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PROTOZOOLOGY - ORIGINAL PAPER

Neospora caninum and *Toxoplasma gondii* infections and their relationship with reproductive losses in farmed red deer (*Cervus elaphus*)

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Abstract

The aims of the present study were to determine the *Neospora caninum* and *Toxoplasma gondii* seropositivity rates in farmed red deer hinds from Argentina and their relationship with reproductive losses. Over a 2-year period, 449 hinds from 4 commercial farms were serologically tested at late gestation for *N. caninum* and *T. gondii* by IFAT. During the first year, a sequential serological analysis was carried out at 3 different time points to analyze antibody dynamics from mating until the end of the gestation period. Fetal and postnatal mortality rates were estimated by 3 successive ultrasound scannings (us) annually and a breeding control carried out after the calving period. Ultrasound fetal measurements were used to estimate conception date and gestational age of abortions. The seropositivity rate for *N. caninum* was 25.5% (37/145) for the yearlings and 34.2% (104/304) for the adults, while for *T. gondii* was 64.3% (93/145) and 78.3% (238/304), respectively. Abortions detected at us1 and us2 were 13/21 (61.9%) with a range of gestational age of 30–87 days, while abortions detected at us3 were 8/21 (38.1%) with a range of gestational age of 49–209 days. The fetal mortality rate was 4% and 5.8%, while the postnatal mortality rate was 18.8% and 4.1% of 101 yearlings and 294 adult pregnant hinds, respectively. Most seropositive hinds to both protozoans showed a stable antibody titer pattern from mating to the end of gestation, and a lower proportion developed an increase in titers suggesting infection recrudescence. Seroconversion during the gestational period was demonstrated in 6 and 50 hinds for *N. caninum* and *T. gondii*, respectively. Hinds with fetal mortality were more likely to be seropositive to *N. caninum* (OR = 3.1) or have *N. caninum* titers ≥400 (OR = 27.4) than hinds that weaned a fawn. No statistical associations were detected for *T. gondii* seropositivity and reproductive losses. The pregnancy rate was not affected by *N. caninum* or *T. gondii* infection, while the serological evidence of *N. caninum* causing postnatal mortality was marginal. Based on serological evidence, *N. caninum* would be a potential abortigenic agent in red deer hinds.

Keywords *Neospora caninum* · *Toxoplasma gondii* · Red deer · Reproductive losses · Abortion · Postnatal mortality

Introduction

Reproductive efficiency is a key variable affecting productivity and profitability in farmed red deer (*Cervus elaphus*). Reproductive performance related to pregnancy and weaning rates have been well documented mainly in New Zealand; however, there are very few studies that have investigated reproductive losses in red deer hinds (Audigé et al. 1999; Audigé et al. 2000; Deer Industry Manual 2000; Patel et al. 2019; Wilson et al. 2012). On the other hand, there is no published information about the detection and quantification of abortion and postnatal mortality rates in red deer commercial herds from Argentina. Among possible causes of reproductive losses, the infection with *Neospora caninum* and *Toxoplasma gondii* should be considered. Both Apicomplexa intracellular protozoans are closely related

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and worldwide distributed, having a facultative heteroxenous life cycle using canids and felids as definitive hosts, respectively. These parasites reproduce asexually in nucleated cells from a wide range of intermediate hosts, both domestic and wild animals, and in addition, *T. gondii* affects humans causing an important zoonosis (Dubey 2010; Dubey et al. 2017). Neosporosis and toxoplasmosis are an important cause of abortions and fetal and perinatal death in cattle and small ruminants (Dubey 2010; Dubey et al. 2017) and have been considered in different deer species as a possible cause of reproductive losses (Basso et al. 2014; Dubey et al. 2014; Patel et al. 2019; Wilson et al. 2012).

The diagnosis of *N. caninum* and *T. gondii* infections in red deer has been generally performed by serological methods (Dubey 2010; Dubey et al. 2017), while there are few studies that have evaluated those results in conjunction with abortion and postnatal mortality diagnosis in order to analyze a possible association between protozoan infections and reproductive losses (Patel et al. 2019; Wilson et al. 2012). Serologic evidence of *N. caninum* infection has been determined in wild and farmed red deer (Almeria et al. 2007; De Craeye et al. 2011; Gozdzik et al. 2010). Wilson et al. (2012) found no serological neither molecular evidence of *N. caninum* involvement in the abortions from several red deer farms from New Zealand with a history of reproductive losses. Therefore, this parasite has not been linked with the occurrence of reproductive losses in red deer. On the other hand, *N. caninum* has been confirmed by microscopic and molecular methods in fetal and stillborn tissues from other cervids as Eld's deer (*Rucervus eldii*) from a zoo in France (Dubey et al. 1996), white-tailed deer (*Odocoileus virginianus*) in the USA (Dubey et al. 2013), fallow deer (*Dama dama*) in Switzerland (Soldati et al. 2004), and axis deer (*Axis axis*) in a zoo from Argentina (Basso et al. 2014). These studies suggested *N. caninum* as the causative agent from fetal and perinatal mortality in the mentioned deer species.

Regarding *T. gondii*, it has been investigated as a potential abortifacient agent in red deer hinds. Wilson et al. (2012) found no association between *T. gondii* seropositivity and abortions in herds with high abortion rates. However, a recent study performed in red deer hinds of different age groups revealed a low association of *T. gondii* seropositivity with abortions in yearling hinds, while no relation was observed in adult hinds (Patel et al. 2019). In addition, both referenced studies detected *T. gondii* DNA in aborted and nonaborted fetuses from some hinds, therefore suggesting that some abortions could be attributed to toxoplasmosis (Patel et al. 2019; Wilson et al. 2012). The findings of the mentioned works demonstrate transplacental infection with *T. gondii*; however, histopathology and/or immunohistochemistry studies were not performed in order to detect the lesions in fetal tissues to confirm this protozoan as a causative of the abortions.

In summary, previous studies in red deer analyzing the relation between *N. caninum* and *T. gondii* infection with abortion are inconclusive, and to our knowledge, there are no investigations that have evaluated a possible association of both protozoan infections with postnatal mortality. Although it is known that these parasites can infect red deer tissues, there is no certainty about the rate and magnitude of related reproductive losses. Considering that other ruminant species evidenced fetal death and perinatal and/or postnatal mortality, associated with neosporosis and toxoplasmosis (Lindsay and Dubey 2020), more studies are needed focusing on identifying the role of *N. caninum* and *T. gondii* infection with reproductive losses in farmed red deer.

The aims of the present study were to determine the *N. caninum* and *T. gondii* seropositivity rates and to evaluate the relationship between both protozoan infections and reproductive losses due to abortion and postnatal mortality in farmed red deer hinds from Argentina.

Materials and methods

Farms and animal management

The work was carried out in 4 red deer commercial farms located in Buenos Aires (farm A, B, and C) and La Pampa (farm D) provinces, Argentina. All the farms have a grazing feeding system based on perennial pastures, green oats and sorghum. Farms A, C, and D performed a winter supply with hay and grains to cover pasture deficit. The animal load was 7, 1.8, 7.7, and 13.8 deer per hectare in farms A, B, C, and D, respectively. There was no joint grazing with other domestic species, while working dogs were present in all the farms.

The study was conducted over 2 consecutive year periods where 449 red deer hinds were mated, of which 145 were yearling hinds (mated at 15 months old) and 304 were adult hinds (≥27 months old, second mating onwards). The total mated hinds per farm were 127, 151, 111, and 60 for farms A, B, C, and D, respectively.

All farms performed a natural mating, using adult males (≥39 months old), and each mating group consisted in 21-35 hinds and 1 stag. The mating period started on 1st to 10th March and ended on 13th to 17th May of each year.

Sequential ultrasonography and reproductive data

The ultrasound scanning (us) was performed by a single operator over the entire period of the study, using a multifrequency linear transducer (Tringa Linear®, Pie Medical, Netherland). It was carried out at 3 different moments throughout the gestation period and named as us1, us2, and us3. The us1 was at the early stage from 2nd to 12th May, the us2 was at the middle stage from 17th June to 4th July, and the us3 was at the late stage from 23rd to 30th October. The us1 was performed 5 to 15 days before the end of the mating period, while the us3 was 9 to 16 days before the start of calving. The average period elapsed between us1 and us2

was 49 days (range 36–63 days) and between us2 and us3 was 124 days (range 111–135 days). The ultrasound pregnancy confirmation was made by visualizing the fetus, fetal parts, or placentomes, and when possible, the fetal heartbeat was recorded to confirm the fetus was alive. A hind was considered not pregnant when an empty uterus was visualized. Ultrasound findings like the absence of heartbeat, scant amount or turbidity of the amniotic fluid, undefined fetal morphology, and undefined intrauterine material were recorded and checked in the next ultrasound to verify the presumption of fetal mortality. In such cases, fetal mortality was recorded in the first ultrasound in which evidence of fetal death was detected. In addition, when in the us2 or us3, the diagnosis was "not pregnant," but with a pregnancy confirmation in the previous ultrasound, the hind was considered as with fetal mortality/aborted. Those hinds diagnosed as "not pregnant" both in the us1 and us2 were considered as hinds that did not conceive.

The gestational age was calculated by fetal measurements according to a previous study (Revol and Wilson 1990) at the moment of pregnancy confirmation. This information was used to estimate the conception date for each pregnant hind according to the following formula: (ultrasound date − fetal age (days) = conception date).

A breeding control was performed between 30 and 45 days after the end of the calving period, where all hinds were locked up in the management shed to record the individual reproductive status (dry or lactating) through direct udder observation. At the same time, each fawn was individually identified using numbered plastic earrings and a hair sample was taken to determine maternal parentage by DNA analysis as previously described (Martinez et al. 2008). The hinds with pregnancy confirmation on us3 and no fawn detected at the breeding control were registered as hinds with postnatal mortality.

Sampling and serology

On the first year of the study, blood samples were collected from 250 mated hinds (60, 91, 59, and 40 from farms A, B, C, and D, respectively) in three different moments: 30 to 36 days before mating, at us2, and at us3. The samples corresponded to 180 adult and 70 yearling hinds.

On the second year of study, all hinds were blood sampled at us3, from a total of 199 mated hinds (67, 60, 52, and 20 from farms A, B, C, and D, respectively), being 124 adults and 75 yearlings. An additional blood sample was collected from each hind detected with fetal mortality or abortion at the moment of the ultrasound confirmation. The blood samples were obtained by jugular punction and centrifuged, and aliquots from serum samples were preserved at −20 °C until serological analysis was performed.

All serum samples were analyzed for antibodies against *N. caninum* and *T. gondii* by indirect fluorescent antibody tests (IFAT) using a rabbit antibovine IgG fluorescein isothiocyanate conjugate, as described previously (Basso et al. 2014; More et al. 2009). Serum was diluted in base 2 and processed to end titer. Complete peripheral fluorescence of the tachyzoites was considered as positive, using the titer 100 as a cut-off. Red deer serum samples with high antibody titer to either *N. caninum* and *T. gondii* by IFAT and confirmed by immunoblot were used as positive controls. A serum sample from an aborted red deer fetus (negative by IFAT at 1 : 25 dilution and by immunoblot) was used as a negative control.

Statistical and data analysis

The following reproductive rates were estimated: pregnancy (pregnant hinds/total mated hinds), fetal mortality (hinds with fetal death/pregnant hinds), postnatal mortality (hinds with postnatal mortality/pregnant hinds), and total loss (hinds with fetal and postnatal mortality/pregnant hinds).

On the first year of the study, the *N. caninum* and *T. gondii* seropositivity rate was determined using the higher IFAT titer obtained within the 3 samples from each hind. In addition, the seroconversion (4 times titer difference between samplings) was analyzed for each protozoan. For *N. caninum* and *T. gondii* antibody titer dynamics, 3 categories were considered with "rising," "stable," and "variable" titers, using the category average antibody titer at each sampling time point.

Based on IFAT results for *N. caninum* and *T. gondii* antigens, yearling and adult hinds were classified into 3 serologic categories: high titer (HT, ≥400), low titer (LT, 100/200), and negative (N, <100). Seropositivity and high titer rates were analyzed over the following performance reproductive groups: hinds mated nonpregnant (hinds that did not conceive), hinds with fetal mortality, hinds with postnatal mortality, and hinds with fawn.

A chi-square for proportions and the odds ratio (OR) was used for the statistical analysis (www.winepi.net). For statistical significance, a p value <0.05 (CI 95%) was used and the OR > 1.6 was considered as indicative of statistical association.

Results

Serology results and the relationship with reproductive losses in the first year of study

Table 1 shows the *N. caninum* and *T. gondii* seropositivity in the 4 studied farms, where is detailed the antibody titer range and the hind age category. Seropositivity for *N. caninum* was significantly higher in farm C, and no statistical differences were observed between young and adult deer. The seropositivity for *T. gondii* was significantly lower in farm C compared to the rest of the farms, while adult hinds had a significantly higher proportion of HT category, being this value mainly influenced by the high proportion of hinds from farm B with this serologic status.

The fetal mortality rate detected in the first year of the study was 5.4% (12/224). Of the 12 hinds with fetal mortality, 8 (66.7%) were seropositive for *N. caninum* and 9 (75%) for *T. gondii*. The postnatal mortality rate was 9.4% (21/224). Of the 21 hinds with postnatal mortality, 13 (61.9%) were seropositive for *N. caninum* and 17 (81%) for *T. gondii*. In counterpart, of the 191 hinds that weaned a fawn, 78 (40.8%) were seropositive to *N. caninum* and 136 (71.2%) to *T. gondii*. The seropositivity to *N. caninum* was significantly higher in hinds with reproductive losses than hinds that weaned a fawn, with the association of *N. caninum* positivity and reproductive losses (OR = 2.5, 95% CI = 1.17–5.45, $p = 0.017$). Contrarily, there was no association between *T. gondii* seropositivity with fetal or postnatal mortality.

The sequential serological analysis carried out at 3 different times allowed to detect positive seroconversion to *N. caninum* in 6 mated hinds (5 adults and 1 yearling), of which 1 did not conceive, 4 weaned a fawn, and 1 had fetal mortality. The aborted hind had the peak of antibodies at the second sampling, reaching a titer of 3200 that was maintained until the third sampling, while the other 4 nonaborted pregnant hinds had the peak of antibodies in the third sampling reaching a titer of 400. In addition, the other 6 pregnant hinds had *N. caninum* high titers (≥400) in at least one of the samplings but not presented seroconversion, of which 4 had fetal mortality. These 4 hinds had an average titer of 11,300, 16,100, and 8100 for the first, second, and third sampling, respectively, while the other 2 nonaborted pregnant hinds had an average titer of 3400, 3600, and 3600 for the first, second, and third sampling, respectively. A strong association was detected between fetal mortality and high titers with or without seroconversion to *N. caninum* (OR = 23.5, 95% CI = 4.9–110.9, $p = 0.0001$).

Seroconversion to *T. gondii* was detected in 50 mated hinds (37 adults and 13 yearlings), of which 5 did not conceive, 44 weaned a fawn, and 1 aborted. Of the 45 pregnant hinds with seroconversion, 31 had the peak of antibodies at the second sampling with an average titer of 1761 (range 400–3200) descending at the third sampling to an average titer of 453 (range 100–1600), while the remaining 14 hinds had the peak in the third sampling with an average titer of 571 (range 400–3200). In addition, 15 pregnant hinds presented high titers (≥400) in at least one of the samplings but without seroconversion, of which 4 aborted, presenting an average antibody titer of 2040, 4453, and 413 for the first, second, and third sampling, respectively. Of the 60 pregnant hinds that presented high titers to *T. gondii* with or without seroconversion, 5 (8.3%) aborted, with no statistical association between high titers and occurrence of fetal mortality.

The *T. gondii* and *N. caninum* antibody dynamics observed from mating until the end of the gestational period is represented in Fig. 1a and b, respectively. These figures show three types of antibody dynamics: stable titers throughout the samplings, constantly rising titers, and variable titers with a peak in the middle of the gestation period. The category of stable titers included the majority of hinds for both protozoans, while rising or variable titer categories represented fewer animals. Despite the three types of dynamics that could be verified in both protozoans, a greater variability in the antibody dynamics was detected for *T. gondii* in relation to *N. caninum.*

Relationship between reproductive performance and *N. caninum* and *T. gondii* serological results in 2 years of study

From 21 abortions detected in the sequential ultrasound scanning over the entire period, 2 were at us1, 11 at us2, and 8 at us3. From 4 abortions in yearling hinds, 1 was at us1, 1 at us2, and 2 at us3, while from 17 abortions in adult hinds, 1 was at us1, 10 at us2, and 6 at us3. The range of gestational age for abortions detected at us1 and us2 was 30–87 days, while for abortions detected at us3 was 49–209 days.

Table 1 Seropositivity to *N. caninum* and *T. gondii* distributed by IFAT titer range, farm, and hind category in the first year of the study

		Yearling hinds (%)				Adult hinds (%)				Total hinds (%)			
	Farm	*n*	Seropositives	100–200	≥400	*n*	Seropositives	100–200	≥400	*n*	Seropositives	100–200	≥400
N. caninum	A	10	3 (30.0)	0 (0)	3 (30.0)	50	25 (50.0)	23 (46.0)	2 (4.0)	60	28 (46.7)^{B}	23 (38.3)^{B}	5 (8.3)^{A}
	B	32	8 (25.0)	8 (25.0)	0 (0)	59	16 (27.1)	15 (25.4)	1 (1.7)	91	24 (26.4)^{C}	23 (25.3)^{B}	1 (1.1)^{B}
	C	13	12 (92.3)	12 (92.3)	0 (0)	46	31 (67.4)	24 (52.2)	7 (15.2)	59	43 (72.9)^{A}	36 (61.0)^{A}	7 (11.9)^{A}
	D	15	4 (26.7)	3 (20.0)	1 (6.7)	25	9 (36.0)	8 (32.0)	1 (4.0)	40	13 (32.5)^{BC}	11 (27.5)^{B}	2 (5.0)^{A}
	Total	70	27 (38.6)^{a}	23 (32.9)^{a}	4 (5.7)^{a}	180	81 (45.0)^{a}	70 (38.9)^{a}	11 (6.1)^{a}	250	108 (43.2)	93 (37.2)	15 (6.0)
T. gondii	A	10	10 (100)	9 (90.0)	1 (10.0)	50	39 (78.0)	35 (70.0)	4 (8.0)	60	49 (81.7)^{A}	44 (73.3)^{A}	5 (8.3)^{A}
	B	32	20 (62.5)	8 (25.0)	12 (37.5)	59	58 (98.3)	5 (8.5)	53 (89.8)	91	78 (85.7)^{A}	13 (14.3)^{C}	65 (71.4)^{B}
	C	13	5 (38.5)	4 (30.8)	1 (7.7)	46	23 (50.0)	16 (34.8)	7 (15.2)	59	28 (47.5)B	20 (33.9)^{B}	8 (13.6)^{A}
	D	15	11 (73.3)	11 (73.3)	0 (0.0)	25	16 (64.0)	16 (64.0)	0 (0.0)	40	27 (67.5)^{A}	27 (67.5)^{A}	0 (0.0)^{A}
	Total	70	46 (65.7)^{a}	32 (45.7)^{a}	14 (20.0)^{a}	180	136 (75.6)^{a}	72 (40.0)^{a}	64 (35.6)^{b}	250	182 (72.8)	104 (41.6)	78 (31.2)

Ref. low IFAT titers = 100/200; high IFAT titers = ≥ 400. Different bold letters indicate significant differences between the same IFAT category from adult and yearling hinds. Different capital letters indicate significant differences between farms

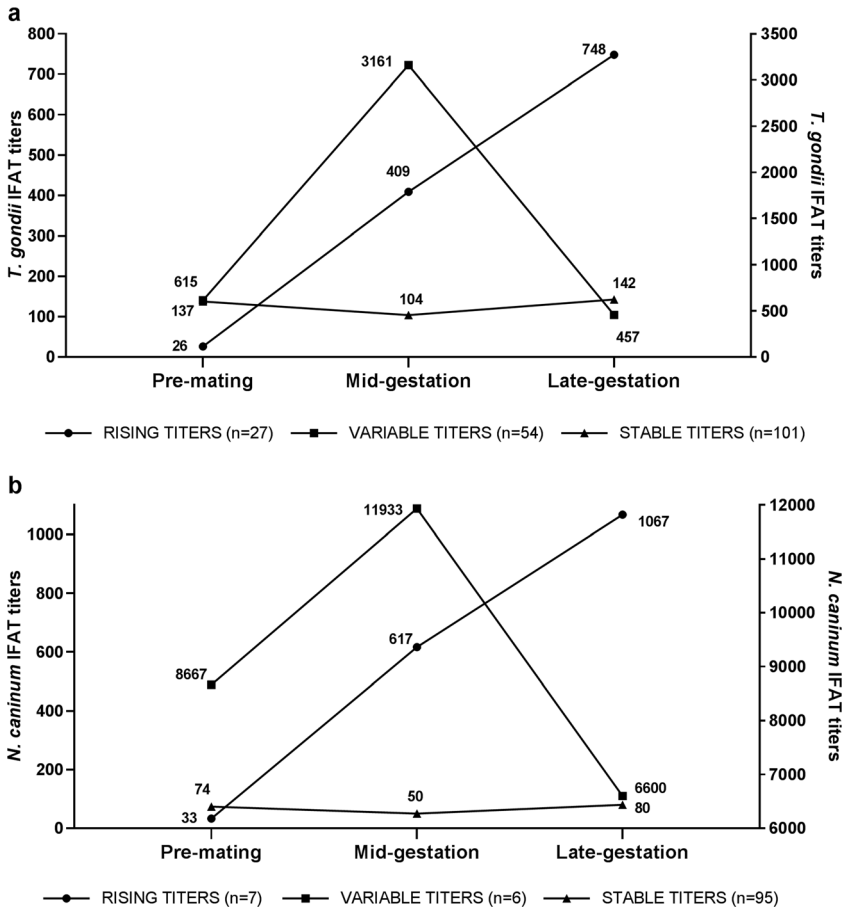

Fig. 1 Dynamics of average *T. gondii* **(a)** and *N. caninum* **(b)** IFAT titers for each group detected from mating until the end of the gestation period in seropositive hinds. **a** Standard deviations for pre-mating, mid-gestation, and late-gestation, respectively. Rising titer group: 112, 862, and 937; stable titer group: 120, 85, and 95; and variable titer group: 72, 2060, and 448. **b** Standard deviations for pre-mating, mid-gestation, and late-gestation, respectively. Rising titer group: 52, 765, and 1150; stable titer group: 67, 64, and 60; and variable titer group: 9495, 11,506, and 9552

A total of 449 hinds (145 yearlings and 304 adults) were evaluated, of which 31.4% (141/449) and 73.7% (331/449) were seropositive for *N. caninum* and *T. gondii*, respectively. The seropositivity rate for *N. caninum* was 25.5% (37/145) for the yearlings and 34.2% (104/304) for the adults, while for *T. gondii* was 64.3% (93/145) and 78.3% (238/304), respectively.

The serological categories of hinds (HT, LT, and N) for *N. caninum* and *T. gondii* were compared based on their reproductive performance over the 2 years of study (Table 2).

The total reproductive loss rate was significantly higher in seropositive hinds to *N. caninum*, compared to seronegatives (20.1%; 26/129 versus 9.8%; 26/266), evidencing an association between seropositivity and total reproductive loss (OR = 2.3, 95% CI = 1.3–4.2, $p = 0.005$). Hinds from *N. caninum* HT category showed higher total reproductive loss than hinds from N category (OR = 8.1, 95% CI = 3.3–19.8, $p < 0.0001$), influenced by the high fetal mortality rate in both yearling and adult hinds (Table 2). In addition, fetal mortality was significantly higher in *N. caninum* seropositive hinds compared to seronegative ones (9.3%, 12/129 versus 3.4%, 9/266), with a marked susceptibility to this reproductive loss in the HT category. Significantly higher fetal mortality rates in *N. caninum* HT category were also detected in each particular farm where HT hinds were present, being 44.4% (4/9), 45.5% (5/11), and 50% (1/2) for farms A, C, and D, respectively, while in the N category was 3.8% (3/79), 2.5% (3/118), 4.3% (2/46), and 4.3% (1/23) for farms A, B, C, and D, respectively.

Neospora caninum seropositive adult hinds had a significantly higher postnatal mortality rate than seronegatives, specifically due to the high postnatal loss that occurred in the LT category. No significant differences were found in postnatal mortality of yearling hinds between *N. caninum* seropositives and seronegatives. The weaning rate of *N. caninum* HT hind category (44%, 11/25) was significantly lower than that of LT (79.3%, 92/116) and N (78%, 240/308) hind categories.

On the other hand, when the *T. gondii* IFAT titer categories were analyzed, no significant differences or associations were found in pregnancy, fetal mortality, postnatal mortality, or weaning rates, both in the global analysis and by hind age (Table 2). The fetal mortality rate in *T. gondii* seropositive hinds was 5.1% (15/292), while in seronegative hinds was 5.8% (6/103). The postnatal mortality rate was slightly higher

Table 2 Reproductive efficiency according to *N. caninum* and *T. gondii* IFAT titers over the 2-year period of the study

Antibody titers		Yearling hinds					Adult hinds					Total hinds				
		Mated	Pregnancy	Fetal mortality	Postnatal mortality	Total loss	Mated	Pregnancy	Fetal mortality	Postnatal mortality	Total loss	Mated	Pregnancy	Fetal mortality	Postnatal mortality	Total loss
		n	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)
N. caninum	HT (≥ 400)	4	2 (50)	1 (50)	1 (50)	2(100)	21	20 (95.2)	9 (45)	0 (0)	9 (45.0)	25	22 (88.0)^{A}	10 (45.5)^{A}	1 (4.5)^{A}	11 (50.0)^{A}
	LT (100/200)	33	25 (75.7)	0 (0)	5 (20.0)	5 (20)	83	82 (98.8)	2 (2.4)	8 (9.7)	10 (12.2)	116	107 (92.2)^{A}	2 (1.9)^{B}	13 (12.1)^{B}	15 (14)^{B}
	N (< 100)	108	74 (68)	3 (4.1)	13 (17.6)	16 (21.7)	200	192 (96)	6 (3.1)	4 (2.1)	10 (5.2)	308	266 (86.4)^{A}	9 (3.4)^{B}	17 (6.4)^{A}	26 (9.8)^{B}
T. gondii	HT (≥ 400)	43	31 (72.1)	2 (6.5)	7 (22.6)	9 (29)	117	112 (95.7)	8 (7.1)	4 (3.6)	12 (10.7)	160	143 (89.4)^{A}	10 (7.0)^{A}	11 (7.7)^{A}	21 (14.7)^{A}
	LT (100/200)	50	31 (62.0)	1 (3.2)	7 (22.6)	8 (25.8)	121	118 (97.5)	4 (3.4)	6 (5.1)	10 (8.5)	171	149 (87.1)^{A}	5 (3.3)^{A}	13 (8.7)^{A}	18 (12)^{A}
	N (< 100)	52	39 (75.0)	1 (2.6)	5 (12.8)	6 (15.4)	66	64 (97.0)	5 (7.8)	2 (3.1)	7 (10.9)	118	103 (87.3)^{A}	6 (5.8)^{A}	7 (6.8)^{A}	13 (12.6)^{A}
	Total	145	101 (69.7)$^{\mathbf{b}}$	4 (4)$^{\mathbf{a}}$	19 (18.8)$^{\mathbf{a}}$	23 (22.8)$^{\mathbf{a}}$	304	294 (96.7)$^{\mathbf{a}}$	17 (5.8)$^{\mathbf{a}}$	12 (4.1)$^{\mathbf{b}}$	29 (9.9)$^{\mathbf{b}}$	449	395 (88.0)	21 (5.3)	31 (7.8)	52 (13.1)

Ref. HT (≥ 400) = high IFAT titers; LT (100/200) = low IFAT titers; N = negative or titer lower than 100. Different bold letters indicate significant differences between the same IFAT category from adult and yearling hinds. Different capital letters indicate significant differences between IFAT titer categories for each protozoan

in *T. gondii* seropositive females (8.2%; 24/292) than in seronegatives (6.8%; 7/103) but without statistical significance. In the same way, *T. gondii* seropositive yearling hinds had a higher rate of fetal mortality (4.8%, 3/62 vs. 2.6% 1/39) and postnatal mortality (22.6%, 14/62 vs. 12.8%, 5/39) in comparison with seronegatives; however, there were no statistically significant differences.

The reproductive status categories of all hinds were correlated with seropositivity and high titers to *N. caninum* and *T. gondii* (Table 3). Hinds with fetal mortality were more likely to be seropositive to *N. caninum* (OR = 3.1, 95% CI = 1.2–7.6, p = 0.013) or have high *N. caninum* titers (OR = 27.4, 95% CI = 9.6–78.0, p = <0.0001) in comparison with hinds that weaned a fawn. The analysis of hinds with coinfection to both protozoans showed a statistical association between fetal mortality and seropositivity (OR = 3.2, 95% CI = 1.3–7.9, p = 0.009). The hinds that presented postnatal mortality had a higher *N. caninum* seropositivity rate than hinds that weaned a fawn; however, this difference was statistically not significant.

The average conception dates were April 26 and April 25 in yearling hinds, and March 3 and April 4 in adult hinds for *N. caninum* seropositive and seronegative categories, respectively. The average conception dates were April 27 and April 23 in yearling hinds, and April 2 and March 31 in adult hinds for *T. gondii* seropositive and seronegative categories, respectively. No statistical differences were found in conception dates between seropositive and seronegative hinds to both protozoans.

Discussion

This is the first study on reproductive losses and their relationship with epidemiological and seroprevalence data of *N. caninum* and *T. gondii* infections in commercial red deer herds from Argentina.

The ultrasonographic methodology applied in this study with the performance of 3 scannings allowed to monitor the entire gestational period and determine a reliable abortion rate, being this information rarely recorded worldwide (Wilson et al. 2012). In none of the investigated farms, it was possible to find any aborted fetus or observe some hind with clinical signs of abortion, showing the difficulty of detection of fetal losses under field conditions. The early gestational age of most of the ultrasound-detected abortions could be an explanation of this finding, considering that 62% of them had ≤87 days. These circumstances suggest that abortions in red deer hinds are possibly underdiagnosed based on clinical/observational criteria and that sequential ultrasound scanning represents a reliable method to diagnose fetal mortality and aborted hinds. The ultrasonographic detection of full-term pregnancies at the end of gestation period (us3) in conjunction with an after calving control allowed to estimate the postnatal mortality rate, resulting in an adequate and safe method to quantify reproductive losses occurred postpartum. As was mentioned for the abortion rate, the postnatal mortality is also a reproductive loss not commonly reported or properly determined (Wilson et al. 2012).

In Argentina, until now, there is no seroprevalence data available for *N. caninum* and *T. gondii* infection in farmed red deer, and the serological results obtained in the present study suggests that these protozoans are present with high frequency in all the analyzed farms, with higher seropositivity rates than those reported in other countries. There are very few reports worldwide of *N. caninum* seroprevalence in farmed red deer. In a previous study conducted in the Czech Republic, a seroprevalence of 6% (24/377) was informed using competitive ELISA and confirmed by IFAT (Bartova et al. 2007). In Poland, an investigation from an area with documented evidence of neosporosis in cattle reported a seroprevalence of 11.3% (12/106) using ELISA and Western blot as a confirmatory test (Gozdzik et al. 2010), while in New Zealand, a 0% (0/42) was informed from aborted and pregnant hinds using a latex agglutination test (Wilson et al. 2012).

Regarding the available data about *T. gondii* infection in farmed red deer, variable seropositivity rates between studies have been published, being of 6.6% (23/348) in Ireland (Halová et al. 2013), 45% (169/377) in the Czech Republic (Bartova et al. 2007), and 30.3% (369/1218) and 42.5% (17/40) in New Zealand (Patel et al. 2019; Wilson et al. 2012), showing that toxoplasmosis is frequent in captive red deer herds around the world.

In the present study, we found that 23.4% of the hinds presented coinfection with *N. caninum* and *T. gondii*. To our knowledge, only one previous study (Rocchigiani et al. 2016) informed coinfection with both protozoans in red deer with a rate of 8% (5/60); however, the animals studied were wild deer, which could explain the lower coinfection rate found. The high seroprevalence recorded in our study for both protozoans could influence the higher coinfection rate detected, especially considering the high seropositivity to *T. gondii*.

A significant association was demonstrated between *N. caninum* seropositivity and fetal mortality, where seropositive hinds are approximately three times more likely to abort than seronegative hinds (OR = 3.1). Similar results were described in cattle by Dubey and Schares (2006), mentioning that chronically infected seropositive cows have an about two- to threefold increased risk of abortion compared to seronegative cows. Our data also showed that, within seropositive hinds, those with high antibody titers (≥400) had a greater probability to abort (OR = 27.4) than hinds with low (100–200) antibody titers, having been observed both globally and at each studied farm, showing repeatability in the results and consequently a stronger statistical significance. Moreover, in the farms where hinds with high antibody titers were detected, the abortion rate from this group of hinds represented more than half of the total abortions (average 55%; range 44.4%–

Table 3 Proportion of seropositivity and high IFAT titers to *N. caninum* and *T. gondii* in relation to each reproductive category group of hinds

Reproductive category	*n*	*N. caninum*		*T. gondii*		*N. caninum* and *T. gondii**
		Seropositive (%)	Titers ≥400 (%)	Seropositive (%)	Titers ≥400 (%)	Seropositive (%)
Mated nonpregnant	54	12 (22.2)^{A}	3 (5.5)^{A}	39 (72.2)^{A}	17 (31.5)^{A}	10 (18.5)^{B}
Hinds with fetal mortality	21	12 (57.1)^{C}	10 (47.6)^{B}	15 (71.4)^{A}	10 (47.6)^{A}	10 (47.6)^{A}
Hinds with postnatal mortality	31	14 (45.2)^{BC}	1 (3.2)^{A}	24 (77.4)^{A}	11 (35.5)^{A}	10 (32.3)^{AB}
Hinds with fawn	343	103 (30.0)^{AB}	11 (3.2)^{A}	253 (73.8)^{A}	122 (35.6)^{A}	75 (21.9)^{B}
Total	449	141 (31.4)	25 (5.6)	331 (73.7)	160 (35.6)	105 (23.4)

Ref. different letters indicate significant differences between reproductive performance categories for each protozoan. * = seropositive to both protozoans (coinfection)

71.4%), demonstrating the high level of reproductive losses that can be caused by neosporosis where hinds with high titers are present in a herd. In the same way, in cattle, it has been reported that cows aborting due to neosporosis often have higher *N. caninum*-specific antibody levels than infected but nonaborting cows (Dubey and Schares 2006). In accordance with this, in the present study, the 83.3% (10/12) of aborted seropositive hinds and 10.2% (12/117) of nonaborted seropositive hinds presented high antibody titers (≥400). The trial carried out during the first year to identify seroconversion determined that within the hinds showing seroconversion to *N. caninum*, the only one that aborted had a peak of antibodies earlier and higher than the nonaborted hinds. In agreement with this, Guy et al. (2001) found that cows presenting the increase of antibody titer during the first half of the gestational period had a higher probability to abort than cows that did it in the second half. In this sense, Innes et al. (2002) suggested that the earlier in gestation the *N. caninum* parasitaemia occurs, the more severe the consequences to the fetus. Therefore, according to the findings discussed before, many similarities were found with cattle in relation to *N. caninum* serologic results and its relationship with fetal mortality.

With regard to postnatal mortality, a significantly higher rate was detected specifically in the *N. caninum* LT category of adult hinds, and interestingly, this finding was not observed in the HT category of adult hinds. On the other hand, despite yearling hinds had a significantly higher postnatal mortality than adults, no statistical association was observed with *N. caninum* serology, suggesting that this high postnatal loss recorded in yearling hinds was not related to neosporosis. According to these results, we consider that it is not possible to confirm or discard *N. caninum* as an agent causing postnatal mortality in red deer hinds, being advisable to continue investigating about it.

The lack of association found between *T. gondii* seropositivity and reproductive losses indicate that this protozoan infection was not related to fetal and postnatal mortality in the studied deer herds. Reinforcing this finding, the hinds that developed seroconversion to *T. gondii* showed no significant reproductive losses. The high seropositivity rates to *T. gondii* found in all the farms suggest a high exposure to this parasite, and the fact that high infection rates were recorded at an early age of 14 months means that most of the animals became infected very young, probably during the first year of life. On the other hand, considering that transplacental transmission of *T. gondii* has been previously demonstrated in red deer hinds (Patel et al. 2019; Wilson et al. 2012) and that most of the fawns are born from seropositive dams due to the high prevalence of toxoplasmosis in all the herds, vertical transmission should be taken into account to explain the high positivity rates observed.

Our data showed that most of hinds resulted seropositives to *T. gondii* at the first blood sampling, meaning that an immune response was developed against this protozoan infection prior to the mating. The presence of protective antibodies could prevent the presentation of abortions from potential *T. gondii* reinfections during the gestational period, as usually occurs in other ruminant species such as sheep (Buxton 1998). Therefore, a solid protective immunity against *T. gondii* in yearling and adult hinds could be considered to explain the lack of association found between seropositivity and reproductive losses. However, the possibility that red deer have a natural resistance to clinical toxoplasmosis as has been described in cattle and water buffaloes (Lindsay and Dubey 2020) should not be ruled out. In previous studies (Patel et al. 2019), no association was found between *T. gondii* seropositivity and abortion in adult females, whereas in yearlings, a low association (OR = 1.6) was reported. Other authors detected the presence of *T. gondii* DNA by PCR in fetal tissues from aborted and nonaborted fetuses (Patel et al. 2019; Wilson et al. 2012); however, in these works, histopathology and immunohistochemistry analyses were not performed to detect the presence of tissue lesions in fetus, placenta, or uterus in order to confirm that abortions were caused by toxoplasmosis. At any case, these studies confirmed the vertical transmission and the

presence of the protozoan in fetal tissues. Therefore, although there is evidence that *T. gondii* can infect the fetus, it still is uncertain the extent to which this protozoan can cause reproductive losses in red deer hinds, and more thorough investigation is required in this field.

The data obtained in relation to seropositivity both to *N. caninum* and *T. gondii* and pregnancy rate indicate that the infection with these protozoans would not affect the hind fertility and hence the conception rate. Furthermore, the similarity in the average conception dates found between seropositive and seronegative hinds for both agents reinforces this assumption. In coincidence with this finding, Patel et al. (2019) also found no association between *T. gondii* infection and pregnancy rate, while to our knowledge, there are no investigations that have evaluated *N. caninum* infection and its relationship with pregnancy rate in red deer.

A significantly higher fetal mortality was found in hinds with coinfection for both protozoans; however, this result could have been influenced by the strong association observed between fetal mortality and *N. caninum* positivity. The similarity in the ORs found between *N. caninum* infection and coinfection is also in agreement with this observation. Therefore, we consider that this result could be due to *N. caninum* infection instead of synergism of both infections. This assumption also suggests that coinfection with both protozoans in red deer would not cause a greater probability of suffering fetal mortality.

Despite seroconversion to *N. caninum* and *T. gondii* during the gestational period was detected both in yearling and adult hinds, an association with a higher probability of fetal mortality was solely observed for *N. caninum*. The antibody dynamics observed for both protozoan infections showed most seropositive hinds with a stable titer pattern from mating to the end of gestation; however, a low proportion of hinds developed an increase in antibody titers doing a peak in the middle or at the end of gestation period, having been this phenomenon more frequent for *T. gondii* antibody dynamics. This characteristic has also been described in other domestic species for both protozoans, which probably is an indirect indicator of infection recrudescence due to parasitic multiplication as a consequence of immunological depression that occurs in the middle gestational period (Innes et al. 2002; Rodriguez et al. 2016). The evaluation of *N. caninum* antibody dynamics also allowed the detection of a hinds group with high antibody titers in the three blood samplings and presenting a high fetal mortality rate (66.7%), not only indicating the presence of a chronic infection but also suggesting a low protective level of antibodies against this protozoan. Similar immunological behavior has been observed in cattle where the *N. caninum* infection does not generate an effective immunity (Innes et al. 2002).

In summary, the lack of association found between seropositivity and reproductive losses indicates that *T. gondii* had not an important role as an abortigenic agent or cause of postnatal mortality. The immunity development observed against this protozoan in most of the hinds was probably effective to avoid reproductive losses; however, this hypothesis should be confirmed in future investigations. It would be advisable to carry out more research to know whether *T. gondii* could have some degree of pathogenicity in first exposed hinds during pregnancy. The *N. caninum* and *T. gondii* antibody dynamics results informed represent the first report of immunological response to these protozoans in red deer hinds during the mating and the whole gestational period. This information is of special interest for the interpretation of serological results in future research works, as well as in control strategies for these diseases in red deer herds. The high association found between *N. caninum* seropositivity and fetal mortality provides strong evidence for the first time that *N. caninum* could be an important abortigenic agent in infected red deer hinds. Serologic evidence showed that approximately half of abortions detected in the present study were attributed to *N. caninum* infection, suggesting that neosporosis could be responsible for considerable reproductive losses in Argentinean farmed red deer. The increased susceptibility to abort in hinds with high *N. caninum* antibody titers would justify a control program based on serological detection and subsequent elimination to reduce fetal mortality rate in red deer farms.

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Declarations

Conflict of interest All the authors declare no conflicts of interest.

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