



First molecular characterization of *Toxoplasma gondii* and molecular analysis of *Neospora caninum* in American mink (*Neogale vison*) introduced in Argentina

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ABSTRACT

The American mink (*Neogale vison*) was introduced to Argentina in the 1930s by the fur industry. Its semi-aquatic habits and foraging behavior facilitates its contact with sporulated oocysts and tissue cysts of apicomplexan parasites such as *Toxoplasma gondii* and *Neospora caninum*. Brain samples from 72 American mink specimens were collected in the province of Neuquén, Argentina. For the detection of *T. gondii* DNA, the quantitative PCR (qPCR) technique was performed, samples positive to qPCR were genotyped by mnPCR-RFLP using 10 genetic markers: SAG1, SAG2 (5'3SAG2, altSAG2), SAG3, BTUB, GRA6, C22-8, C29-2, L358, PK1 and Apico markers. In addition, ROP18/ROP5 allelic combination analysis was performed on samples successfully genotyped. A conventional PCR for the detection of *N. caninum* was performed. Fifteen percent (11/72) of the samples had *T. gondii* DNA. A new non-archetypical genotype was characterized, named AmMink01Arg (ToxoDB #347). The ROP18/ROP5 results identified the 3/4 combination of alleles. Phylogenetic analysis evidenced that the genotype ToxoDB #347 is related to non-archetypical genotypes of high gene variability (ToxoDB #15, #17, #283 and #286). No *N. caninum* DNA was detected in any of the samples. The results of the present study confirm *T. gondii* infection in American mink present in northern Patagonia, where they may favor its dissemination and persistence in the environment acting as a new host of *T. gondii* in the invaded community. This is the first molecular report for Argentina and the first genotypic characterization of *T. gondii* in American mink for the American continent.

1. Introduction

The apicomplexan protozoan parasites *Toxoplasma gondii* and *Neospora caninum* are globally distributed and can infect wild and domestic animals and, in the case of *T. gondii*, humans as well. The presence of definitive hosts (DH) favors the dissemination of oocysts in the environment. Domestic and wild felids act as DH for *T. gondii* and in the case

of *N. caninum* the DH are domestic canids and wild species like grey wolves (*Canis lupus*), coyotes (*Canis latrans*) and Australian dingoes (*Canis familiaris dingo*) (Dubey, 2021; Dubey et al., 2017). The infection can be established by vertical or horizontal route. During sporulation in the environment, oocysts become infectious and available to DH and intermediate hosts (IH) by contaminating pastures and water sources (Dubey, 2021; Dubey et al., 2017).

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The American mink (*Neogale vison*) is native to North America and has been introduced in Europe, Asia and South America (Claverie et al., 2023; Fasola et al., 2021). In Argentina American mink were introduced by the fur industry in the 1930s. Deliberate releases and escapes, together with its ability to adapt to different environments and its generalist diet, have allowed mink populations to become established in the province of Buenos Aires and throughout much of Argentinian Patagonia (Claverie et al., 2023; Fasola et al., 2021). It is considered detrimental for native species conservation mainly because it predares terrestrial mammals, fish, birds, crustaceans, amphibians and reptiles. It also has a negative impact on the development of economic activities such as fish farming, poultry production, and bird-watching and fishing tourism (Fasola et al., 2021). In order to counteract this, population control plans are conducted for this species in various regions of introduction.

The semi-aquatic habits and foraging behavior of the American mink facilitates its contact with the sporulated oocysts and tissue cysts of apicomplexan parasites, and for this reason it is recognized as a sentinel species in natural environments (Sepúlveda et al., 2011). However, information on the health risk it poses to other wild and domestic mammals and humans is still limited. Molecular detection of *T. gondii* in brain samples of American mink was higher in free-living individuals from Spain (Ribas et al., 2018), Poland (Sroka et al., 2019) and United Kingdom (Burrells et al., 2013) than in farmed mink from China (Zheng et al., 2016). The genotypes of *T. gondii* reported in American mink are the archetypal type II present in the United Kingdom, characterized by five markers (Burrells et al., 2013), and the archetypal type II (#3) and non-archetypal genotypes (#9) identified in China using 10 markers (Zheng et al., 2016). Neosporosis in this species has been poorly studied, the only report being in Great Britain, where it showed a prevalence of 4.6 % in brain samples analyzed by molecular methods (Bartley et al., 2013). In Argentina there is only one serological report for *T. gondii* (23 %) and *N. caninum* (3 %) in mink and, so far, the presence of both parasites in this invasive species has not been studied by molecular methods (Martino et al., 2017).

The aim of the present study was to detect and molecularly characterize *T. gondii* and *N. caninum* in wild American mink established in Northern Patagonia, Argentina.

2. Materials and methods

2.1. Samples collection

Brain samples from 72 American mink individuals were collected in southern Neuquén province, Argentina, by the Terrestrial Ecology Group of Neuquén (INIBIOMA-CEAN) over a four-year period (2019–2022) (Figure 1; appendix). Data on sex and age category were recorded for each captured animal: 48 adult males, 11 juvenile males, 2 adult females, and 11 juvenile females (see **Supplementary Material**). Individuals were captured using live traps, anaesthetized and subsequently euthanized. None showed signs of disease and only one American mink was found road-killed. Trapping was conducted in riverbanks and wetlands in natural, rural and urbanised areas in close proximity to recreational areas used for swimming, fishing and birdwatching, ranches with livestock and poultry farming, piscicultures, and residential areas where free-ranging domestic and wild canids and felids are frequent. Necropsies and sample collection were performed at the CEAN facilities. Samples were preserved at -20°C and sent to the Laboratory of Immunoparasitology (LAINPA), Faculty of Veterinary Sciences, UNLP, for planned processing and analysis. The field and laboratory protocols were evaluated and approved by the CICUAL (Institutional Committee for the Care and Use of Laboratory Animals) of INIBIOMA (Prot. 2020/028) and the School of Veterinary Sciences, UNLP (Prot.115–5–21 T), and authorised by the regional wildlife agency (Disp. 008/21, Secretaría Desarrollo Territorial y Ambiente, Neuquén).

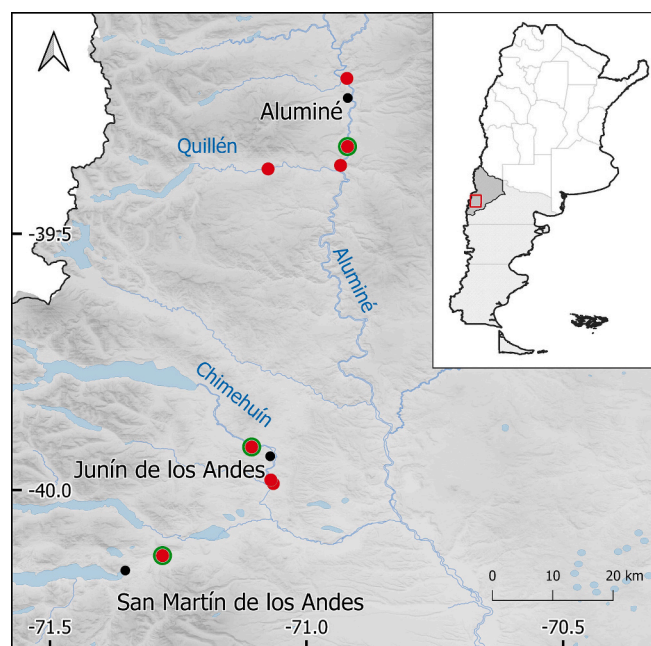


Figure 1. Study area showing the location of sites of collection of American mink (red circles), indicating sites where *Toxoplasma gondii* was detected (green circles). The three cities and towns of this area are indicated (black circles, names in black) together with the rivers sampled (names in blue). The inset shows the study area (red square) in the Neuquén province (dark grey) located in Patagonia (light grey), Argentina. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. DNA extraction

Brains were individually homogenized, a sample of 25 mg was taken from each homogenate and total DNA extraction was performed using a commercial kit, the DNA PuriPrep-T (Inbio Highway, Argentina), according to the manufacturer's instructions. The DNA obtained was stored at -20°C until molecular analysis. An extraction control was used in each round of extraction to ensure no contaminations.

2.3. *Toxoplasma gondii* molecular analysis

For the detection of the specific 529 bp sequence of *T. gondii* DNA, the quantitative PCR (qPCR) technique was performed according to the methodology described by Bier et al. (2019). The cycling protocol includes a step of $50^{\circ}\text{C}/2\text{ min}$, followed by an initial denaturation step of $95^{\circ}\text{C}/10\text{ min}$, and then 45 amplification cycles of $95^{\circ}\text{C}/15\text{ s}$, $58^{\circ}\text{C}/20\text{ s}$, and a final extension of $72^{\circ}\text{C}/30\text{ s}$. For each detection cycle, samples were considered positive if they crossed the initial fluorescence cycle threshold (Ct) number 35. Positive samples to qPCR were genotyped by mnPCR-RFLP using 10 genetic markers: SAG1, SAG2 (5'3'SAG2, alt-SAG2), SAG3, BTUB, GRA6, C22–8, C29–2, L358, PK1 and Apico markers (Pardini et al., 2019). Amplicons were visualized after electrophoresis in 2.5 % agarose gels for all markers with the exception for Apico (3 %) and stained with Ecogel staining solution (Inbio Highway, Argentina). Samples successfully genotyped were analyzed for the ROP18/ROP5 profile. The mnPCR-RFLP was done as described by Bernstein et al. (2024). Simplex external and internal PCRs were run for each locus (ROP18 DEL, ROP18 UPS, and ROP5) to avoid contamination. As in the genotyping procedure, the restriction patterns were visualized in the same manner. Reference strains GT1, PTG, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64 and TgToucan were used as reference controls for both mnPCR-RFLP.

2.4. *Neospora caninum* molecular analysis

The conventional PCR technique for the molecular detection of *N. caninum* was performed using the pair of specific primers Np6+/Np21+ for the amplification of a 337 bp fragment (Campero et al., 2018; Müller et al., 1996). The reaction product was revealed by an electrophoretic run on a 1.5 % agarose gel containing Ecogel staining solution (Inbio Highway, Argentina). The amplified fragments were read with a blue light transilluminator (Safe Imager™, Invitrogen, USA).

2.5. Phylogenetic network analysis

Phylogenetic network was inferred using the software SplitsTree 6.0 CE.0.alpha (Bernstein et al., 2018; Huson and Bryant, 2006). Genotypes used included ToxoDB genotypes from Argentina (Bernstein et al., 2024; Bernstein et al., 2018), reference strains RH (#10), ME49 (#1), VEG (#2) (Khan et al., 2007), and genotyping results from the present study.

2.6. Statistical analysis

Multivariate logistic regression was performed using the categorical variables sex (male/female) and age category (adult/juvenile) to evaluate possible risk factors for *T. gondii* and *N. caninum* infection in American mink. Results were expressed as odds ratios (OR) with their 95 % confidence interval (95 % CI) and corresponding *p*-values. The probability of infection was calculated for each sex and age combination using the ORs obtained. The analysis was performed in Rstudio (R version 2024.12.1.563), using the stats packages for the glm and broom functions (R Core Team, 2025).

3. Results

3.1. *Toxoplasma gondii* molecular analysis

Fifteen percent (11/72; 95 % CI 8.8–25 %) of the analyzed samples were positive for the molecular detection of *T. gondii*. Complete genotyping was only possible in one sample (Ct 24), named AmMink01Arg, which was characterized as a new non-archetypical genotype and designated #347 according to ToxoDB. The ROP18/ROP5 results identified the 3/4 allele combination. All markers are described in Table 1.

3.2. *Neospora caninum* molecular analysis

All brain samples were negative for the molecular detection of *N. caninum* DNA in American mink.

3.3. Phylogenetic network analysis

The similarity of the new genotype of *Toxoplasma gondii*, ToxoDB #347, obtained from an American mink in this study is shown in relation to the phylogenetic network of the genotypes previously reported in Argentina and the reference strains (Figure 2). The ToxoDB #347 genotype clustered with samples of non-archetypical genotypes reported in chickens from Northern Argentina (TgCk14-6Arg (#283) and TgCk14-7Arg (#283) from the province of Misiones, TgCkAr25 (#15) from the province of Entre Rios, and TgCkAr27 (#17) and TgCkAr28 (#17) from an unknown province of Argentina).

3.4. Statistical analysis

Although males and adults were found to be more prone to infection by *T. gondii* than females and juveniles, respectively, no statistically significant differences were found between infection and the variables of sex (*p* = 0.747) and age category (*p* = 0.653). The estimated probabilities of *T. gondii* infection for each group were: 7.7 % for juvenile females, 11.1 % for juvenile males, 11.3 % for adult females, and 16.1 % in adult males. As *N. caninum* infection was not detected in any of the samples analyzed, no statistical analysis was performed in this regard.

4. Discussion

The results of the present study confirm *T. gondii* infection in wild American mink present in Argentina, this being the first molecular report for the country and genotypic characterization of *T. gondii* in this species for the American continent. The molecular prevalence obtained in brain samples (15.2 %) was lower than that detected in wild mink in Poland (25 %) and the United Kingdom (29.2 %), but higher than that observed in wild mink in Spain (9.2 %) and in fur farmed mink in China (8.6 %) (Burrells et al., 2013; Ribas et al., 2018; Sroka et al., 2019; Zheng et al., 2016). Captured mink appeared to be healthy and showed no evident clinical symptoms. Although there has been one report of fatal toxoplasmosis in a juvenile mink in native range (Jones et al., 2006), acute infections in this species have been associated with the occurrence of abortions in farmed mink (Dubey, 2021; Dubey et al., 2020). The organs most frequently parasited by *T. gondii* in chronic infections are brain and heart (Dubey, 2021). Molecular detection in brains of free-living individuals in this study provides new valuable input regarding the sanitary conditions of mink inhabiting Patagonian rivers and ponds.

Exposure to the infecting forms of the studied protozoan parasites, oocysts and tissue cysts, varies considerably according to the living conditions of individuals and their trophic level. The contact of captive American mink with apicomplexan parasites depends on the hygiene of the enclosure provided by the keepers, the type of food supplied and the sanitary control of food and water. On the contrary, individuals in the wild may acquire the infection from the environment and/or their food as different terrestrial-aquatic scenarios are present and mink can consume preys or carrion with tissue cysts or use water bodies contaminated with oocysts. For this reason, the American mink is considered a sentinel species (Dubey et al., 2020; Sepúlveda et al., 2011) since it can reflect the sanitary status of the environment, allowing to detect the presence of apicomplexans such as *T. gondii* and *N. caninum* and their corresponding circulating genotypes.

In turn, characteristics such as sex and age have been evaluated as potential risk factors for *T. gondii* infection in this invasive species. Although the present study shows a tendency for adult males to have a higher probability of infection, the *p*-values obtained (greater than 0.05) from comparisons of the categorical variables age (adult vs. juvenile) and sex (male vs. female) were not statistically significant. The observed trend is consistent with previous findings in free-living American mink in Chile, where no association was found between *T. gondii* seroprevalence and sex or age (Barros et al., 2018; Sepúlveda et al., 2011). In the present study, the tendency may be related to the higher proportion of male and adult individuals captured, despite known differences in movement ranges between male and female American mink, which could influence the likelihood of contact with infectious stages of

Table 1
Toxoplasma gondii genotyping results from brain samples of American mink in Northern Patagonia, Argentina.

Sample	Markers												ToxoDB ID	Genotype
	SAG1	5-SAG2	alt. SAG2	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	Apico	ROP18/ROP5		
AmMink01Arg	II or III	I or II	II	III	III	III	III	I	I	u-1	I	3/4	#347	Non-archetypical

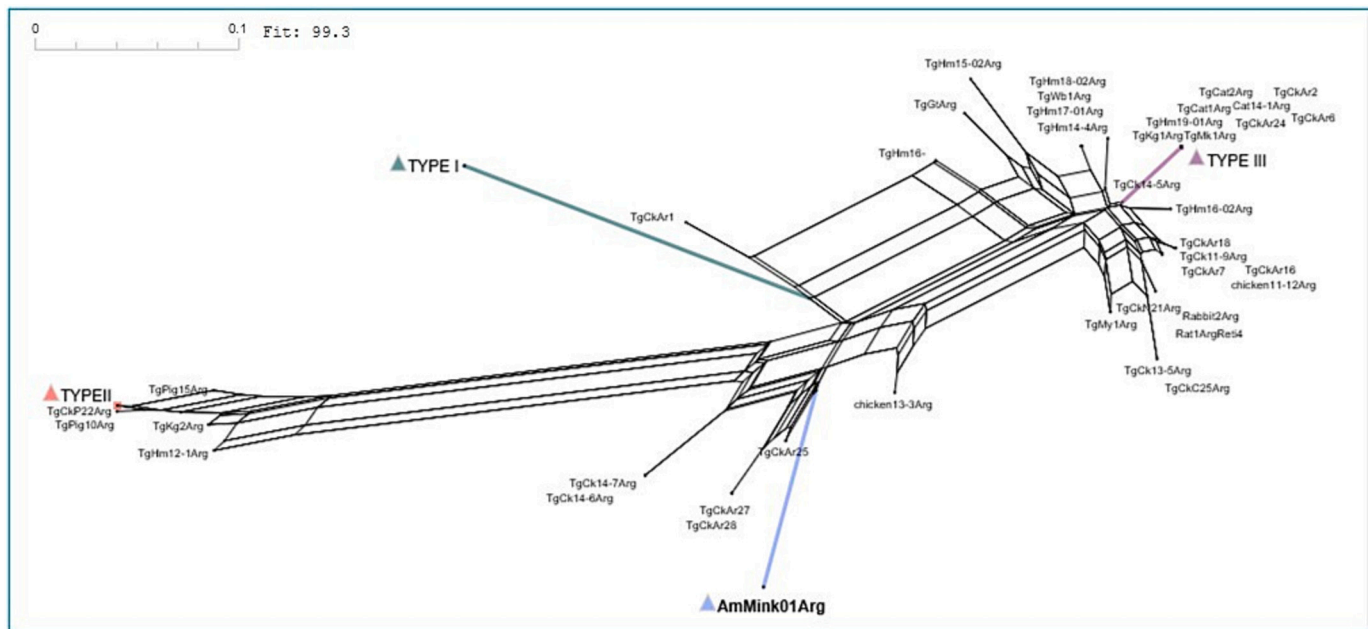


Figure 2. Phylogenetic network of the previously reported genotypes of *Toxoplasma gondii* in Argentina, reference strains and new genotype identified in this study (AmMink01Arg). Phylogenetic network was inferred using the software SplitsTree 6.0 CE.0_alpha (Huson and Bryant, 2006).

T. gondii (Zabala et al., 2007). Further studies with larger and more balanced sample sizes are required to draw definitive conclusions.

The non-archetypical genotype characterized in the present work (ToxoDB #347) has not been previously reported. The *T. gondii* genotypes previously identified in American mink were archetypical type II in the United Kingdom (Burrells et al., 2013) and archetypical type II (ToxoDB #3) and non-archetypical (ToxoDB #9) in China (Zheng et al., 2016), the latter being the predominant genotype in China and phylogenetically distant from genotype #347 although both are non-archetypical genotypes. In addition, our molecular characterization was performed for 10 allelic markers, whereas the genotype described in the United Kingdom only analyzed five markers and its characterization was found to be incomplete. The phylogenetic analysis showed that genotype ToxoDB #347 is far from archetypical genotypes and is related to non-archetypical genotypes with high genetic variability (ToxoDB#283 and #286 (chicken 13-3Arg)) previously described in backyard chickens in Northern Argentina (Misiones province), which are phylogenetically close to medium/high virulence strains from Brazil (BrI and BrIV) and are associated with cases of human ocular toxoplasmosis in Argentina (Bernstein et al., 2018; Pardini et al., 2016). In turn, the observed ROP18/ROP5 allelic combination would indicate that the ToxoDB #347 genotype presents non-lethal virulence in mouse model, suspecting that in natural infections this genotype would act similarly. Bernstein et al. (2018) described a regionalization of *T. gondii* genotypes in Argentina, with archetypical type II genotypes concentrated in central Argentina (Buenos Aires province), and archetypical type III genotypes and non-archetypical gene variants shared with Brazil due to geographical proximity in Northeastern Argentina. However, genotype #347 was the first to be found in Patagonia, an ecoregion located more than 1500 km away from where genotypes #15, #283 and #286 were detected, evidencing the presence of non-archetypical genotypes in other regions of Argentina. The movement of *T. gondii* genotypes between distant regions may be due to the transport of animals or through the movement of migratory birds or other long distance migratory species, allowing the parasite to spread to new areas (Dubey et al., 2021). In turn, invasive exotic species, given their great capacity for geographic dispersion, could promote the dissemination of the apicomplexan when infected (Bezerra-Santos et al., 2023; Mendoza Roldan and Otranto, 2023). Studies on toxoplasmosis in migratory birds have

described seroprevalences between 15 and 30.4 % in Svalbard, Russia, Italy and Canada (Dini et al., 2023; Elmore et al., 2015; Sandström et al., 2013). Due to the predatory behavior of the American mink on waterfowl and passerines, *T. gondii* could be transported by migratory birds, although more studies are needed to further evaluate the presence of the *T. gondii* in American mink and their prey to understand their role in the life cycle of the parasite. The movement of parasitized domestic animals such as cats and poultry between regions could also allow the interaction of mink with new genotypes of *T. gondii* and the consequent perpetuation of the parasite's life cycle. It should be noted that this is the first genotyping of *T. gondii* reported for Argentine Patagonia, being unknown the predominant genotypes in this region.

The prevalences of *N. caninum* previously reported in American mink are lower than those reported for *T. gondii*, in agreement with the results of the present study. While *N. caninum* DNA has been detected in American mink brains in Great Britain (Bartley et al., 2013), it was not detected in the present study, despite the sampling sites being frequently used by domestic dogs, livestock, and wildlife. This result does not rule out the possibility that the apicomplexan is present in the environment (Campero et al., 2021). Due to its semi-aquatic habits, the American mink is associated with waterbodies, where the presence of domestic canids is less frequent than in urban and livestock areas, and therefore is less likely to be exposed to *N. caninum* oocysts in feces. However, infection can be acquired by ingesting tissue cysts from birds, as these have been described as intermediate hosts of *N. caninum* and from the remains of infected mammalian tissues (de Barros et al., 2018).

Together with native species, invasive exotic species participate in the wildlife cycles of apicomplexan protozoa, thus increasing the number of hosts available in the environment and, favoring their dissemination and persistence in the environment in which they develop. To date little is known about the role of free-living animals in the epidemiological context of *T. gondii* infection in Patagonia. Introduced species, particularly semi-aquatic carnivores such as the American mink, could act as important reservoirs increasing the circulation of *T. gondii* in the environment and as sentinel species. Wild and domestic carnivores in this region move freely between sylvatic and synanthropic environments (e.g. cats (*Felis catus*), dogs (*Canis lupus familiaris*), American mink, foxes (*Lycalopex gymnocercus*, *Lycalopex culpaeus*). Therefore, the finding of *T. gondii* in American mink in Northern Patagonia indicates

the circulation of this parasite in the region and the potential contribution of mink to its spread in both natural and anthropic environments. Management actions to control populations of introduced species facilitate access to a large number of biological samples that can be used for sanitary studies of wild individuals. This can provide key environmental information for the development and implementation of preventive measures in One Health, thus mitigating the damage caused by apicomplexans.

CRedit authorship contribution statement

Marina Runco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **María Laura Gos:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Lais Pardini:** Writing – review & editing, Methodology. **Mariana Bernstein:** Writing – review & editing, Methodology, Formal analysis. **M. Laura Guichón:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **María Virginia Rago:** Writing – review & editing, Resources, Investigation, Data curation. **Luciana Piudo:** Resources, Investigation, Funding acquisition, Conceptualization. **Alejandro González:** Visualization, Investigation. **Martín Monte Verde:** Investigation. **Lucía María Campero:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **María Cecilia Venturini:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2025.105929>.

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