and that it is extremely important to have behavioral, clinical, and genetic information on the animals to be reintroduced, as well as on the recipient populations. The release of an animal whose condition is unknown or doubtful can affect natural populations with consequences that are difficult to assess. Therefore, to make a final decision about the release of an animal, a rigorous and conservative framework must be adopted that tends to protect the species above all things and prioritize the populations above the individuals (Aprile and Bertonatti 1996). Espunyes Nozières (2011) proposed a framework to develop recovery and conservation plans for species considered endangered and vulnerable. They suggest that strategies must consider, mainly: (1) identification of the species and their threats; (2) delimitation of the geographical scope of application; (3) guiding criteria on the compatibility between the requirements of the species and the uses and exploitation of the land; and (4) recommended actions to eliminate or mitigate the effect of the identified limiting or threat factors, and evaluation of the actions carried out.

In this context, in 2016, the non-Governmental Organization (NGO) called *Protectora Rosario* (PJ 130/12) of Rosario city, Argentina, started an awareness campaign under the slogan The Tortoise is Not a Pet inviting all those people who had tortoises as pets in their homes to leave them, voluntarily, at the NGO facilities to be released to their natural habitat. The NGO received 33 tortoises, then, NGO members contacted environmental government authorities to coordinate the continuity of the program. An interdisciplinary group of specialists from government environmental areas (Ministerio de la Producción, Ciencia y Tecnología, and Ministerio de Ambiente y Cambio Climático of Santa Fe Province), from the Center for the Reception and Rehabilitation of Wildlife known as La Esmeralda of Santa Fe city, from the Universidad Nacional del Litoral, and from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), was established to evaluate the possibility of reintroducing tortoises into the wild. La Esmeralda is a government institution that receives animals belonging to different wild species from seizures, complaints, and voluntary deliveries, to recover them, evaluate their reintroduction, and find, where it is possible, potential release sites according to the characteristics of the species and its area of distribution.

The IUCN proposes that the reintroduction of individuals into the wild must be carried out: (1) in an area where the species is native within its historical distribution; (2) that it can be guaranteed, at least in principle, that the place of release has the conditions necessary for the survival and development of the species; and (3) that the threats, beyond the natural ones, are minimal or non-existent. The justification for reintroducing animals must be rigorously determined and the feasibility of reintroduction evaluated by balancing the benefits and risks for conservation. Frequently, the release of an animal back into nature triggers arduous discussions about whether or not this action should be taken. This reflects the widespread mistrust that still exists about reintroduction activities because many releases have been carried out without taking appropriate precautions. For these reasons, sanitary and biological controls must be strict to avoid negative impacts on wild populations (Weeks et al. 2011) and demonstrate that it is possible to develop an effective re-introduction project (Aprile and Bertonatti 1996; Soorae 2013; Barongi et al. 2015). In this context, the objectives of our study were: (1) to evaluate tortoises clinically and behaviorally to analyze their feasibility for release; (2) determine the genetic population of origin of each tortoise, by identifying its mitochondrial haplotype for its potential reintroduction into the nature; and (3) generate educational outreach to prevent hunting and commercialization of this species.

Materials and Methods

The staff of the government environmental areas transferred 33 tortoises from the NGO facilities to the La Esmeralda Center for Reception and Rehabilitation of Wildlife, in Santa Fe city. We transported the animals in container boxes, taking into account their sizes and maintaining safe conditions to ensure their welfare. Then, following internal protocols for the reception and handling of the animals that arrived at the La Esmeralda Center, we determined the sex and the approximate age of the tortoises, took morphometric measurements, and evaluated their general health status through a complete clinical review. We determined the sex of each individual using morphometry and based on sexual dimorphism in the curvature of the carapace and plastron (Pritchard and Trebbau 1984). We separated tortoises according to their size and characteristics and placed them in pens for quarantine.

We enriched the facilities by providing physical and mental stimulation, encouraging species-typical behaviors that allow animals to have more control over their environment (Frederick 2016). The physical enrichment consisted of shelters, barriers, and hiding places to generate complex environments, and to maintain a better physical condition based on the natural history of the species (Ferrie et al. 2014). We added areas for a retreat (mounds, logs, etc.) and visual barriers (clumps of grass, large stones, etc.) to reduce stress and competition among animals (Ousterhout and Semlitsch 2016), allowing them to choose the best conditions. We used additional enrichment to create vertical and horizontal spaces to achieve greater motor stimulation (Frederick 2016) and different types of substrates (water, sand, wet paper, etc.).

Clinical evaluation.—The quarantine process followed the guidelines of Resolution No. 731/11 of the National Service for Animal Health and Food Quality (Servicio Nacional de Sanidad y Calidad Agroalimentaria 2011). This period began with the identification of individuals by a combination of marks on the dorsal plates (Nagle et al. 2017). The assessment of the health status of the tortoises included a clinical evaluation, through physical handling. We carry out an inspection and palpation of each animal, in addition to the typical diagnostic procedures: (1) radiology; (2) complete blood biochemical studies; (3) blood smears (to test blood parasites); (4) flotation and sedimentation techniques to test gastrointestinal parasites according to the methods of Satorhelyi and Sreter (1993); (5) gram stain of fecal matter; nostrils, conjunctival and cloacal swabs for cytology and bacteriological cultures (Jacobson, 2007); and (6) two serum samples from each animal separated by two weeks to test Herpesvirus (Chitty and Raftery 2013). If we found health problems, we applied treatments. For vitamin A deficiency, we used intracelomic injectable vitamin A (IC) 5,000 IU, repeated at 14 d. For the treatment of endoparasites, we used Albendazole (10%) 50 mg/kg, repeated at 30 d post coprology positive. We used Fipronil + Methoprene spray to eliminate ticks, and for the treatment of *Salmonella* sp. and *Escherichia coli*, we used Enrofloxacin (5%) 5 mg/kg every 24 h for 10 d. To treat *Pseudomonas aeruginosa*, we used Ceftazidine at a rate of 20 mg/kg intramuscularly (IM) every 48 h, making a total of three applications.

Behavioral evaluation.—We perform all behavioral assessments non-invasively to promote animal welfare. The evaluation consisted of several tests including individual ethograms, group behavioral observations to assess alertness, rest, agonism, refuge, locomotion, courtship, copulation and eating habits in the enclosures. We performed the characterization of the use of space through an index of dispersion, which is the ratio between the variance between number of individuals occurring per sampling unit (s2) and the average number of individuals in randomly sampled units of area within the landscape (Fath 2019). We provided a balanced diet, consisting of 80% vegetables (zucchini, carrots, etc.), 18% leafy vegetables, and 2% fruits (Pellett et al. 2015), to allow the nutritional enrichment of the animals. To make the diet, we substituted commercial fruits and vegetables with wild vegetables with equal nutritional value (i.e., cactus, *Opuntia* sp., dandelions, *Taraxacum* sp., purslane, *Portulaca* sp., etc.) whose fruits and flowers generate visual stimuli that promote sensory enrichment in the tortoises.

Genetic analysis.—To carry out genetic analyses, we obtained blood samples by puncturing the subcarapacial vein, and we conserved the blood in a lysis buffer (Longmire et al. 1988). We processed the samples in the laboratory to extract DNA following protocols developed by our working group (Amavet et al. 2012). We carried out the PCR amplification of the cytochrome b region using primers developed by Sánchez (2012): Cyt b A (5´-TTACGAAAAACCCACCCAAT-3´) and Cyt b H (5´-GGTTTACAAGACCAATGCTT-3´), which allow amplification of the entire gene. We developed PCR reactions applying our own protocols (Amavet et al., 2017), and PCR products were sequenced in an Applied Biosystems 3730 XL automatic sequencer at Macrogen Inc., Seoul, Korea.

The obtained sequences were identified as *Chelonoidis chilensis* cytochrome b sequences by BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI (Genbank). We aligned the sequences using the ClustalW algorithm implemented in MAFFT online service (Katoh et al. 2019) and edited them using Mega 7.0 software (Kumar et al. 2016). From the 33 sequences obtained, we estimated the number of haplotypes (*n*), diversity of haplotypes (*h*), number of segregating sites between sequences (S), and nucleotide diversity (π) using the DnaSP 5 program (Librado and Rozas 2009). Based on a matrix that included the sequences obtained (33) plus the 21 haplotypes described by Sánchez (2012), we built a haplotype network using POPART (Leigh and Bryant 2015), using the TCS option.

Evaluation of release sites.—We evaluated different sites for the release of the tortoise specimens. The evaluation considered recent records of populations of *C. chilensis* using surveys of local people. From these surveys we identified populations of tortoises in the rural area near Villa Minetti city, in the northwest of Santa Fe Province. We evaluated different areas for release, taking into account the environmental characteristics including: (1) presence of native vegetation that ensures availability of food for tortoises; (2) locations at least 5 km away from crops or livestock production; (3) climatic factors that correspond to the Dry Chaco region (low rainfall, high average temperatures in summer); and (4) sites with little modification or anthropic intervention. Once we selected sites for release, we had meetings with local authorities where we informed them about the reintroduction and gave them posters for educational purposes. Prior to release, we verified the identification of each released tortoise, and we also explained the identification system to the property owners for subsequent monitoring.

Educational component.—We also started an educational campaign prior to release, to enhance public knowledge on the prohibitions on keeping *C. chilensis* as pets and marketing their specimens in the provincial territory. Our program used graphic materials (Fig. 3). Our campaign was especially focused on the staff of the two educational institutions in Villa Minetti city. We talked with the authorities and teachers, and we explained the conservation problems that the tortoise is going through, and we publicized our program to release the tortoises in that area. They promised to spread these actions by talking with their students using the materials we provided them.

Results

Clinical evaluation.—We evaluated 19 males and 14 female tortoises, and most had signs of being in captivity, showing behaviors conditioned by food, approaching people to be fed. All individuals showed signs compatible with vitamin A deficiency (blepharitis, extreme lethargy, tearing, rhinitis, and anorexia), endoparasite eggs (nematodes, *Strongylus* sp.), and 10% of the animals had ectoparasitic tick, *Amblyoma testudinis*. We isolated two positive samples for *Salmonella* sp. in the blood cultures of the 33 tortoises analyzed. In addition, we found other pathogens, such as *Escherichia coli* and *Pseudomonas aeruginosa* in approximately 70% of the animals. The samples were negative for *Mycoplasma* sp., herpesvirus, and *Pasteurella* sp. The quarantine process ended 120 d after receiving the tortoises, and after verifying the correct recovery of all the animals.

Behavioral evaluation.—Tortoises showed the correct use of the available space and were basking and seeking shade according to environmental conditions. We also observed that their dependence on humans for their food was eliminated. This indicated to us their change in feeding behavior, and that the animals responded positively to the naturalization of the diet.

Figure 3. Educational campaign poster to promote the protection of the Argentinian Tortoise (*Chelonoidis chilensis*). The legend states "Tortoise: it is one of the endangered wild species due to its commercialization in the illegal pet market. Don't be an accomplice. A house does not replace its house. Do not buy or give them away."

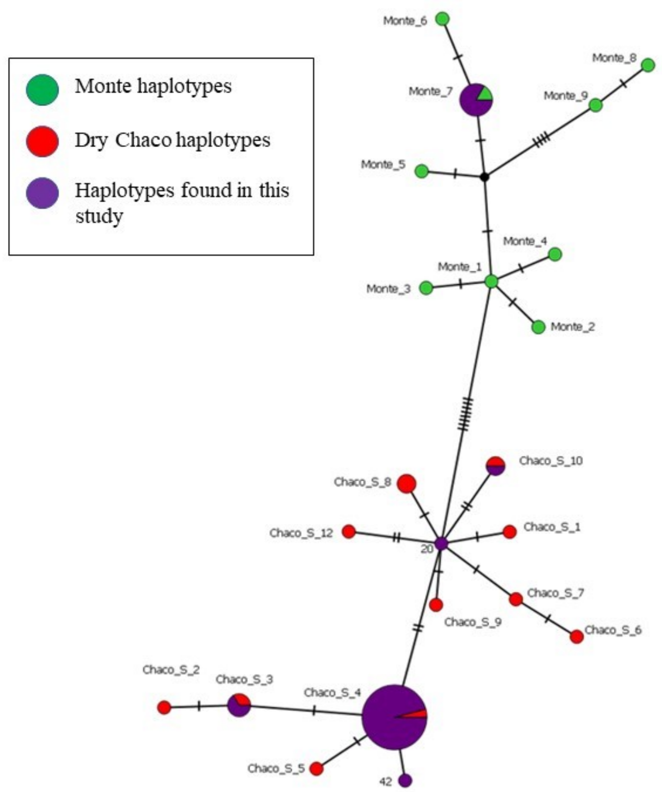

Figure 4. Haplotype network of the Argentinian Tortoise (*Chelonoidis chilensis*) obtained using all the samples under analysis and haplotype sequences from Sanchez (2012).

Genetics.—We obtained 33 good quality sequences of the Cyt b gene, of 1035 bp. From the analysis with the DnaSP program, we determined a value of segregating sites between sequences (S) = 15, number of haplotypes (h) = 6, and haplotype diversity (Hd) = 0.50 with a variance of 0.00951, a standard deviation of 0.098, and nucleotide diversity (π) = 0.00350. Of the six haplotypes we found, four correspond to haplotypes described by Sanchez (2012): three from the Dry Chaco (Chaco_S_3, Chaco_S_4, and Chaco_S_10), one from Monte (Monte_7), and two new haplotypes for the species. Then, of the 33 sequences analyzed in this study, 26 correspond to the Dry Chaco haplogroup, five to the Monte haplogroup, and only two new haplotypes appeared (sequences 20 and 42), which are genetically closed to Dry Chaco haplogroup (Fig. 4). Based on these haplotypes, we decided to release 28 of the 33 tortoises, 26 of which had Dry Chaco haplotypes and two of which had haplotypes closely related to that haplogroup, in an area of the province of Santa Fe that is part of the Dry Chaco region.

Releases.—We transported tortoises from the city of Santa Fe to Villa Minetti. We selected two fields that were part of large cattle ranches but were located far from the areas where cattle were found. We released 12 tortoises in one cattle ranch and 16 in the second ranch, both of which had similar environmental characteristics. Immediately after their release, we recorded the behavior of each tortoise when they came into contact with the natural environment, and we verified that some of them began to feed on the local vegetation. The rest of the tortoises could not be released and remained in the facilities of the Wild Fauna Reception and Rehabilitation Center.

Monitoring.—We designed a monitoring program with land owners and their employees to follow up the behavior and survival of the released tortoises. Through these contacts, which took place once a month, we obtained videos and photos of the animals and were able to identify 23 different live and tagged animals during the 6 mo following release (Table 1). A gradual decrease in the number of observations can be observed over time, specifically for site number 1. We recorded only animals having different identifying marks each month. We did not see any damage (evidence of predator or human attack) on any tortoise.

Education program.—We interviewed the local authorities in the city of Villa Minetti, near the reintroduction site. They reported a change in attitude that

Table 1. Number of Argentinian Tortoises (*Chelonoidis chilensis*) from Villa Minetti, Santa Fe, Argentina, recorded at both release sites each month after release for 6 mo.

Month After Release	Site 1	Site 2
1	4	2
2	3	1
3	2	3
4	2	1
5	1	2
6	1	1

occurred in the local population after our reintroduction. This was determined because there was no income reported by people with the intention of capturing or harming tortoises, as was the case previously. Thus, we consider the results of the educational program to be positive. We believe that the awareness that teachers carried out with their students had an impact in changing the behavior in their families.

Discussion

We developed our pre-release screening protocol based on strategies defined by Espunyes Nozières (2011). These strategies include identification of threats to the species, delimitation of the geographical scope of application, compatibility between the requirements of the species and the uses of the land (due to the fact that there are currently no pristine areas and the releases frequently occur in areas modified or used by anthropic activities), recommended actions to mitigate the effect of the identified threat factors, and follow-up evaluation of the actions carried out. Our work achieved the proposed objectives mainly due to the interdisciplinary nature of our workgroup because it included scientists, academics, and workers from government entities. We had frequent communication with local authorities and educational entities in the areas where we released the animals. This facilitated the development of reintroduction protocols that we consider successful thanks to the contribution of the local inhabitants, who became involved in the conservation and monitoring of the released tortoises. We consider the reintroduction to be successful because we were able to relocate 23 of the 33 released tortoises during 6 mo of monitoring after release, and we did not find any signs of disease or damage in the released tortoises. We believe that the animals, after some time, may have moved to the most protected sites, with denser vegetation within the release area where they would be more difficult to observe during monitoring. The effectiveness of the reintroduction actions carried out will continue to be monitored in the future through field observations, every month, for 2 y to determine if the released animals have adapted to the release site, if there is effective reproduction between them and/or wild animals, and if there is growth in the size of the population. Additional education and monitoring will be continued with the collaboration of the authorities of the city of Villa Minetti.

Because there are no prior examples for the reintroduction of *C. chilensis* in their natural environment that include all of the aspects evaluated in this study, we consider that our data to be valuable to establish standard protocols for the reintroduction of this species. The genetic analytic methods that we used allowed us to identify the population of origin of the release candidates. Further, the genetic sequences obtained in this study increase the pool of genetic data available in Argentina for this poorly studied species.

When tortoises of different species and different origins are housed in the same facility, there is a high risk of disease transmission, especially viral pathogens (Henk Zwartepoorte, unpubl. report). It is essential consider sanitary practices when transferring animals, reintroducing them to captive populations, and handling members of an endangered species (Bertolero 2010). Highly contagious diseases, such as mycoplasmosis, herpesvirosis, pasteurellosis, and salmonellosis have been described in tortoises, and these diseases have the potential to cause high mortality in captive populations (Chitty and Raftery 2013). These diseases are not frequent in free-living animals but could become a potential problem if they were introduced into a wild population. Although the tortoises in our studies tested negative for rhinitis-causing herpes, previous reports in other species in wildlife rescue centers indicated that asymptomatic herpesvirus carriers developed the clinical signs of infection due to stress caused by transport and captivity (Bicknese et al. 2010) or were affected by a herpesvirus that they were not habituated to and that was present in the other species in the collection (Salinas et al. 2011). The accidental introduction of sick animals or carriers of these viruses could have detrimental effects on Argentine tortoise populations, especially when we consider that there is already a history of these problems in reptile species (Jacobson et al. 1985).

In addition to diseases that exclusively affect tortoises, there are other pathogens with zoonotic potential, for example, bacteria such as *Salmonella* sp., *Escherichia coli*, vancomycin-resistant enterococci, *Chlamydophila*, mycobacteria and different parasites such as Pentastomids and *Cryptosporidium* sp. and viruses such as West Nile Virus and various fungi (Mitchell 2011). For this reason, a complete clinical review of the animals is essential, as was carried out in this study, to evaluate the presence of these pathogenic agents, and if they are detected, corresponding treatments must be applied to affected animals. We think the repatriation of an animal can be considered successful when the released animal does not negatively impact natural populations, survives in its habitat, overcomes dependence on man for food, remains free of disease, and eventually has reproductive success (Aprile and Bertonatti 1996). This was verified in the animals reintroduced in the framework of this study because of all the evaluation and planning activities carried out prior to release.

In a framework undoubtedly surrounded by innumerable difficulties, the example described here can provide a stimulus to increase the cases of reintroduction, although this must be decided in a rational and consensual manner. In most cases of reintroduction, it is evident that the success is not only verified by improvements in the conservation of biodiversity, but also in the well-being of the communities directly related to the natural resources. We consider that the methods used in the diagnosis and evaluation of these animals generate a baseline for the establishment of standardized protocols to analyze future reintroductions of this species in Argentina and elsewhere within its range.

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