Exploring the epidemiological role of the Eurasian lynx (*Lynx lynx*) in the life cycle of *Toxoplasma gondii*

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A R T I C L E   I N F O

A B S T R A C T

Toxoplasma gondii is a successful coccidian parasite able to infect all warm-blooded animals and humans, causing one of the most common zoonoses worldwide. The Eurasian lynx (*Lynx lynx*) is one of the feline potential hosts of *T. gondii* in Switzerland, but little is known about its epidemiological role as a definitive or intermediate host. Serum samples from 183 Eurasian lynx collected from 2002 to 2021 were tested for antibodies to *T. gondii* by ELISA, IFAT and in case of inconclusive results, immunoblot. Antibodies to *T. gondii* were found in 150 of 183 (82%) Eurasian lynx. Older age, good health status and a low-altitude habitat were found to be significant predictors for seropositivity. *T. gondii* oocysts were detected in 3 of 176 (1.7%) faecal samples, indicating the Eurasian lynx as a definitive host. In addition, *T. gondii* DNA was detected in skeletal muscle (7/88), heart muscle (2/26) and/or brain tissue (2/36) from 10 different lynx by real-time PCR. In one animal, a *T. gondii*-like tissue cyst was observed in heart muscle and confirmed as *T. gondii* by immunohistochemistry (1/20) and real-time PCR. With an adapted nested-PCR-multilocus-sequence typing (MLST) and *in silico* restriction-fragment-length-polymorphism analysis (RFLP) approach two different *T. gondii* genotypes were detected: a lineage II variant (ToxoDB #3) in three animals (two oocyst samples and one heart muscle sample) and a novel genotype exhibiting both type II and III alleles in a further animal (skeletal muscle). The present results indicate that *T. gondii* infection is widespread in the Swiss lynx population. The Eurasian lynx may contribute to environmental contamination with oocysts and is able to harbour the parasite in different tissues. Genotyping revealed the presence of both a common *T. gondii* lineage in Europe and a previously unknown genotype and thus shedding more light on the complex molecular epidemiology of *T. gondii*.

1. Introduction

Toxoplasma gondii (Apicomplexa, Sarcocystidae) is a globally widespread protozoan parasite able to infect wildlife, domestic animals and humans alike (Dubey, 2022). In humans, disease is mainly associated with congenital infections or immunosuppression, e.g. AIDS and transplant patients (Porter and Sande, 1992; Guerina, 1994). However, reports of symptomatic infections in immune-competent individuals are increasing (Grigg et al., 2001). The parasite undergoes a facultative indirect life cycle, with all warm-blooded animal species as potential intermediate hosts and felids as definitive hosts. Although the parasite may persist through carnivorism among intermediate hosts, it relies on definitive hosts for sexual reproduction, which leads to shedding of millions of oocysts with the faeces. Excretion of environmentally resistant oocysts provides an important source of infection for many intermediate hosts, which become reservoirs of the parasite. Oocyst uptake is the main infection route for herbivores, including domestic and wild ruminants, which may serve as a source of infection for humans through

meat consumption (Dubey et al., 2021a; Fanelli et al., 2021; Conrady et al., 2022). Besides the domestic cat (*Felis catus*) and the wild cat (*Felis silvestris*), the Eurasian lynx (*Lynx lynx*) is the only further potential definitive host of *T. gondii* in Switzerland, able to contribute to environmental contamination with oocysts. Definitive hosts may generate novel *T. gondii* genotypes through genetic recombination during sexual reproduction in the intestine. After ingestion, infectious *T. gondii* stages invade intestinal and extraintestinal tissues of the host, multiply as tachyzoites during the acute phase of infection and as bradyzoites contained in tissue cysts in later infection stages. Tissue cysts develop mainly in the central nervous system (CNS) and heart and skeletal muscular tissue of both, intermediate and definitive hosts (Hill et al., 2014).

The Eurasian lynx distribution ranges from Europe to Asia, with only highly fragmented populations prevailing in Central and Western Europe (https://www.kora.ch/en/species/lynx/distribution, KORA, accessed 20.12.2022) and an estimated population size in Europe (excluding Russia and Belarus) of 9′000 to 10′000 individuals (http://www.catsg.org/m/index.php?id=99, CatSG, accessed 13.12.2022). The Eurasian lynx vanished from Switzerland during the 19th century and was reintroduced in the 1970s, giving raise to two distinct populations, one in the Jura Mountains, the other in the Alps (Breitenmoser and Breitenmoser-Würsten, 2008). Since then, these populations have slowly grown to an estimated number of 254 (± 12) individuals older than a year in 2019 (https://www.kora.ch/en/species/lynx/abundance, KORA, accessed 20.12.2022).

Free-ranging felid populations often exhibit high antibody prevalences against *T. gondii*. Such high seroprevalences have been reported in the Eurasian lynx (75.6% in Sweden, (Ryser-Degiorgis et al., 2006); 86.1% in Finland (Jokelainen et al., 2013),), the Iberian lynx (*Lynx pardinus*; 66.7% in Spain (García-Bocanegra et al., 2010),), the bobcat (*Lynx rufus*, 40–83% in the US (Labelle et al., 2001; Mucker et al., 2006),) and the Canada lynx (*Lynx canadensis*; 44% in Canada (Labelle et al., 2001)). In Switzerland, seroprevalence in domestic cats is estimated at 42% (Schreiber et al., 2021) but there are no figures for the Eurasian lynx population. Although there were high seroprevalence rates in *Lynx* spp. in the studies conducted in Sweden, Finland and Spain, *T. gondii* oocyst excretion has not yet been identified in these species (Ryser-Degiorgis et al., 2006; García-Bocanegra et al., 2010; Jokelainen et al., 2013). By contrast, it has been demonstrated that captive bobcats have shed oocysts (Marchiondo, 1976).

T. gondii shows high genetic variabilities. Besides the well-known archetypal genotypes I, II and III, a series of studies showed that there are new variant and non-clonal genotypes, which may show different virulence phenotypes (Grigg et al., 2001; Deiró et al., 2021). In Europe, type II is most prevalent not only in animals but also in humans (Fernández-Escobar et al., 2022a); however, non-clonal genotypes might occur, which may be associated with severe clinical outcome (Boothroyd and Grigg, 2002; Elbez-Rubinstein et al., 2009; Pomares et al., 2011).

In several studies dealing with genetic characterization of *T. gondii* from bobcats in the US, genotypes ToxoDB #1, #2, #3, #5 (most cases) and #24 were found (Dubey et al., 2004, 2015; Yu et al., 2013; Shwab et al., 2014; Verma et al., 2017). In Switzerland, information on *T. gondii* genotypes circulating in felids is scarce. Genotyping of *T. gondii* from domestic cats revealed ToxoDB #3 (Berger-Schoch et al., 2011; Spycher et al., 2011), which is a frequently found genotype in Europe (Fernández-Escobar et al., 2022b). Molecular characterisation of *T. gondii* in wild felids in Switzerland has not been reported so far.

Given the lack of information about *T. gondii* occurrence in Eurasian lynx, its epidemiological role is currently unclear. To address this knowledge gap, we assessed 1) prevalence of *T. gondii*-antibodies and risk factors for seropositivity in the Swiss lynx population, 2) frequency of oocyst shedding, 3) occurrence of *T. gondii* in tissues and 4) circulating *T. gondii* genotypes.

2. Material and methods

2.1. Sample collection

According to the lynx management plan implemented by the Swiss Federal Office of Environment (FOEN), all dead lynx (found dead or culled due to health-related issues or repeated predation on domestic livestock) are subject to a post-mortem examination at the Institute for Fish and Wildlife Health (FIWI). From 2002 to 2021, 297 lynx were necropsied and sampled according to a standard protocol in the framework of the lynx health monitoring programme funded by the FOEN (Ryser-Degiorgis and Segner, 2015; Ryser-Degiorgis et al., 2021). Serosanguinous fluid collected from the heart or thoracic cavity and tissue samples (including skeletal muscle) were stored at −20 °C. Tissue samples were fixed in 4% buffered formalin, cut and embedded in paraffin. Faecal samples were systematically collected for coprological analysis and immediately processed at the Institute of Parasitology (IPB) of the Vetsuisse Faculty, University of Bern (see below). In 2021, the set of samples was increased with brain and heart specimens for the purpose of this study.

Only animals from which serosanguinous samples were available, were included in this study (n = 183 from 2002 to 2021). Previously frozen fluid samples were thawed, centrifuged, and sera stored at −20 °C until further analysis. Additionally, relevant frozen tissue samples (heart, skeletal muscle, brain) were selected, and a corresponding set of metadata (including sex, age, geographical region, altitude, health status, body condition) was compiled.

Lynx age was estimated based on dentition (i.e., milk dentition or counting of tooth cementum annuli) and morphometric characteristics and grouped into three age classes as previously described (Marti and Ryser-Degiorgis, 2018a, 2018b): juveniles (<1 year old, n = 105), subadult (n = 34) and adult (≥2 years for females and ≥3 years for males, n = 44). Animals were given a body condition evaluation based on body fat reserves and musculature (good, moderate, emaciated). Health status was defined as "healthy" or "diseased". Healthy marked the absence of significant pathological findings and a moderate to good nutritional status. The geographical region of origin and altitude (meters above sea level) were documented using the coordinates of where the

Table 1
Percentage of lynx serum samples positive for antibodies against *T. gondii* (apparent prevalence) by variable category and odds-ratios (OR) with 95% CI and P-values based on univariate testing.

Variable	N_{Pos}/N_{Tot}	Apparent prevalence (95% CI)	OR (95% CI)	P-value
Sex				
Male	69/85	81.2 (72.3–89.5)	Reference	0.796
Female	81/98	82.7 (75.2–90.2)	1.1 (0.52–2.36)	
Age				
Juvenile	73/105	69.5 (60.7–78.3)	Reference	<0.0005
Subadult	33/34	97.1 (91.4–100)	NA	
Adult	44/44	100% (92.0–100)	NA	
Region of origin				
Alps	84/111	75.7 (67.7–83.7)	Reference	0.006
Jura	66/72	91.7 (85.3–98.1)	3.54 (1.46–9.92)	
Health status				
Healthy	96/105	91.4 (86.1–96.8)	Reference	<0.0005
Diseased	54/78	69.2 (59.0–79.5)	0.21 (0.09–0.48)	
Altitude	NA	NA	NA	0.068

Abbreviation: CI, confidence interval; NA, not applicable.

carcasses were found. Sample composition is provided in supplementary material (Suppl. Table 1).

2.2. Serological tests

All sera were tested by two commercial assays, namely ELISA and Indirect Fluorescent Antibody Test (IFAT) for detecting antibodies against *T. gondii*. Samples with inconclusive or conflicting results were additionally tested by immunoblot.

2.2.1. ELISA

A commercially available ELISA kit: ID Screen® Toxoplasmosis Indirect (ID.vet, Grabels, France), designed for detection of specific antibodies against *T. gondii* in serum, plasma or meat juice from multiple species, including dogs, cats, goats, sheep, cattle and pigs (TOXO-MS) was used. ELISA plates precoated with cell culture-derived *T. gondii* tachyzoite antigen (TgSAG1/P30) and a multi-species conjugate were provided in the kit. According to the manufacturer's instructions, for each serum sample, a sample-to-positive ratio (S/P ratio) based on optical density was calculated. Samples with an S/P% $\leq$ 40% were considered negative, 40% < S/P% < 50% inconclusive and S/P% $\geq$ 50% positive. Internal serum controls from a *Toxoplasma*-positive and *Toxoplasma*-negative cat were included in each plate.

2.2.2. IFAT

As a second indirect diagnostic method, an IFAT was performed. A fluorescein isothiocyanate-labelled anti-cat-IgG antibody produced in goat (Sigma, F4262) was used as conjugate. Sera were diluted two-fold from 1:40 up to 1:2560 and a titre $\geq$1:80 was used as the cut-off. Titres of 1:40 were considered inconclusive, and titres <1:40 negative. For a dilution to be considered positive, a majority of tachyzoites had to show complete peripheral fluorescence. All slides were assessed by the same two observers (four-eyes principle) to ensure consistency of interpretation.

2.2.3. Immunoblot

To clarify conflicting (positive by one of both tests) and inconclusive ELISA and IFAT results, an *in-house* (IPB) *T. gondii* tachyzoite surface antigen TgSAG1 (P30)-based immunoblot was performed. The protocol was used as previously described (Basso et al., 2020), with the exceptions of using goat anti-feline IgG (H + L) alkaline phosphate (AP) conjugate (Southern Biotech, Birmingham, USA) as secondary antibody, and an AP-buffer and NBT/BCIP Stock Solution (Roche, Mannheim, Deutschland) mixture as a substrate. The sample was considered positive if a sole band of a relative molecular mass of 30 kDa was visible.

2.3. Coproscopy

The collected rectal faecal samples were processed by a sedimentation/zinc chloride flotation method (Deplazes et al., 2020). When *T. gondii*-like oocysts (oocysts measuring 10–13 μm) were detected, a concentration method based on flotation with sugar solution (specific gravity 1.3) and washing by centrifugation (Ortega-Mora et al., 2007) was performed to purify the oocysts from the rest of the faecal matter. Subsequently, molecular investigation was carried out to confirm identification of *T. gondii* oocysts and/or differentiate them from morphologically similar oocysts of *Hammondia hammondi*. In addition, in these cases, histological sections of small intestine were screened for the occurrence of *T. gondii* development stages.

2.4. PCR

2.4.1. DNA extraction

DNA was extracted from fresh tissues (heart muscle, skeletal muscle and brain) as follows: 500 mg of tissue were homogenized with 900 μl Buffer ATL and 100 μl Proteinase K and incubated overnight at 56 °C.

Subsequently, 200 μl of the homogenate were used to complete further DNA extraction steps as indicated by the manufacturer (DNeasy Blood and Tissue Kit, QIAGEN, Hilden, Germany). Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue as described (Müller et al., 2003). DNA was extracted from oocyst samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions.

2.4.2. Real-time PCR for T. gondii and conventional PCR for Hammondia hammondi DNA

A Taq-Man-based real-time qPCR was performed in a CFX96 qPCR instrument (Bio-Rad Laboratories AG, Cressier, Switzerland) to detect and quantify *T. gondii* DNA from fresh tissue samples and oocysts from faeces. For analysis of the PCR results, CFX manager software version 1.6 was used. The specific Taq-Man based real-time qPCR targeting the 529 bp repetitive genomic sequence of *T. gondii* (Homan et al., 2000) was used. The reaction mixture (10 μl per reaction) contained 5 μl of 2 × Mastermix (SensiFAST™ Probe NO-ROX Kit; Bioline Meridian Lifescience, Memphis, TN, USA), 0.25 μL of 20 μM-forward primer Tox-9 (5' – AGGAGAGATATCAGGACTGTAG – 3') and 0.25 μL of 20 μM-reverse primer Tox-11 (5' – GCGTCGTCTCGTCTAGATCG – 3') (Reischl et al., 2003), 0.1 μL of 10 μM detection probe Tox-HP-1 labelled with fluorescein amidite (FAM) on the 5' end as previously described by Reischl et al. (2003) but additionally containing Black Hole Quencher 1 (BHQ1) in 3' position as an essential element of the TaqMan hydrolysis probe (5' –(FAM)-GAGTCGGAGAGGGAGAAGATGTT-(BHQ1)-3'), 0.3 μL of 10 mM-dUTP (supplementary to dTTP included in the 2 × Mastermix) and 0.1 μL (one unit) of heat-labile uracil DNA glycosylase (UDG; both from Bioline Meridian Lifesciences). For UDG-mediated decontamination, the temperature profile included an initial 10 min incubation at 40 °C that was followed by a 5 min denaturation period at 95 °C. Subsequently, DNA amplification was achieved during 50 cycles of 10s at 95 °C and 20s at 62 °C. After each cycle, light emission by the fluorescent dye was measured at 62 °C. 2 μL of DNA template was used and the volume was completed with 2 μL of H_2O. As negative control, 4 μL of H_2O were used.

In addition, a conventional PCR for *Hammondia hammondi* was performed on DNA extracted from *T. gondii*-like oocysts from faeces as previously described (Schares et al., 2008).

2.4.3. n-PCR-MLST and in silico RFLP analysis

Genotyping was performed by a multilocus sequence typing (MLST) approach, including amplification of 10 genetic markers (SAG1, SAG2 (5' and 3' SAG2 amplified separately, and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29–2, L358, PK1 and Apico) as described (Su and Dubey, 2020), but using a Multiplex PCR Kit (QIAGEN), including a Hot-StarTaq® DNA polymerase instead. Amplified PCR products were purified (DNA Clean & Concentrator-5, Zymo Research, Irvine, USA) and sequenced in both directions with the same primers as those used in the second step of the nested PCR (Microsynth, Balgach, Switzerland). The obtained sequences were investigated for SNPs, and analysed by *in silico* RFLP using the NEBcutter V2.0 programme (Vincze et al., 2003) as described (Castro et al., 2020), to compare them with the *T. gondii* genotypes deposited in ToxoDB databank (https://toxodb.org/toxo/app, ToxoDB, accessed 09.08.2022).

2.5. Histology, immunohistochemistry and PCR on FFPE tissue samples

Heart muscle, skeletal muscle and brain (FFPE tissue samples) were sectioned and stained with hematoxylin and eosin following the protocols of the Institute of Animal Pathology (Vetsuisse Faculty, University of Bern) and screened for the occurrence of *T. gondii*-like stages and potential inflammatory reaction. Screening was performed with a light microscope at 200x magnification.

When putative protozoan stages were detected, immunohistochemical (IHC) staining for *T. gondii* was performed on freshly prepared recuts from the same paraffin blocks. For this, unstained histological sections

were deparaffinised and hydrated in a descending alcohol series, and endogenous peroxidase and alkaline phosphatase were blocked with a commercial Dual Endogenous Enzyme Block (Agilent Technologies Switzerland AG, Basel, Switzerland). Nonspecific binding was blocked by dissolving the primary antibody in a solution of PBS and bovine serum albumin (PBS-BSA). Subsequently, sections were incubated for 60 min with whole *T. gondii* extract rabbit antiserum as primary antibody diluted 1:2′000 (Winzer et al., 2015) at room temperature. Sections were treated with Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) as recommended by the manufacturer. DAB+ (diaminobenzidine tetrahydrochloride, Agilent Technologies Switzerland AG, Basel, Switzerland) was used as the chromogen to reveal parasitic stages. In the end, all samples were counterstained with hematoxylin (Artechemis AG, Bern, Switzerland). Histological sections of cat lung and liver positive for *T. gondii* were used as positive controls for the IHC technique, and the primary antibody was omitted for negative controls. Samples were considered positive when parasite stages were stained in brown by DAB. Further sections of FFPE specimens with putative protozoan stages were additionally tested by qPCR for *T. gondii*-DNA (protocol as mentioned above), and by conventional PCRs for *Hepatozoon* sp. (Kegler et al., 2018) and *Sarcocystis* sp. DNA (Basso et al., 2020a). All samples were also tested by a PCR targeting the α-actin gene to confirm the presence of amplifiable DNA (Müller et al., 2015).

2.6. Data analyses and statistics

Results of the three serological tests were interpreted as follows: a sample was classified as positive if both ELISA and IFAT were positive and as negative if both were negative. In case of inconclusive or conflicting results in any of both tests, samples were tested by immunoblot and then classified as positive or negative according to the immunoblot result. Apparent seroprevalence was then estimated with a free online-tool (https://www.sample-size.net/, accessed 30.11.2022) (Kohn and Senjak, 2021).

Parasite detection was considered positive when *T. gondii* was detected by immunohistochemical staining or when *T. gondii* DNA was amplified from frozen or FFPE tissues.

All statistical analyses were performed using R (R Core Team, 2021). Level of significance was set at 0.05. In a first step, univariate comparison between serology results (positive, negative) and the explanatory variables, i.e., sex (male, female), age (juvenile, subadult, adult), geographical region of origin (Alps, Jura), altitude (in meters over sea), health status (healthy, diseased) was performed to identify potential risk factors for seropositivity, by (i) Pearson's chi-square test when the explanatory variable consisted of two categories (i.e., sex, geographical region of origin, health status), (ii) the Kruskal-Wallis rank sum test with post hoc Mann-Whitney-Wilcoxon test followed by Holm-Bonferroni correction for explanatory variables with more than two categories (i.e., age) and (iii) Spearman's rank correlation for the continuous variable altitude (non-normally distributed). In a next step, we examined the effects of sex, age, geographical region of origin, altitude, health status on the serological results by fitting a generalised linear model (glm). Best model selection was done using Akaike's Information Criterion (AIC) and considering that models with a ΔAIC of 0–2 provide similar support (Anderson et al., 2001). An inter-rater agreement (kappa) was calculated to evaluate the agreement between the ELISA and IFAT tests (https://www.graphpad.com/quickcalcs/kappa1/, accessed 29.11.2022). The obtained kappa values were interpreted as follows: slight agreement (κ = 0–0.20), fair agreement (κ = 0.21–0.40), moderate agreement (κ = 0.41–0.60), substantial agreement (κ = 0.61–0.80) or almost perfect agreement (κ = 0.81–1.00) (Landis and Koch, 1977). A map showing the geographical distribution of Eurasian lynx tested for *T. gondii* antibodies was drawn with the free software QGIS version 2.18.7 (QGIS Development Team (2017). QGIS Geographic Information System. Open Source Geospatial Foundation Project http://qgis.osgeo.org).

3. Results

3.1. Serology

Specific anti-*T. gondii* IgG antibodies were found in 150 of the 183 serum samples (82.0%, 95% CI: 76.4–87.5%) when the results from all serologic tests were taken into consideration. The Swiss-wide distribution of seropositive and seronegative individuals is shown in Fig. 1. Almost all of the seronegative samples were obtained from juvenile animals, while subadult and adult lynx showed very high seroprevalences: 97.1% (95% CI: 91.4–100%) and 100% (95% CI: 92–100%) respectively.

Apparent prevalences according to the selected explanatory variables are shown in Table 1. Univariate analysis showed highly significant differences (p < 0.001) for the factors age and health status and significant difference for geographical region of origin (p < 0.05). Probability for being seropositive increased with age and in healthy animals, and in animals from the Jura region. By contrast, no effect was found for altitude (p = 0.068) and sex (p = 0.796). However, in the best fitting glm, only health status and altitude were retained as significant predictors of serostatus (Table 2). In the multivariate regression analysis, lynx found at higher altitudes or categorized as diseased were less likely to be seropositive (Table 2).

When comparing the performance of the two main serologic tests, 138/183 of the tested lynx showed a positive result for *T. gondii* in ELISA, while IFAT revealed 141/183 positive serum samples with a titre of ≥1:80. The obtained weighted kappa-value of 0.741 indicated a substantial agreement between the two tests. A summary of results of the two tests is shown in Table 3. Immunoblot results of samples with inconclusive and conflicting results are shown in the supplementary material (Suppl. Table 2).

3.2. Detection of T. gondii oocysts and enteroepithelial stages

T. gondii-like oocysts were found in 3/176 (1.7%, 95% CI: 0–3.6) faecal samples (Fig. 2). All three samples tested positive for *T. gondii*-DNA and negative for *H. hammondi*. All the three lynx with oocysts in faecal samples were juvenile animals, with two of them being antibody-positive. *T. gondii* enteroepithelial stages were detected in small intestine sections from two of the animals (W20_8385 and W21_4446) (Fig. 2). In the third animal (W14_3480) such evaluation was compromised by autolysis.

3.3. PCR on fresh tissue samples

A total of 11 out of 150 analysed fresh tissue samples from 10 out of 92 (10.9%, 95% CI: 4.5–17.2) lynx, from which fresh tissue samples were available, were positive for *T. gondii*-DNA by qPCR. Positive samples were 7/88 skeletal muscle, 2/26 heart muscle and 2/36 brain tissue. One lynx tested positive in two tissues (skeletal and heart muscle).

3.4. Sequencing

Multilocus and nested PCR was positive for samples from seven lynx. Successful amplification and sequencing of all 10 markers was achieved in four samples (two oocyst, one skeletal muscle and one heart muscle samples from four different animals). For the other three samples, only five or less markers could be amplified and sequenced. Allele patterns by *in silico* restriction enzyme digestion and fragmentation are shown in Table 4. Both oocyst samples and *T. gondii* from the heart sample corresponded to the same lineage II variant strain ToxoDB #3. In one of the heart muscle samples, sequencing revealed a single nucleotide polymorphism (SNP) in the SAG3 marker sequence (OQ230332, sequence included primers). The sequence showed 100% identity with isolates having an SNP at 187 with a change from G to T (e.g., feline KU599488, ovine MT361126, canine KU599459) compared to the clonal type II
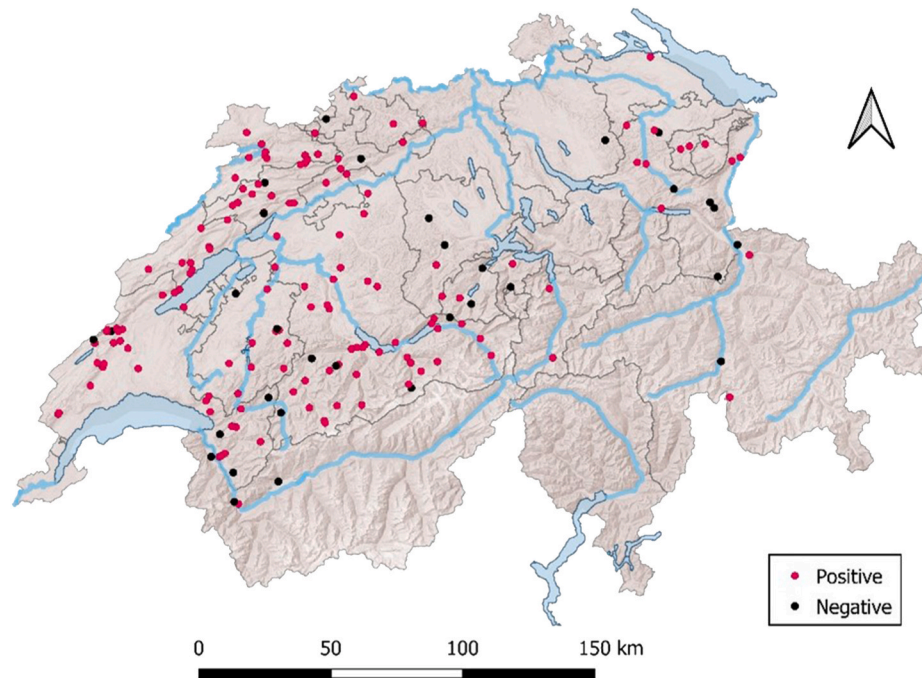
Fig. 1. Map of Switzerland showing the geographical distribution of Eurasian lynx tested for *T. gondii* antibodies. Shades of grey correspond to the relief and main rivers and lakes are in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Parameters of best glm obtained for serology of Eurasian lynx (*Lynx lynx*) from Switzerland and odds-ratios (OR) with 95% CI and P-values based on multivariate testing.

Model	Term	OR (95% CI)	P-value
Model: Serology Age + Altitude + Status	Intercept	7.095e+9 (0.000–9.789e+232)	0.988
	Age		
	Juvenile	0.000 (0.000–1.830e+22)	0.989
	Subadult	0.000 (0.000–2.003e+24)	0.991
	Altitude	0.998 (0.996–0.999)	0.008**
	Status		
	Diseased	0.230 (0.087–0.567)	0.002**

AIC: 130.7.

Table 3
Serological results obtained for Eurasian lynx from Switzerland (2002–2021) by ELISA and IFAT.

		TOXO-MS (ELISA)			
		Positive	Inconclusive	Negative	Total
T. gondii IFAT	Positive	130	7	4	141
	Inconclusive	7	4	4	15
	Negative	1	2	24	27
	Total	138	13	32	183

reference strain (ME49). Parasites in the skeletal muscle sample represented a novel previously undescribed genotype (data submitted to ToxoDB for inclusion), consisting of a combination of type III and II alleles, as well as a type I allele in the Apico marker (Table 4).

3.5. Histology, immunohistochemistry and PCR from FFPE-tissues

Protozoan stages were detected in 20 out of 187 histological specimens (10.7%, 95% CI 6.3–15.1). Inflammation was observed in 49 samples but never in direct association to putative *T. gondii* stages. In two samples, *Sarcocystis* sp. cysts were identified morphologically. In the remaining 18 samples, the observed stages were very small (<20 μm)

and the protozoan species not easily identifiable by morphology. Quantitative PCR for *T. gondii* was positive in a heart muscle sample, with a single tissue cyst. This sample was also the only one with positive IHC-labelling (Fig. 3). In two other heart muscle sections with parasitic tissue stages that were IHC-negative, PCR was positive for *Hepatozoon* sp. DNA, which was further identified as *H. silvestris* (OQ207707, OQ207708). A summary of all histology and immunohistochemistry results is provided in Table 5.

4. Discussion

In agreement with previous studies in *Lynx* spp. populations (Labelle et al., 2001; Mucker et al., 2006; Ryser-Degiorgis et al., 2006; García-Bocanegra et al., 2010; Jokelainen et al., 2013), the reported investigations carried out in Switzerland revealed a very high *T. gondii* seroprevalence. However, the present study is the first to document oocyst shedding, tissue cyst occurrence and *T. gondii* genotypes in the Eurasian lynx.

4.1. Seroprevalence

The sample size of 183 lynx, though sampled over 19 years is high considering the protected status of the species (no hunting) and the small population size of around 255 individuals in Switzerland (https://www.kora.ch/en/species/lynx/abundance, KORA, accessed 20.12.2022). On a methodological point of view, the indirect multispecies ELISA was selected for practicality and its easy use for a large number of samples. As this commercial ELISA kit is validated for domestic cats but not for lynx, a second serologic test, IFAT, was performed to compare the results and provide more reliable seroprevalence data. Both tests are well known and widely accepted for *T. gondii* antibody detection and were previously used for serologic testing also in other wild animal species (Dubey et al., 2021b). The two serologic tests performed equally well as illustrated by the kappa-test results, which revealed a substantial agreement to detect positive, inconclusive, and negative samples.

As already observed in other studies on lynx, sex had no effect on
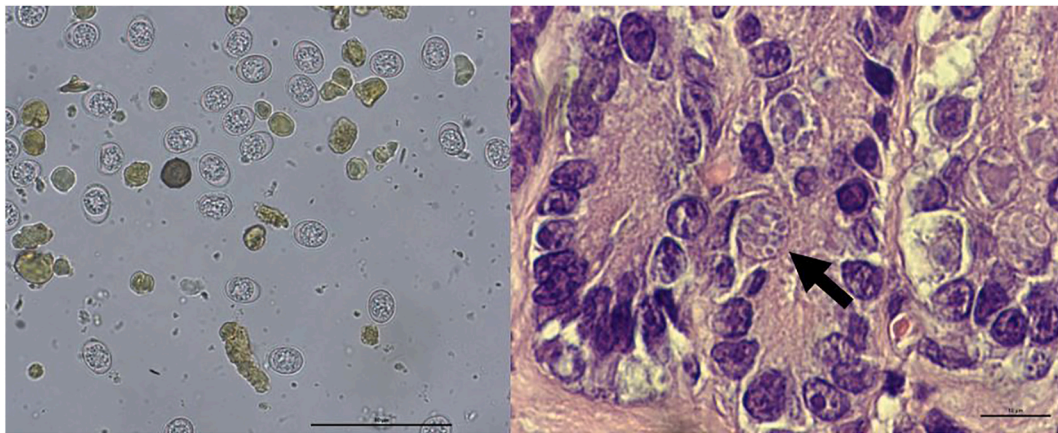
Fig. 2. Unsporulated *T. gondii* oocysts, with a diameter of 10–12 μm, after flotation from a faecal sample of a juvenile lynx (left) (ID W20_8385). *T. gondii* development stage (meront, arrow) in a histological section of small intestine of a lynx (right) (ID W21_4446).

Table 4

Genotypes of *T. gondii* obtained in this study. The first column shows the sample ID. Remaining columns show the *in silico* RFLP results for each marker.

Sample ID	Source	PCR-RFLP markers											
		SAG1	3′-SAG2	5′-SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
W06_1532	Skeletal muscle	II or III	I or III	III	III	III	III	III	II	III	II	III	I
W20_8385	Oocysts	II or III	II	I or II	II	II	II	II	II	II	II	II	I
W21_0845	Heart muscle	II or III	II	I or II	II	II[a]	II	II	II	II	II	II	I
W21_4446	Oocysts	II or III	II	I or II	II	II	II	II	II	II	II	II	I
W15_7213	Skeletal muscle	II or III	II	NA	NA	NA	II	II	NA	NA	NA	NA	I
W20_9086	Brain tissue	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	I
W21_4032	Skeletal muscle	II or III	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviation: NA: marker region not amplified by PCR.

Note: GenBank accession numbers of the marker sequences obtained in this study: SAG1: OQ230355-OQ230360; 3′-SAG2: OQ230342-OQ230346; 5′-SAG2: OQ230347-OQ230350; alt. SAG2: OQ230351-OQ230354; SAG3: OQ230330-OQ230333; BTUB: OQ230380-OQ230384; GRA6: OQ230361-OQ230365; c22-8: OQ230334-OQ230337; c29-2: OQ230366-OQ230369; L358: OQ230370-OQ230373; PK1: OQ230338-OQ230341; Apico: OQ230374-OQ230379.

[a] Single nucleotide polymorphism (SNP) in the position 187 respective to the *T. gondii* reference strain ME49, implying a substitution of thymine in place of guanine.
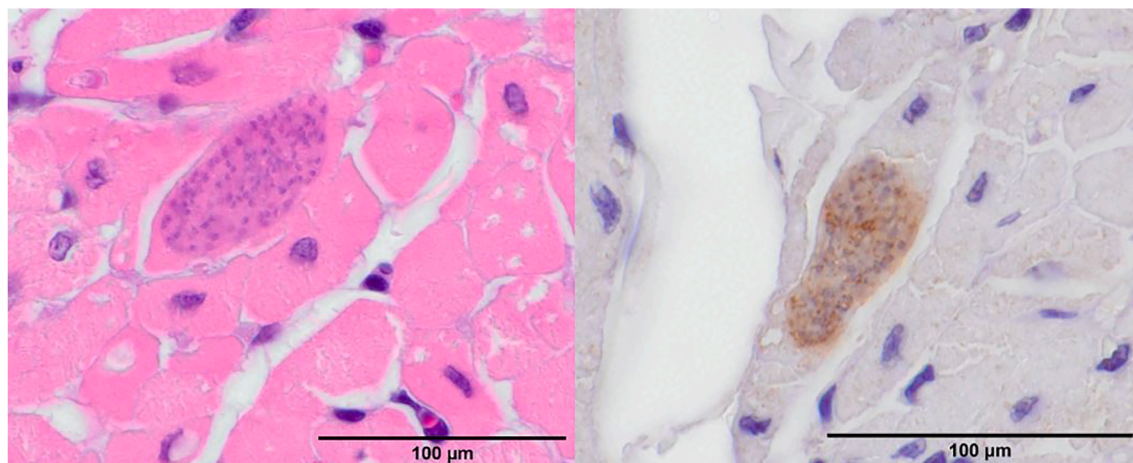
Fig. 3. *T. gondii* tissue cyst in a histological section of heart muscle (hematoxylin-eosin stain (left), immunohistochemistry (right)) from a juvenile lynx (ID W06_1532).

seropositivity, whereas age was a significant factor (Ryser-Degiorgis et al., 2006; García-Bocanegra et al., 2010; Jokelainen et al., 2013). Animals older than a year showed significantly higher antibody prevalence than juvenile lynx. This finding is not surprising as antibodies can persist for several years and likelihood of seroconversion increases during the lifetime of an animal (Zarnke et al., 2001; Ryser-Degiorgis et al., 2006; Roelke et al., 2008; Basso et al., 2020b). Even though univariate analysis did not show a significant effect of altitude, the glm-fitted model retained altitude as a protective factor, i.e., animals from higher altitudes were less likely to be seropositive. This was also observed in domestic sheep taken to summer grazing pastures in the Swiss Alps (Basso et al., 2022). Lower cat density and thus lower environmental contamination with oocysts, combined with more extreme weather conditions with lower temperatures and more intensive sunlight, which could negatively affect oocyst survival, are possible explanations for this finding (Dubey, 2022). Similarly, a very low

Table 5

Occurrence of parasite stages in histological specimens (evaluation in H&E stained section, IHC and PCR).

	Samples with putative protozoan stages (n)	*T. gondii* qPCR	*T. gondii* immunohistochemistry	*Hepatozoon* sp. PCR	*Sarcocystis* sp. PCR
Skeletal muscle	5[a]/183	0/5	0/5	0/5	0/5
Heart muscle	15[b]/183	1/15	1/15	2/15	0/15
Brain tissue	0/12	ND	ND	ND	ND
Total	20/378	1/20	1/20	2/20	0/20

Abbreviation: ND: not done.

[a] Fresh tissue was available from four animals, 1/4 samples was positive by *T. gondii* qPCR.

[b] Fresh tissue was available from five animals, 2/5 samples were positive by *T. gondii* qPCR.

seroprevalence (0.9%; 95% CI 0.3–2.1%) was documented in Alpine ibex (*Capra ibex ibex*) occurring at high altitudes in the Swiss Alps (Marreros et al., 2011). Lynx from mountain areas live on average at a higher altitude than lynx with home ranges in lowlands, and are therefore less exposed to domestic cats. However, given that home ranges of lynx can be very large (90–150 km² (https://www.kora.ch/en /species/lynx/profile, KORA, accessed 12.12.2022)), results on the effect of altitude have to be interpreted with caution.

Of note is that "diseased" animals were less often seropositive than "healthy" animals. This is partially due to a bias in the dataset. Indeed, diseased animals were often juveniles and juvenile animals were mostly seronegative. They were a majority of orphans, which are generally very emaciated and were therefore placed in the "diseased" category.

4.2. Epidemiology of T. gondii in the wild

Transmission of *T. gondii* can occur in a wide variety of ways, including oral (carnivorism), faeco-oral and transplacental transmission. In felids, ingestion of tissue cysts from intermediate hosts is the most efficient route of transmission (Dubey, 2006). The diet of the Eurasian lynx in western and central Europe comprises mainly larger ungulates (Breitenmoser and Breitenmoser-Würsten, 2008). In Switzerland, roe deer (*Capreolus c. capreolus*) are the undisputed main prey, followed by chamois (*Rupicapra r. rupicapra*) and, to a minor extent, foxes (*Vulpes vulpes*) (Molinari-Jobin et al., 2007). Although no studies have been carried out in Switzerland on *T. gondii* infections on the main lynx prey yet, its occurrence in roe deer and chamois was demonstrated in Italy and France by high seroprevalences of 13–43.7% and 3.8–16.8%, respectively (Gaffuri et al., 2006; Gotteland et al., 2014; Crotta et al., 2022). Similar numbers might be expected in Switzerland. Another possibility of infection is through contact with domestic cats or wildcats, which typically encroach into lynx habitat.

4.3. Detection of oocysts in faeces

To our knowledge, this study is the first to demonstrate oocyst shedding in free-ranging *Lynx* spp. In earlier studies, demonstration of oocyst excretion failed both in coprological investigations of free-ranging Eurasian lynx (Sweden, Finland) and Iberian lynx (Spain), and even in an experimental infection in a Eurasian lynx (Oksanen et al., 1997). However, oocyst excretion has been documented in captive bobcats and various free-ranging wild felids (Miller, 1972). Domestic cats usually shed oocysts for one to three weeks after ingestion of tissue cysts and seroconversion often occurs after the initial shedding period (Dubey, 2022). Besides, re-shedding of oocysts was reported both in domestic cats and in several wild felid species (Lukešová and Literák, 1998; Basso et al., 2005; Zulpo et al., 2018). Nothing is known about the duration of the shedding period, or if re-shedding of oocysts after re-infection does occur in the Eurasian lynx. In our study, *T. gondii* oocysts were detected in faecal samples of three lynx. In addition, also the presence of enteroepithelial stages was observed in two of the animals (ID W20_8385 and W21_4446), which confirms Eurasian lynx as a definitive host of *T. gondii*. In the third animal in which oocysts in the faeces were observed (ID W14_3480) this confirmation was not possible

due to autolytic changes in the intestine. Interestingly, two of these animals were seropositive for *T. gondii* at the time of shedding (ID W14_3480 and W21_4446). This may be explained by three scenarios: (i) the shedding period in Eurasian lynx might be longer than in domestic cats, (ii) these two juvenile animals got infected with oocysts from the environment (as prepatency after an infection with oocysts is longer than after ingestion of tissue cysts) (Dubey, 2006), or (iii) this corresponded to re-shedding events. Although only three animals were found to excrete oocysts, seropositive animals may have previously excreted oocysts. A similar picture has also been described in domestic cats. It is estimated that approximately 1% of the entire cat population worldwide excretes oocysts at one time point (Dubey, 2022). Domestic cats mainly excrete oocysts in the first two or three weeks after infection, and are thought to remain seropositive for long periods or even life-long (Schreiber et al., 2021). Due to the relatively narrow birth period of lynx in Switzerland, the age of the three young lynx with oocysts in their faeces could be determined to the month (Breitenmoser-Würsten et al., 2007). One lynx (ID W20_8385) was about 3.5 months old and the other two animals were 6 months old. As with domestic cats, we assume that the animals most frequently become shedders after initial infection, which then mostly applies to young animals. This is underlined by the fact that in most studies of Eurasian and Iberian lynx in Europe, the larger proportion of animals tested were adult lynx and no shedders were found. In our study, the proportion of juvenile animals was higher, which also increased the chance of finding oocyst-shedding animals. Another reason why oocysts might be found in faeces is a previous intestinal passage. This could be due to predation of domestic or wild cats, which would excrete oocysts at exactly this timepoint. However, this is a rather rare occurrence, which was also shown in a review of five studies about lynx predation in Switzerland where the total number of predation cases of domestic and wild cats was three cases and in comparison, the main prey of roe deer, alpine chamois and red fox accounted for more than 1000 cases (Molinari-Jobin et al., 2007). In this study we could confirm intraepithelial development of *T. gondii* at least in two cases, indicating that the presence of oocysts in the faeces was not a remnant of intestinal passage.

4.4. Occurrence of T. gondii tissue stages

Tissue stages were rarely found in this study. Tissue cyst abundance in felids is indeed known to be low. At the FIWI, lynx organs are systematically screened by histology for general health evaluation (Ryser-Degiorgis et al., 2021) but tissue cysts of any kind were rarely observed (FIWI, unpublished data). Re-evaluation of selected cases in this study revealed a higher detection of protozoan tissue stages than initially recorded in routine diagnostics. Tissue cysts are small in early stages of infection and therefore easily missed. While mature *T. gondii* and *Sarcocystis* tissue cysts and *Hepatozoon* schizonts are easily identifiable in histological sections, early stages of these parasites might be occasionally misidentified, which makes diagnosis solely based on morphological criteria challenging. PCR techniques represent an adequate aid for parasite identification in histological sections. However, it has a low sensitivity, as parasite stages may be not present in all histological sections. Moreover, formalin fixation may cause DNA

damage and a molecular diagnosis might be not possible if tissue samples were fixed for long periods. Immunohistochemical techniques also aid in the morphological diagnosis of protozoan tissue stages. In this study, *T. gondii* was specifically detected by IHC staining in one animal. *Sarcocystis* tissue cysts (morphologically identified in H&E stained sections) did not stain by IHC for *T. gondii*; therefore, cross-reactions with early stages of *Sarcocystis* were unlikely. In two further IHC-negative cases with protozoan parasite stages that were not clearly identifiable in H&E, subsequent PCR and sequencing enabled their attribution to *H. silvestris*. This *Hepatozoon* species was already identified as cause of fatal myocarditis in a domestic cat in Switzerland before (Kegler et al., 2018). *Hepatozoon* is frequently found in European wild cats (Hodžić et al., 2017), which share lynx habitat in Switzerland. *Hepatozoon* spp. were also reported in free-ranging Iberian lynx, bobcats and ocelots (*Felis pardalis*) (Mercer et al., 1988; Metzger et al., 2008; Allen et al., 2011).

In this study, observed tissue cysts/parasite stages were not associated with inflammatory or necrotic processes. This is in accordance with felids not being highly susceptible for clinical toxoplasmosis. Only a few cases of toxoplasmosis with fatal outcome are reported in wild felids, all in captive individuals, including a newborn bobcat that died one week after birth (Dubey et al., 1987) and juvenile Pallas' cats (*Otocolobus manul*) (Riemann et al., 1974; Kenny et al., 2002; Basso et al., 2005).

4.5. Genotyping T. gondii in lynx

Recent studies, especially in Brazil, have shown that there is greater genetic variability in *T. gondii* than initially believed (Vitaliano et al., 2014; Witter et al., 2020). An overview of the known allele-type combinations is provided by the ToxoDB database (https://toxodb.org/toxo/app, ToxoDB, accessed 09.08.2022).

This is the first *T. gondii* genotyping study in wild carnivores from Switzerland. A complete multilocus genotyping could only be achieved for four animals, though *T. gondii* was identified in more individuals. The main difficulty was to obtain samples with sufficient DNA amount. The well-known and widespread genotype #3 (type II variant; Shwab et al., 2014) was detected in three of the animals. Toxo DB #3 has previously been isolated from European wildlife several times. This genotype was detected in arctic foxes from Norway, wildcats and Eurasian beavers (*Castor fiber*) from Germany and even dolphins from the Mediterranean Sea (Prestrud et al., 2008; Herrmann et al., 2013; Fernández-Escobar et al., 2022b). Also in Switzerland, genotype ToxoDB #3 was already detected in domestic cats and voles (Berger-Schoch et al., 2011; Spycher et al., 2011; Pardo Gil et al., 2023). Genotype II is typically considered intermediate to non-virulent in mice, causing mostly subclinical infections (Sibley and Boothroyd, 1992; Wendte et al., 2011). Nevertheless, genotype II was associated with fatal infections in 32 animals from altogether five different species (Jokelainen, 2012), including a fatal case in a domestic cat from Switzerland (Spycher et al., 2011). Genotype III is strongly represented and largely considered of low virulence (Sibley and Boothroyd, 1992).

Furthermore, a new, unknown genotype was found in skeletal muscle of a juvenile lynx. In the newly discovered allele pattern, six of the 10 tested markers corresponded to type III alleles, two to type II, one to type I (Apico), as well as one marker (SAG1) where a type II or III allele was possible. The obtained sequences for all markers were of a very good quality and did not show the presence of double peaks, which could suggest a coinfection with two *T. gondii* genotypes in the same analysed tissue sample. Therefore, this genotype could represent a II x III recombinant strain, as it was observed in several studies in Europe (Fernández-Escobar et al., 2022b).

It is known that RFLP is a good tool for tracing ancestry from historical archetypes, as certain sequences are required for enzyme digestion. However, SNPs can only be detected with complete sequencing of the marker sequence. Strictly speaking, occurring SNPs would lead to a new classification or to description of more non-clonal genotypes

(Wendte et al., 2011). In this study, due to sequencing and *in silico* digestion, complete marker sequences were available for analysis and were compared to reference sequences of archetypal *T. gondii* types I (GT1; ToxoDB genotype #10), II (ME49; ToxoDB genotype #1) and III (VEG; ToxoDB genotype #2). The observed SNP in the SAG3 sequence of a lynx (ID: W21_0845; OQ230332) has also been detected in sheep in Spain (Fernández-Escobar et al., 2020). There, it was also noticed that this mutation leads to a change in the codon at this position. Whether this results in a change in pathogenicity has not been investigated in detail, but abortions with this genotype and mutation have been observed in the same sheep. Whether the occurrence of this SNP might be frequent throughout Europe is currently unknown because most of the studies performed traditional RFLP analysis without sequencing of the obtained amplicons. There were no deviations from the original types I, II and III in the rest of the complete sequences.

An important question is the relationship between *Toxoplasma* detection (whether in faeces or tissue) and the animal clinical status. In the case of the young lynx with the novel genotype, *T. gondii* cysts were also detected in the heart but they were not associated with inflammatory reaction. This animal was an orphan in a wildlife rescue centre that died of a severe purulent peritonitis with concomitant *Yersinia pseudotuberculosis* infection (Morend et al., 2022). This infection may have happened either via preying on rodents or eating contaminated food. It is unclear whether *Toxoplasma* found its way into the organism via the same route. In this case, the infection was an incidental finding. Pathological changes associated with *Toxoplasma* cysts or DNA were not found in the other animals in this study either. These findings suggest that despite the presence of the parasite, clinical and pathological changes would be exceptional. Congenital toxoplasmosis can occur (Dubey et al., 1987) and therefore neonates would be most likely to develop toxoplasmosis but there were no neonatal animals in this study. Indeed, such young animals are hardly ever found under free-ranging conditions.

In conclusion, this study showed that the Eurasian lynx can act as an intermediate and final host of *T. gondii* by demonstrating oocyst shedding and tissue cyst occurrence. Genotyping revealed the presence of both a locally common *T. gondii* lineage and a previously undescribed genotype. Investigation of lynx prey in the same study area including genotyping would increase the understanding of *T. gondii* epidemiology in Swiss wildlife.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.03.005.

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