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Dose-dependent effects of experimental infection with the virulent *Neospora caninum* Nc-Spain7 isolate in a pregnant mouse model

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ABSTRACT

Pregnant BALB/c mice have been widely used as an in vivo model to study *Neospora caninum* infection biology and to provide proof-of-concept for assessments of drugs and vaccines against neosporosis. The fact that this model has been used with different isolates of variable virulence, varying infection routes and differing methods to prepare the parasites for infection, has rendered the comparison of results from different laboratories impossible. In most studies, mice were infected with similar number of parasites (2×10^6) as employed in ruminant models (10^7 for cows and 10^6 for sheep), which seems inappropriate considering the enormous differences in the weight of these species. Thus, for achieving meaningful results in vaccination and drug efficacy experiments, a refinement and standardization of this experimental model is necessary. Thus, 2×10^6, 10^5, 10^4, 10^3 and 10^2 tachyzoites of the highly virulent and well-characterised Nc-Spain7 isolate were subcutaneously inoculated into mice at day 7 of pregnancy, and clinical outcome, vertical transmission, parasite burden and antibody responses were compared. Dams from all infected groups presented nervous signs and the percentage of surviving pups at day 30 postpartum was surprisingly low (24%) in mice infected with only 10^2 tachyzoites. Importantly, infection with 10^5 tachyzoites resulted in antibody levels, cerebral parasite burden in dams and 100% mortality rate in pups, which was identical to infection with 2×10^6 tachyzoites. Considering these results, it is reasonable to lower the challenge dose to 10^5 tachyzoites in further experiments when assessing drugs or vaccine candidates.

1. Introduction

The obligate-intracellular protozoan parasite *Neospora caninum* (Apicomplexa: Sarcocystidae) is a major cause of reproductive failure in cattle which causes substantial economic losses (Dubey and Schares, 2011; Reichel et al., 2013). *N. caninum* has a heteroxenous life cycle, in which dogs and other canids have been shown to act as definitive hosts, and cattle and other ungulates represent natural intermediate hosts (McAllister et al., 1998; Dubey and Schares, 2011; Dubey et al., 2011). Neosporosis is generally latent and asymptomatic in non-pregnant cattle, yet the outcome of either primo-infection or recrudescence in a pregnant cow can be abortion, birth of weak calves or of clinically healthy but congenitally infected calves (Dubey et al., 2007).

Although cattle is the most important target species, the mouse has been the most widely used experimental model to study biological aspects of *N. caninum* infection. Despite the obvious limitations and physiological differences between mice and ruminants, the pregnant mouse model has also been applied extensively to provide initial results on the efficacy of novel drugs or vaccines of *N. caninum* infection (Benavides et al., 2014; Monney and Hemphill, 2014). Indeed, the use of laboratory mice is of advantage in terms of low cost, easy handling, short gestation period and litter size (López-Pérez et al., 2006). Following experimental infection, mice exhibit acute-disease symptoms such as rough hair coat, inactivity, anorexia within 6–12 days after infection, or chronic neurological disease symptoms later on, such as head tilt, circular movements, ataxia, hind limb weakness or paralysis (Collantes-Fernández et al., 2006). Furthermore, when inoculation is performed during pregnancy, the parasite is efficiently transmitted from dam to foetus

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causing foetal loss and/or similar clinical signs in offspring mice (López-Pérez et al., 2008). Unfortunately, the large number of reported experimental infections conducted in mice differ with regard to different parameters such as the preparation of parasites used for infection, the route of administration, the isolate, dose or timing of infection and the mouse strains used. All of this results in a high degree of variability, rendering meaningful comparison of results from different laboratories an impossible task. Thus, the standardization of a *Neospora* mouse model would enable more robust comparisons of results among experiments (Benavides et al., 2014; Monney and Hemphill, 2014).

One of the most commonly employed models for the preliminary assessment of intervention strategies are pregnant BALB/c mice experimentally infected at mid-gestation, typically with 2×10^6 tachyzoites of the Nc-Liv or Nc-1 isolate (e.g., Liddell et al., 1999; Nishikawa et al., 2001; López-Pérez et al., 2008; Aguado-Martínez et al., 2009; Debache et al., 2009; Zhang et al., 2010; Marugán-Hernández et al., 2011; Monney et al., 2012; Pastor-Fernández et al., 2015). However, there are clear indications that this infection dose is much too high: (i) mice were infected with a similar number of parasites (2×10^6) as normally employed in ruminant models such as 10^7 for cows and 10^6 for sheep, which are sufficient to cause foetal death when inoculated during the first term of gestation (Williams et al., 2007; Caspe et al., 2012; Regidor-Cerrillo et al., 2014; Arranz-Solís et al., 2015); (ii) in terms of body weight mice differ from cattle and sheep to a much higher degree; (iii) in many of these mouse studies dams developed clinical signs of disease, something which does not occur in ruminants. This could give rise to biased results in vaccine screenings, and might lead to a premature dismissal of potential active formulations, even more if a highly virulent isolate, such as Nc-Liv, is used.

The aim of this study was to standardize and refine the pregnant BALB/c mouse model for *N. caninum* infection. For that purpose, pregnant BALB/c mice were experimentally infected with 2×10^6, 10^5, 10^4, 10^3 or 10^2 *N. caninum* tachyzoites. Challenge was carried out using the Nc-Spain7 isolate, which had been earlier obtained from an asymptomatic calf (Regidor-Cerrillo et al., 2008). This isolate has a controlled low number of passages in cell culture and its high degree of virulence has been demonstrated in vitro (Regidor-Cerrillo et al., 2011) and in vivo (Regidor-Cerrillo et al., 2010; Caspe et al., 2012; Collantes-Fernández et al., 2012; Dellarupe et al., 2014; Regidor-Cerrillo et al., 2014; Arranz-Solís et al., 2015). Our findings could provide the basis for a standardized pregnant mouse model for the evaluation of drug and vaccine candidates under unified experimental criteria.

2. Material and methods

2.1. Parasite culture and dose preparation

N. caninum tachyzoites of the Nc-Spain7 isolate (Regidor-Cerrillo et al., 2008) were propagated by continuous passages in Vero cell culture maintained in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml at 37 °C with 5% CO_2 in tissue culture flasks. For the challenge, tachyzoites (passage 15) were recovered from culture flasks when they were still largely intracellular (>90% of undisrupted parasitophorous vacuoles) and infected cells were repeatedly passed through a 25-gauge needle at 4 °C. The number of viable tachyzoites was estimated by Trypan blue exclusion (typically 95–99%) followed by counting the viable tachyzoites in a Neubauer chamber. Subsequently, viable tachyzoites were adjusted to the required dose (2×10^6, 10^5, 10^4, 10^3, 10^2) by dilution in culture medium, and were subcutaneously injected in a final volume of 200 μl per mouse. Infection took place within 30 min of harvesting from tissue culture.

2.2. Mice and ethics statement

Animal procedures were approved by the animal welfare committee of the Canton of Bern (approval No. BE 105/14) and followed the corresponding guidelines. 118 BALB/c females and 59 males were purchased from Charles River Laboratories (Sulzheim, Germany) at the age of 6 weeks and were maintained in a common room under conventional day/night cycle housing conditions, according to the standards approved by the animal welfare legislation of the Swiss Veterinary Office. Animals were used for experimentation after 4 weeks of acclimatization.

2.3. Experimental design, sampling and data collection

Pregnancy was achieved after synchronization of oestrus by the Whitten effect (Whitten, 1957) followed by mating (1 male housed with 2 females) for three nights. Subsequently, female mice were randomly distributed in six groups and subcutaneously challenged with 2×10^6 (G1; $n = 20$), 10^5 (G2; $n = 20$), 10^4 (G3; $n = 19$), 10^3 (G4; $n = 19$) and 10^2 (G5; $n = 20$) tachyzoites of the Nc-Spain7 isolate at mid gestation (days 7–10 after mating), while mice from group 6 (G6; $n = 20$) were left unchallenged and received a culture media inoculation. Pregnancy was confirmed by weighing at days 15–18 of gestation, and pregnant mice were then allocated separately to rear their pups. Dams and their offspring were evaluated twice a day from birth to day 30 postpartum (pp). Data on pregnancy rate (percentage of female mice housed with males that became pregnant), litter size (number of delivered pups per dam), early pup mortality (number of full-term dead pups from birth until day 2 pp), post-natal mortality (number of dead pups from day 3 to 30 pp) and clinical signs of dams and non-pregnant mice were recorded during this time. Clinical signs were scored according to the description made by Pastor-Fernández et al. (2015). Briefly, the general appearance of mice and the presence of clinical signs compatible with neosporosis were recorded and scores of 0 (no alterations), 1 (ruffled coat), 2 (rounded back), 3 (noticeable loss of body condition/severe weight loss) or 4 (nervous signs such as activity decrease, hind limb paralysis, walking in circles or head tilt) were given depending on the severity of the clinical signs. Non-pregnant mice were weighed once a week after the challenge, whereas pregnant mice were weighed at days 15 and 30 pp and neonates every second day from day 15 pp onwards until the end of the experiment (day 30 pp). Day 15 pp was chosen as a starting point for weight monitoring in order to avoid excessive handling of the pups during the first 2 weeks after birth, which might result in rejection by the dams. As a humane endpoint, mice exhibiting evident loss of body condition (score of 3) or nervous signs (score of 4) were culled to limit unnecessary suffering. Surviving dams, non-pregnant mice and pups were euthanized in a CO_2 chamber at 30 days pp. Blood from dams and non-pregnant mice was recovered by cardiac puncture and sera were obtained to test humoral immune responses. Brains from dams, non-pregnant mice and surviving pups were also sampled for parasite detection and quantification by quantitative real time PCR. Samples were stored at −20 °C until further analysis.

2.4. Humoral immune responses

N. caninum-specific IgG1 and IgG2a serum isotypes were determined by ELISA in dams and non-pregnant mice as previously described (Marugán-Hernández et al., 2011). Briefly, ELISA was performed in plates coated with a soluble *N. caninum* tachyzoite antigen (Álvarez-García et al., 2002), using a 1:100 dilution of sera

Table 1
Summary of the effects of *Neospora* Nc-Spain7 infection in pregnant mice.

Group[a]	Pregnancy rate[b]	Litter size[c]	Morbidity[d]	Mortality[e]	Time of euthanasia (days pp)[f]	Parasite detection[g]
G1 (2×10^6)	11/20 (55%)	4.91 ± 2.43	10/11 (91%)	6/11 (54%)	11,12,17,17,24,25	11/11 (100%)
G2 (10^5)	15/20 (75%)	6.27 ± 2.28	8/15 (53%)	6/15 (40%)	14,19,23,23,24,25,25	15/15 (100%)
G3 (10^4)	9/19 (47%)	5.78 ± 2.05	8/9 (89%)	5/9 (56%)	17,18,24,28,29	9/9 (100%)
G4 (10^3)	11/19 (58%)	6.55 ± 2.16	10/11 (91%)	6/11 (54%)	14,17,23,29,29,29	11/11 (100%)
G5 (10^2)	11/20 (55%)	5.45 ± 1.69	4/11 (36.4%)	2/11 (18.2%)	15,19	9/11 (81.8%)
G6 (unchallenged)	12/20 (60%)	5.50 ± 1.68	0/12 (0%)	0/12 (0%)		0/12 (0%)

[a] Mice challenged with Nc-Spain7 tachyzoites.
[b] No. of pregnant mice/mice housed with males (percentage).
[c] Average number of full-term delivered pups (±SD).
[d] No. dams showing clinical signs (score ≥ 1)/no. of dams in the group (percentage).
[e] No. of dead or culled dams before day 30 pp/no. of dams in the group (percentage).
[f] Days pp when sacrifice of dams that displayed clinical signs (score 3 and 4) were performed.
[g] No. of brain PCR positive dams/no. of dams in the group (percentage).

samples and an anti-mouse IgG1 or IgG2a peroxidase-conjugated as secondary antibody (1:5000, Southern biotechnology). Sera from mice experimentally infected with Nc-Liv and non-infected mice from previous experiments (Pastor-Fernández et al., 2015) were used as positive and negative controls, respectively. For each plate, values of the optical density read at 405 nm wavelength (OD_{405}) were converted into a relative index percent (RIPC) using the following formula RIPC = (OD_{405} sample − OD_{405} negative control)/(OD_{405} positive control − OD_{405} negative control) × 100.

2.5. Parasite detection and quantification in brains

DNA extraction from brain tissue was carried out as previously described (Monney et al., 2011). DNA concentration was measured using the Quantifluor® dsDNA kit (Promega) following manufacturer's recommendations and was adjusted to 5 ng/μl. Neospora-specific quantitative real-time PCR was performed from 20 ng of DNA as described by Müller et al. (2002), using the Light Cycler™ Instrument (Roche Diagnostic, Basel, Switzerland). Parasite burden was calculated by interpolation from a standard curve with DNA equivalents from 1000, 100 and 10 tachyzoites included in each run. Parasite load was expressed as parasite number/μg host DNA.

Tissues from pups that succumbed to infection from days 3 to 30 pp were not analyzed and considered as PCR-positive according to previous findings (Dellarupe et al., 2014).

2.6. Statistical analysis

Differences in pregnancy rates, early pup mortality, morbidity and parasite presence in brains were analyzed by Chi-square (χ^2) and Fisher *F*-tests. Post-natal mortality was analysed by the Kaplan–Meier survival method (Bland and Altman, 1998) to estimate the percentage of surviving animals at each time point. The Log-rank test was applied to compare the survival curves between different groups (Bland and Altman, 2004) and the median survival time, i.e., the day at which 50% of the pups died, was calculated. For pair-wise comparisons, a value of $P < 0.05/k$ was considered statistically significant, where *k* corresponded to the number of groups. One-way ANOVA followed by Tukey's multiple test were employed to compare anti-*N. caninum* antibody levels, litter size and body weights. In addition, unpaired two-tailed *t*-test was used for comparisons between IgG1 and IgG2a levels within each group. Parasite burdens were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups. To further comparisons of parasite loads and antibody levels between dams and non-pregnant mice, the *U* Mann-Whitney test and unpaired two-tailed *t*-test, respectively, was applied. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were carried out using GraphPad Prism 6 (v.6.01) software.

3. Results

3.1. Evaluation of N. caninum infection in dams

Data on pregnancy rates, litter size, morbidity, mortality and parasite presence in dams are summarized in Table 1.

3.1.1. Pregnancy rate and litter size

For all groups, pregnancy rates ranged from 47 to 75%, with no significant differences among them. Similarly, no differences between the groups were found regarding the litter sizes (4.91–6.55 delivered pups), suggesting that pregnancy was not noticeably altered by infection with different doses.

3.1.2. Morbidity and mortality

Skin lesions at the site of parasite inoculation (interscapular region) were observed after 1–2 weeks post-infection (p.i.) in 4/11 dams from G1 (2×10^6), 5/15 from G2 (10^5) and 2/11 from G5 (10^2). These consisted of dermal nodules and small scabs that eventually resolved throughout the experiment. No other lesions were found in the remaining groups. On the other hand, dams from all infected groups exhibited clinical signs such as ruffled coat (score = 1), rounded back (score = 2) and severe weight loss (score = 3) from the second week p.i. onwards. Moreover, 6/11 dams from G1 (2×10^6), 6/15 from G2 (10^5), 5/9 from G3 (10^4), 6/11 from G4 (10^3) and 2/11 from G5 (10^2) had to be culled prior to day 30 pp due to the severity of the clinical signs (Fig. 1A, Table 1). These dams were euthanized from day 11 pp (22–25 days p.i.) onwards (Table 1). For all of them, no surviving pups remained in their litter at the time of euthanasia. No clinical signs were observed in the unchallenged group (G6).

3.1.3. Body weight

Significantly lower body weights compared to the unchallenged group were found at day 15 pp in dams infected with 2×10^6 (G1), 10^5 (G2), 10^4 (G3) and 10^3 (G4) tachyzoites ($P < 0.05$), while infection with 10^2 tachyzoites did not have such an impact (Fig. 1B).

3.1.4. Quantification of cerebral parasite load

N. caninum DNA was detected in the brain of all dams from infected groups, with the exception of 2 mice in G5 (10^2). Moreover, when comparing parasite burden between groups, no significant differences were found among infected groups (G1-G5). (Fig. 1C).

3.1.5. Humoral immune responses

All infected dams developed *Neospora*-specific humoral immune responses at day 30 pp, with IgG1 and IgG2a antibody levels significantly increased in comparison to the unchallenged

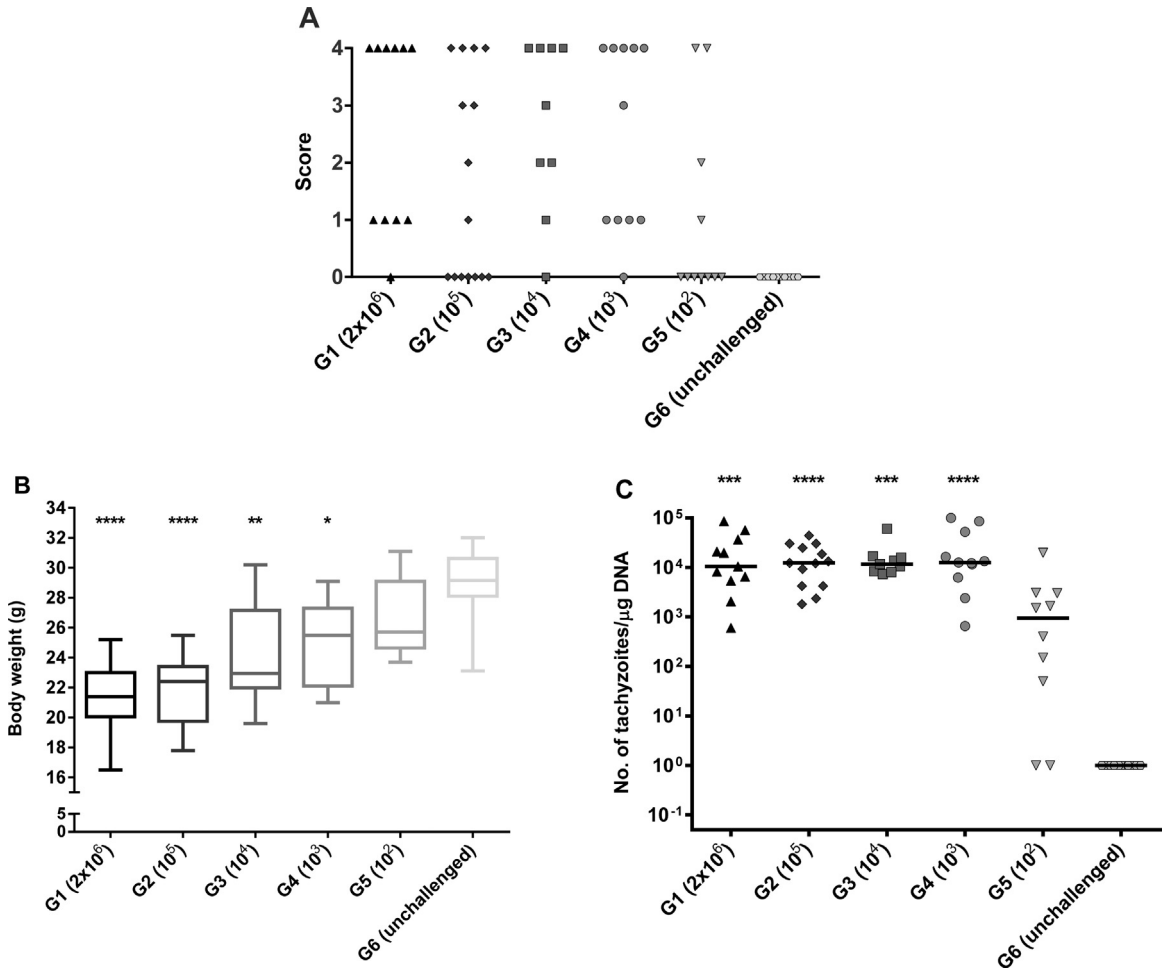

Fig. 1. Effect of *N. caninum* Nc-Spain7 isolate inoculation in pregnant mice.
(A) Morbidity in dams. Scores were based on the detection and severity of clinical signs after challenge (1, ruffled coat; 2, rounded back; 3, severe weight loss or 4, nervous signs). Each point represents a single animal. (B) Body weights in dams after 15 days pp. Box-plot graphs represent the median weight in grams, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers). (****), (**) and (*) above box-plots indicate significant differences ($P < 0.0001$; $P < 0.01$ and $P < 0.05$, respectively) in comparison to the unchallenged group (G6). (C) Cerebral parasite burden in dams. Each dot represents individual values (numbers of parasites per µg of DNA), and medians are represented as horizontal lines. Taking into account that the *N. caninum* detection limit by real-time PCR is 10 parasites, negative samples (0 parasites) were represented on the log scale as <10 (i.e., 10^0). (****) and (***) indicate $P < 0.0001$ and $P < 0.001$, respectively, significant higher levels in comparison to unchallenged group (G6).

group ($P < 0.0001$). However, IgG1 levels from G5 (10^2) were significantly lower than those from the other infected groups (G1–G4) ($P < 0.01$). On the other hand, no differences were observed between infected groups regarding IgG2a levels other than a significantly higher production in dams infected with the highest dose (2×10^6, G1) in comparison to those infected with the lowest dose (10^2, G5) ($P < 0.01$). Finally, comparisons between IgG1 and IgG2a levels within each group revealed an IgG2a biased immune response in the group infected with the lowest dose (10^2, G5) ($P < 0.05$) (Fig. 2A), which was the only infected group showing an IgG1/IgG2a ratio <0.9.

3.2. N. caninum *infection in offspring mice*

Data on early pup mortality, post-natal mortality, vertical transmission and median survival time for each group are summarized in Table 2.

3.2.1. *Morbidity, weight analysis and mortality in pups*

Early pup mortality rates, i.e., percentages of those pups that were born and died within 2 days pp, were similar among all groups (4–13%).

A high number of pups from all infected groups displayed a delay in growth and coat development, weight loss (Additional file 1) and exhibited nervous signs (ataxia, hind limb weakness, head tilt and walking in circles). In fact, high post-natal mortality rates were observed from days 3 to 30 pp in all infected groups, ranging from 76% in G5 (10^2) to 100% in G2 (10^5). Mortality in G2 (10^5) was significantly higher in comparison to G3 (10^4) ($P < 0.01$), G4 (10^3) ($P < 0.001$) and G5 (10^2) ($P < 0.0001$), and the mortality in G1 (2×10^6) was significantly higher than in G5 (10^2) ($P < 0.05$). No clinical signs nor death were detected in the offspring of the uninfected group (G6) throughout the experiment. Survival curves showed that in G5 (infected with 10^2 tachyzoites) survival was significantly higher than in all the other infected groups (G1–G4; $P < 0.01$) (Fig. 3A). In addition, groups infected with the highest doses (G1 -2×10^6- and G2 -10^5-) exhibited a significantly lower offspring survival rate compared to the remaining infected groups (G3–G5) ($P < 0.01$).

Data for monitoring the body weights of pups (starting at day 15 pp) in G1 (2×10^6) and G2 (10^5) came from only one litter from day 17 and 19 pp onwards, respectively, and therefore these groups were excluded from statistical analysis. Bearing this in mind, the offspring of the uninfected group (G6) showed significantly higher body weights than those from the infected groups G3 (10^4) at days 15, 17 and 19 pp; G4 (10^3) at day 15 and 17 pp; and G5 (10^2) at days 15, 17, 19, 23, 25, 27 and 29 pp (Fig. 3B).

Table 2
Summary of the effects of *Neospora* Nc-Spain7 infection in delivered pups

Group[a]	Early pup mortality[b]		Post-natal mortality[c]		Litters with 100% post-natal mortality	Vertical transmission[d]	Median survival time[e]
	Per pup	Per litter	Per pup	Per litter			
G1 (2×10^6)	7/54 (13%)	5/11 (45%)	44/47 (93.6%)	9/9 (100%)[f]	8/9 (88.9%)*	44/47 (93.6%)	11
G2 (10^5)	10/94 (11%)	7/15 (47%)	84/84 (100%)	15/15 (100%)	15/15 (100%)	84/84 (100%)	13
G3 (10^4)	2/52 (4%)	2/9 (22%)	45/50 (90%)	9/9 (100%)	7/9 (77.8%)	45/50 (90%)	15.5
G4 (10^3)	3/72 (4%)	2/11 (18%)	59/69 (85.5%)	11/11 (100%)	7/11 (63.6%)	63/69 (91.3%)	15
G5 (10^2)	6/60 (10%)	3/11 (27%)	41/54 (75.9%)	11/11 (100%)	6/11 (54.5%)	44/54 (81.5%)	18
G6 (unchallenged)	3/66 (5%)	2/12 (17%)	0/63 (0%)	0/12 (0%)	0/12 (0%)	0/63 (0%)	NA

[a] Mice challenged with Nc-Spain7 tachyzoites.
[b] Number of stillborn and dead pups until day 2 pp/total delivered pups (percentage).
[c] Number of dead pups from day 3 PP onwards/no. of pups alive by day 2 pp (% pup mortality).
[d] PCR-positive surviving pups plus those which succumbed to infection from day 3 pp/no. pups alive from day 3 pp (percentage).
[e] Day pp at which 50% of pups succumbed to infection.
[f] Number of litters were reduced since in two of them all pup died before day 3 pp.

3.2.2. Vertical transmission

Detection of vertical transmission ran in parallel to post-natal mortality. *Neospora* DNA was only detected in 4/10 and 3/13 surviving pups from the groups inoculated with 10^3 (G4) and 10^2 (G5) tachyzoites, respectively. All but one of these PCR-positive surviving pups exhibited clinical signs at 30 days pp.

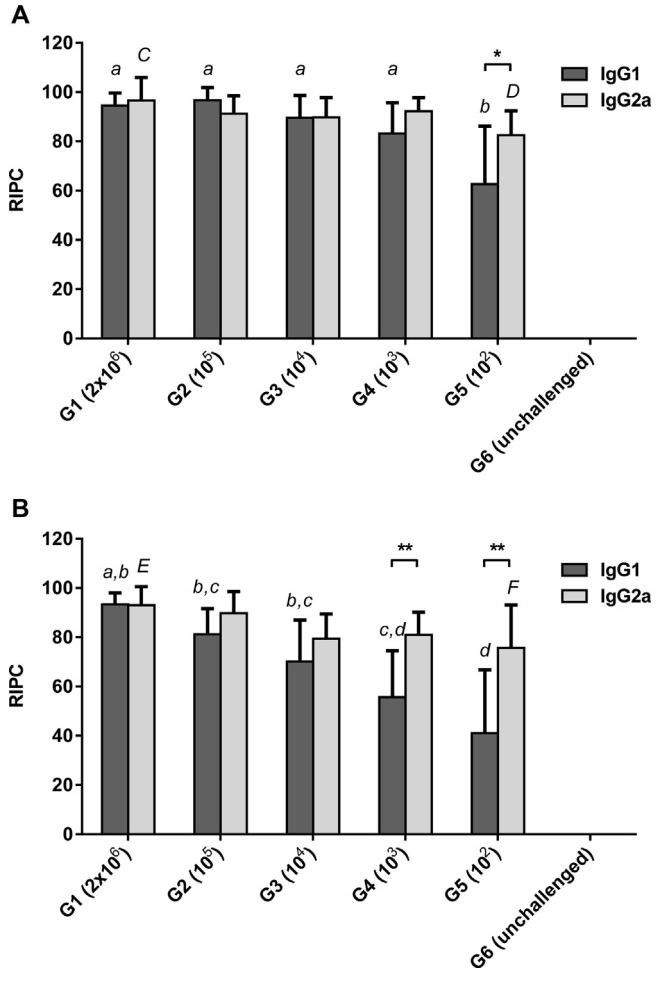

Fig. 2. Humoral immune responses in challenged mice.
Anti-*N. caninum* immunoglobulins (IgG1 and IgG2a isotypes) generated in (A) dams and (B) non-pregnant mice after infection with 2×10^6 (G1), 10^5 (G2), 10^4 (G3), 10^3 (G4) and 10^2 (G5) tachyzoites of the Nc-Spain7 isolate. Bars represent the average RIPC (relative index percent) and error bars represent standard deviations for each group. (*) indicates $P < 0.05$ and (**) $P < 0.01$ significant differences between IgG1 and IgG2a within each group. Different letters (upper case for IgG1 and lower case for IgG2a) above each column indicate significant differences among groups ($P < 0.05$).

3.3. Evaluation of N. caninum infection in non-pregnant mice

3.3.1. Morbidity and mortality

In contrast to pregnant mice, only non-pregnant mice infected with the highest doses (2×10^6 -G1- and 10^5 -G2-) displayed clinical signs. However, in general, these were more severe than those from pregnant mice, since all but one of the non-pregnant mice

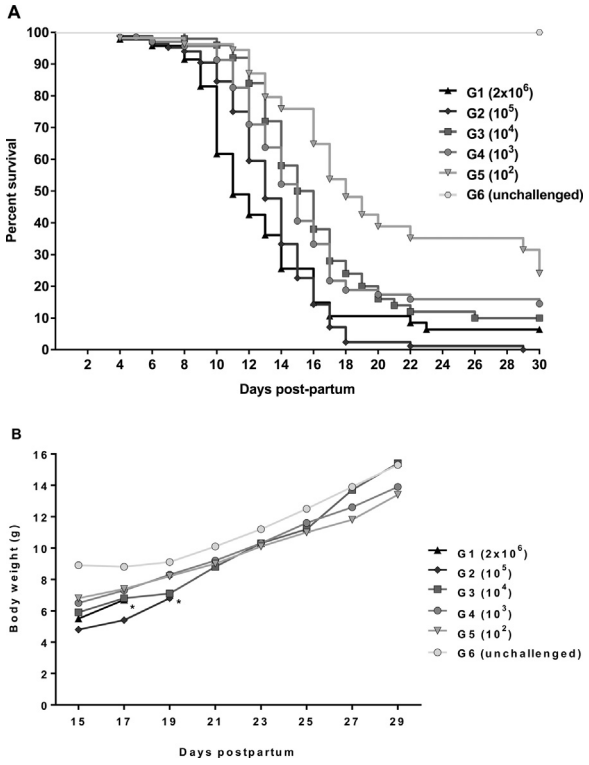

Fig. 3. Effect of *N.caninum* Nc-Spain7 isolate inoculation in the offspring.
(A) Kaplan–Meier survival curves for neonates born from dams infected on day 7–10 of pregnancy with 2×10^6 (G1), 10^5 (G2), 10^4 (G3), 10^3 (G4) and 10^2 (G5) tachyzoites from the *N. caninum* Nc-Spain7 isolate and the uninfected group. Each point represents the percentage of surviving animals at that day and downward steps correspond with observed deaths. (B) Body weight progression of neonates born from dams infected on day 7–10 of pregnancy with 2×10^6 (G1), 10^5 (G2), 10^4 (G3), 10^3 (G4) and 10^2 (G5) tachyzoites from the *N. caninum* Nc-Spain7 isolate and the uninfected group. Each point represents the average body weight of all animals per group (an asterisk denotes data obtained from a sole litter that had pup/s which did not succumb to infection. These data were therefore not considered for the statistical analyses).

from G1 and G2 showed severe loss of body condition and nervous signs (mainly lethargy, walking in circles and head tilt). As a consequence, these mice had to be euthanized between days 28 and 35 p.i. (Additional file 2A). Moreover, significantly lower body weights were recorded 4 weeks p.i. in G1 (2×10^6) ($P < 0.0001$) and G2 (10^5) ($P < 0.01$) compared to the uninfected group (G6) (Additional file 2B). On the other hand, mice from the remaining groups showed no clinical signs throughout the experiment other than a small ulcerated skin lesion in the site of inoculation that eventually healed in 1/8 non-pregnant mice from G4 (10^3) and 2/9 from G5 (10^2).

3.3.2. Quantification of cerebral parasite load

Neospora DNA was detected in all infected non-pregnant mice except in 2/9 mice from G5 (10^2). Moreover, no differences were found when comparing parasite burden in the uninfected group (G6) and mice infected with 10^3 (G4) and 10^2 (G5) tachyzoites, nor among infected groups (G1-G5) (Additional file 2C).

Further analysis comparing the cerebral parasite burden in pregnant mice with the parasite load in non-pregnant mice revealed a significant higher number of tachyzoites in the CNS of dams from G3 (10^4) and G4 (10^3) in comparison to the respective groups in non-pregnant mice ($P < 0.05$ and $P < 0.01$, respectively).

3.3.3. Neospora-specific antibody levels in non-pregnant mice

Compared to the unchallenged group (G6), all infected non-pregnant mice elicited significantly higher levels of both IgG1 and IgG2a, with the exception of one mouse from G5 (10^2) which did not produce IgG1 antibodies. IgG1 levels were found to be significantly lower in G5 (10^2) compared to G1 (2×10^6) ($P < 0.0001$), G2 (10^5) ($P < 0.01$) and G3 (10^4) ($P < 0.05$), and in G4 (10^3) compared to G1 (2×10^6) ($P < 0.001$). IgG2a levels in G5 (10^2) were significantly lower than in G1 (2×10^6) ($P < 0.05$). In addition, when comparing IgG1 and IgG2a levels within groups, mice from G4 (10^3) and G5 (10^2) showed significantly higher IgG2a levels than those of IgG1 ($P < 0.01$) (see Fig. 2B). Finally, IgG1/IgG2a ratio ranged from 1.01 in G1 (2×10^6) to 0.50 in G5 (10^2), being significantly lower in the latter compared to G1 ($P < 0.001$), G2 and G3 ($P < 0.05$).

Further analysis comparing antibody levels between pregnant and non-pregnant mice revealed significant higher IgG1 levels in dams from G2 (10^5), G3 (10^4) and G4 (10^3) ($P < 0.01$) and IgG2a levels in dams from G3 (10^4) and G4 (10^3) ($P < 0.05$) in comparison to those from non-pregnant mice.

4. Discussion

Mice are the most commonly employed in vivo experimental model in biomedical research, and this also holds true for studies on neosporosis despite the fact that the most economically relevant *N. caninum* infections occur in cattle. While considerable insights into *Neospora* host-parasite interactions have been gained in mice, results from different laboratories cannot be reliably compared due to variable parameters such as mouse breed, parasite isolates and in vitro culture conditions, timing and handling of parasite preparations, inoculation routes and timing of infection, and other experimental settings. In addition, very different outcomes have been observed between non-pregnant and pregnant mice (reviewed in Monney and Hemphill, 2014). BALB/c mice are the most widely employed mouse breed for studies on *Neospora* infection biology (López-Pérez et al., 2010; Regidor-Cerrillo et al., 2010; Dellarupe et al., 2014) and as proof-of-concept model for the assessment of drugs (e.g., Debache et al., 2011; Debache and Hemphill, 2012; Schorer et al., 2012; Ojo et al., 2014). Most notably, the pregnant BALB/c model has demonstrated various degrees of efficacy for a number of vaccine candidates (e.g., Aguado-Martínez et al., 2009; Debache et al., 2009; Zhang et al., 2010; Marugán-Hernández et al., 2011; Monney et al., 2012; Rojo-Montejo et al., 2012), and enabled researchers to assess the effects of parasite infection and respective vaccines on both progeny and dams (López-Pérez et al., 2008). Nevertheless, the lack of standardization of this model in different laboratories remains a major obstacle and hinders more efficient research. Therefore, this study was conducted to evaluate the effects of various infectious doses of a virulent *N. caninum* isolate in pregnant and non-pregnant BALB/c mice, with the aim to optimize the handling of the parasite and the challenge dose, and thus to contribute to the refinement and standardization of the BALB/c mouse model for its use in further studies.

The Nc-Spain7 isolate was selected due to its biological traits, as it has been shown to be highly virulent in vitro (Regidor-Cerrillo et al., 2011) and in vivo (Regidor-Cerrillo et al., 2010; Caspe et al., 2012; Collantes-Fernández et al., 2012; Dellarupe et al., 2014; Regidor-Cerrillo et al., 2014; Arranz-Solís et al., 2015). Moreover, the timing of parasite culture passage has been acknowledged to play an important role, since attenuation due to prolonged in vitro maintenance of *N. caninum* isolates has been previously described (Long et al., 1998; Bartley et al., 2006). In this study, parasites with a low number of passages (15) were employed, which ensured that no, or only minimal, loss of virulence occurred. Besides the "standard" dose for experimental infection of 2×10^6 tachyzoites, inoculations of 10^5, 10^4, 10^3 and 10^2 tachyzoites were also assessed, and the outcome of infection was evaluated by measuring morbidity, mortality, cerebral parasite load and humoral immune responses. Surprisingly, the differences between highest and lowest doses were much lower than expected, and we here demonstrate that experimental infection with as little as 100 tachyzoites could induce high mortality in both dams and offspring.

An important aspect is the manipulation of parasites during inocula preparation. First, parasites were collected from cell cultures when the great majority of tachyzoites were still intracellular, thus assuring optimal invasion capacities (Regidor-Cerrillo et al., 2011). We have shown earlier that extracellular maintenance of *N. caninum* tachyzoites induces rapid loss of infectivity (Naguleswaran et al., 2003). Thus, all procedures were undertaken rapidly and at low temperature, and mice were infected within 30 min of parasite isolation.

As expected, results obtained in mice inoculated with 2×10^6 tachyzoites showed high levels of morbidity, mortality and cerebral parasite load in both non-pregnant and pregnant mice, confirming the high capacity of the Nc-Spain7 isolate to spread widely, persist in dams, cross the placenta and infect the offspring. This mirrors findings reported in previous studies using the same pregnant mouse model and isolate (Regidor-Cerrillo et al., 2010; Collantes-Fernández et al., 2012; Dellarupe et al., 2014), and those employing the virulent isolate Nc-Liv (Marugán-Hernández et al., 2011; Dellarupe et al., 2014; Pastor-Fernández et al., 2015). Importantly, reduction of tachyzoite numbers by a factor 20 (from 2×10^6 to 10^5) did not alter median survival time, parasite burden and immune responses in both pregnant and non-pregnant mice, and was still causing 100% of pup mortality until day 30 pp. Interestingly, even dams infected with lower numbers of tachyzoites were able to transmit the parasite to their offspring, as illustrated by pup mortality rates of 76–90% in the groups infected with 10^2, 10^3 and 10^4 tachyzoites.

All infected dams and non-pregnant mice elicited a specific humoral immune response, as shown by ELISA. Nevertheless, IgG1 and IgG2a isotypes profiles varied in some groups. Those groups inoculated with lower tachyzoite numbers (below 10^4 in non-pregnant and 10^2 in pregnant mice) had diminished IgG1 production compared to other groups, whereas IgG2a levels remained similar in all infected groups, which finally lead to an IgG2a biased immune response in those animals infected with a lower tachyzoite numbers. This dose-dependent modulation of the immune

response is consistent with previous reports, in which mice administered with low number of parasites appears to degrade the IgG1 response (Lundén et al., 2002; Rojo-Montejo et al., 2012). As reported by Rojo-Montejo et al., (2012), when a high parasite number is administered, a large number of tachyzoites may remain extracellular, eliciting a humoral immune response, while the inoculation of a low number of parasites might lead to the internalization of most of the tachyzoites inside the early antigen presenting cells, enhancing a cell-mediated immunity.

The cerebral parasite burden in infected mice was determined by real time PCR, in most cases at 4–6 week p.i.. Remarkably, all groups infected with 10^3 up to 10^6 tachyzoites exhibited similar parasite loads, irrespective of the infection dose. Only in the mice infected with 100 tachyzoites parasite loads were consistently lower. These data correlate with the nervous signs, which were more frequently observed in mice inoculated with 2×10^6 to 10^3 tachyzoites. Also in earlier studies increased infection in the brain was associated with the appearance of neurological symptoms (Long et al., 1998; Collantes-Fernández et al., 2006; López-Pérez et al., 2008). In the group infected with 100 tachyzoites, two dams were found to be PCR-negative, but vertical transmission also occurred in these two PCR-negative dams, since their pups died and parasites were detected in pup brain samples. These findings suggest that tachyzoites rapidly disseminate following infection, cross the placenta and reach foetal tissues, while crossing the blood brain barrier might be a more time-consuming undertaking, and could also be impaired by the low numbers of tachyzoites injected. The IgG2a-dominated antibody profile is suggestive for a more Th1-biased cellular immune response, which could also prevent tachyzoite proliferation in dams and limit the number of tachyzoites reaching the brain. However, more investigations on cytokine expression profiles are needed to clarify this point.

Although non-pregnant mice infected with 2×10^6 and 10^5 tachyzoites displayed severe signs of neurological disease at an even higher number than dams, no clinical signs were noted and good body condition without significant weight loss was maintained in those animal infected with the lower doses. Thus, infection with a lower number of tachyzoites (10^4–10^2) renders non-pregnant mice clearly less susceptible to disease compared to pregnant mice. This was consistent with the data on cerebral parasite burden, which was higher in pregnant mice compared to non-pregnant mice inoculated with 10^3 and 10^4 tachyzoites. This is probably due to the immune modulation that occurs during pregnancy (Aguado-Martínez et al., 2009; Pastor-Fernández et al., 2015). Differences in antibody responses in pregnant versus non-pregnant mice were also detected: higher IgG2a and (more markedly) IgG1 levels in pregnant mice are indicative for a predominantly humoral immune response. This rather Th2-driven immune response in pregnant mice protects foetal viability, with the caveat of a less efficient immune response against an invading pathogen. However, upon infection with 2×10^6 and 10^5 tachyzoites, even non-pregnant mice suffered severe clinical signs and exhibited similar parasite burden in the brain.

In summary, we hereby describe the outcome of *Neospora* infection in a pregnant BALB/c mouse model by performing experimental infections using different numbers of tachyzoites of the virulent Nc-Spain7 isolate. We found that an infectious dose of 10^5 tachyzoites, cultured and isolated in vitro under defined conditions and administered subcutaneously, was sufficient to maintain 100% of infection in pregnant and non-pregnant mice, vertical transmission and high mortality in non-pregnant mice, dams and pups. This infectious dose is 20 times lower than the 2×10^6 tachyzoites usually applied by most researchers in the field. In addition, experimental infection with only 100 tachyzoites is sufficient to cause significant rates (76%) of postnatal mortality in pups during the first 30 days after birth, but leaves dams relatively unaffected. This clearly mimics a more realistic scenario in terms what would happen in pregnant cattle infected with this parasite. In terms of use of this model for vaccine development and/or drug design, it becomes evident that most studies have been carried out with an exceedingly high challenge dose that no immune system could have controlled, hence the number of promising vaccine and drug candidates has remained consistently low. Future studies should be carried out using lower infection doses, which will then allow to obtain more accurate and realistic conclusions in such studies. This work may lay the foundations for the refinement of a standardized *Neospora* pregnant mouse model, which might be used widespread by different research groups for further assessments of drug or vaccine candidates against neosporosis.

Author contributions

LMOM and AH conceived and designed the experiments. DAS and AAM prepared the inocula, carried out the infections and participated in clinical examination of the animals, necropsies and sampling. DAS and JM performed PCR. DAS performed serological assays, statistical analysis and interpreted the results. DAS wrote the paper, with results interpretation and discussion inputs from AAM, JRC, LMOM and AH. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2015.05.021

References

Aguado-Martínez, A., Álvarez-García, G., Fernández-García, A., Risco-Castillo, V., Marugán-Hernández, V., Ortega-Mora, L.M., 2009. Failure of a vaccine using immunogenic recombinant proteins rNcSAG4 and rNcGRA7 against neosporosis in mice. Vaccine 27 (52), 7331–7338.

Álvarez-García, G., Pereira-Bueno, J., Gómez-Bautista, M., Ortega-Mora, L.M., 2002. Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Vet. Parasitol. 107 (1–2), 15–27.

Arranz-Solís, D., Benavides, J., Regidor-Cerrillo, J., Fuertes, M., Ferre, I., Ferreras, M.C., Collantes-Fernández, E., Hemphill, A., Pérez, V., Ortega-Mora, L.M., 2015. Influence of the gestational stage on the clinical course, lesional development and parasite distribution in experimental ovine neosporosis. Vet. Res. 46 (1), 19.

Bartley, P.M., Wright, S., Sales, J., Chianini, F., Buxton, D., Innes, E.A., 2006. Long-term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum* in vivo. Parasitology 133, 421–432.

Benavides, J., Collantes-Fernández, E., Ferre, I., Pérez, V., Campero, C., Mota, R., Innes, E., Ortega-Mora, L.M., 2014. Experimental ruminant models for bovine neosporosis: what is known and what is needed. Parasitology 141 (11), 1471–1488.

Bland, J.M., Altman, D.G., 2004. The logrank test. BMJ 328 (7447), 1073.

Bland, J.M., Altman, D.G., 1998. Survival probabilities (the kaplan-meier method). BMJ 317 (7172), 1572.

Caspe, S.G., Moore, D.P., Leunda, M.R., Cano, D.B., Lischinsky, L., Regidor-Cerrillo, J., Álvarez-García, G., Echaide, I.G., Bacigalupe, D., Ortega Mora, L.M., Odeon, A.C.,

Campero, C.M., 2012. The *Neospora caninum*-spain 7 isolate induces placental damage, fetal death and abortion in cattle when inoculated in early gestation. Vet. Parasitol. 189 (2–4), 171–181.
Collantes-Fernández, E., Arrighi, R.B., Álvarez-García, G., Weidner, J.M., Regidor-Cerrillo, J., Boothroyd, J.C., Ortega-Mora, L.M., Barragán, A., 2012. Infected dendritic cells facilitate systemic dissemination and transplacental passage of the obligate intracellular parasite *Neospora caninum* in mice. PLoS One 7 (3), e32123.
Collantes-Fernández, E., López-Pérez, I., Álvarez-García, G., Ortega-Mora, L.M., 2006. Temporal distribution and parasite load kinetics in blood and tissues during *Neospora caninum* infection in mice. Infect. Immun. 74 (4), 2491–2494.
Debache, K., Alaeddine, F., Guionaud, C., Monney, T., Müller, J., Strohbusch, M., Leib, S.L., Grandgirard, D., Hemphill, A., 2009. Vaccination with recombinant NcROP2 combined with recombinant ncmic1 and ncmic3 reduces cerebral infection and vertical transmission in mice experimentally infected with *Neospora caninum* tachyzoites. Int. J. Parasitol. 39 (12), 1373–1384.
Debache, K., Guionaud, C., Kropf, C., Boykin, D., Stephens, C.E., Hemphill, A., 2011. Experimental treatment of *Neospora caninum*-infected mice with the arylimidamide db750 and the thiazolide nitazoxanide. Exp. Parasitol. 129 (2), 95–100.
Debache, K., Hemphill, A., 2012. Effects of miltefosine treatment in fibroblast cell cultures and in mice experimentally infected with *Neospora caninum* tachyzoites. Parasitology 139 (7), 934–9441.
Dellarupe, A., Regidor-Cerrillo, J., Jiménez-Ruiz, E., Schares, G., Unzaga, J.M., Venturini, M.C., Ortega-Mora, L.M., 2014. Clinical outcome and vertical transmission variability among canine *Neospora caninum* isolates in a pregnant mouse model of infection. Parasitology 141 (3), 356–366.
Dubey, J.P., Jenkins, M.C., Rajendran, C., Miska, K., Ferreira, L.R., Martins, J., Kwok, O.C., Choudhary, S., 2011. Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*. Vet. Parasitol. 181 (2–4), 382–387.
Dubey, J.P., Schares, G., 2011. Neosporosis in animals – the last five years. Vet. Parasitol. 180 (1–2), 90–108.
Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20 (2), 323–367.
Liddell, S., Jenkins, M.C., Collica, C.M., Dubey, J.P., 1999. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. J. Parasitol. 85 (6), 1072–1075.
Long, M.T., Baszler, T.V., Mathison, B.A., 1998. Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*. J. Parasitol. 84 (2), 316–320.
López-Pérez, I.C., Collantes-Fernández, E., Aguado-Martínez, A., Rodríguez-Bertos, A., Ortega-Mora, L.M., 2008. Influence of *Neospora caninum* infection in balb/c mice during pregnancy in post-natal development. Vet. Parasitol. 155 (3-4), 175–183.
López-Pérez, I.C., Collantes-Fernández, E., Rojo-Montejo, S., Navarro-Lozano, V., Risco-Castillo, V., Pérez-Pérez, V., Pereira-Bueno, J., Ortega-Mora, L.M., 2010. Effects of *Neospora caninum* infection at mid-gestation on placenta in a pregnant mouse model. J. Parasitol. 96 (5), 1017–1020.
López-Pérez, I.C., Risco-Castillo, V., Collantes-Fernández, E., Ortega-Mora, L.M., 2006. Comparative effect of *Neospora caninum* infection in BALB/c mice at three different gestation periods. J. Parasitol. 92 (6), 1286–1291.
Lundén, A., Wright, S., Allen, J.E., Buxton, D., 2002. Immunisation of mice against neosporosis. Int. J. Parasitol. 32 (7), 867–876.
Marugán-Hernández, V., Ortega-Mora, L.M., Aguado-Martínez, A., Jimenez-Ruiz, E., Álvarez-García, G., 2011. Transgenic *Neospora caninum* strains constitutively expressing the bradyzoite ncsag4 protein proved to be safe and conferred significant levels of protection against vertical transmission when used as live vaccines in mice. Vaccine 29 (44), 7867–7874.
McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28 (9), 1473–1478.
Monney, T., Debache, K., Grandgirard, D., Leib, S.L., Hemphill, A., 2012. Vaccination with the recombinant chimeric antigen recNcMIC3-1-R induces a non-protective Th2-type immune response in the pregnant mouse model for *N. caninum* infection. Vaccine 30 (46), 6588–6594.
Monney, T., Hemphill, A., 2014. Vaccines against neosporosis: what can we learn from the past studies? Exp. Parasitol. 140, 52–70.
Monney, T., Rutti, D., Schorer, M., Debache, K., Grandgirard, D., Leib, S.L., Hemphill, A., 2011. Recncmic3-1-r is a microneme- and rhoptry-based chimeric antigen that protects against acute neosporosis and limits cerebral parasite load in the mouse model for *Neospora caninum* infection. Vaccine 29 (40), 6967–6975.
Müller, N., Vonlaufen, N., Gianinazzi, C., Leib, S.L., Hemphill, A., 2002. Application of real-time fluorescent pcr for quantitative assessment of *Neospora caninum* infections in organotypic slice cultures of rat central nervous system tissue. J. Clin. Microbiol. 40 (1), 252–255.
Naguleswaran, A., Muller, N., Hemphill, A., 2003. Neospora caninum and *toxoplasma gondii*: a novel adhesion/invasion assay reveals distinct differences in tachyzoite-host cell interactions. Exp. Parasitol. 104 (3–4), 149–158.
Nishikawa, Y., Tragoolpua, K., Inoue, N., Makala, L., Nagasawa, H., Otsuka, H., Mikami, T., 2001. In the absence of endogenous gamma interferon, mice acutely infected with Neospora caninum succumb to a lethal immune response characterized by inactivation of peritoneal macrophages. Clin. Diagn. Lab. Immunol. 8 (1071–412; 4), 811–816.
Ojo, K.K., Reid, M.C., Kallur Siddaramaiah, L., Müller, J., Winzer, P., Zhang, Z., Keyloun, K.R., Vidadala, R.S., Merritt, E.A., Hol, W.G., Maly, D.J., Fan, E., Van Voorhis, W.C., Hemphill, A., 2014. *Neospora caninum* calcium-dependent protein kinase 1 is an effective drug target for neosporosis therapy. PLoS One 9 (3), e92929.
Pastor-Fernández, I., Arranz-Solís, D., Regidor-Cerrillo, J., Álvarez-García, G., Hemphill, A., García-Culebras, A., Cuevas-Martín, C., Ortega-Mora, L.M., 2015. A vaccine formulation combining rhoptry proteins NcROP40 and NcROP2 improves pup survival in a pregnant mouse model of neosporosis. Vet. Parasitol. 207 (3–4), 203–215.
Regidor-Cerrillo, J., Arranz-Solís, D., Benavides, J., Gómez-Bautista, M., Castro-Hermida, J.A., Mezo, M., Perez, V., Ortega-Mora, L.M., González-Warleta, M., 2014. *Neospora caninum* infection during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. Vet. Res. 45 (10), 9716.
Regidor-Cerrillo, J., Gómez-Bautista, M., Del Pozo, I., Jiménez-Ruiz, E., Aduriz, G., Ortega-Mora, L.M., 2010. Influence of neospora caninum intra-specific variability in the outcome of infection in a pregnant BALB/c mouse model. Vet. Res. 41 (4), 52.
Regidor-Cerrillo, J., Gómez-Bautista, M., Pereira-Bueno, J., Aduriz, G., Navarro-Lozano, V., Risco-Castillo, V., Fernández-García, A., Pedraza-Díaz, S., Ortega-Mora, L.M., 2008. Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in spain. Parasitology 135 (14), 1651–1659.
Regidor-Cerrillo, J., Gómez-Bautista, M., Sodupe, I., Aduriz, G., Álvarez-García, G., Del Pozo, I., Ortega-Mora, L.M., 2011. In vitro invasion efficiency and intracellular proliferation rate comprise virulence-related phenotypic traits of *Neospora caninum*. Vet. Res. 42 (1), 41.
Reichel, M.P., Alejandra Ayanegui-Alcerreca, M., Gondim, L.F., Ellis, J.T., 2013. What is the global economic impact of *Neospora caninum* in cattle – the billion dollar question. Int. J. Parasitol. 43 (2), 133–142.
Rojo-Montejo, S., Collantes-Fernández, E., López-Pérez, I., Risco-Castillo, V., Prenafeta, A., Ortega-Mora, L.M., 2012. Evaluation of the protection conferred by a naturally attenuated *Neospora caninum* isolate against congenital and cerebral neosporosis in mice. Vet. Res. 43, 62.
Schorer, M., Debache, K., Barna, F., Monney, T., Müller, J., Boykin, D.W., Stephens, C.E., Hemphill, A., 2012. Di-cationic arylimidamides act against *Neospora caninum* tachyzoites by interference in membrane structure and nucleolar integrity and are active against challenge infection in mice. Int. J. Parasitol. Drugs Drug Resist. 2, 109–120.
Whitten, M.K., 1957. Effects of exteroceptive factors on the oestrous cycle of mice. Nature 180 (4599), 1436.
Williams, D.J., Guy, C.S., Smith, R.F., Ellis, J., Bjorkman, C., Reichel, M.P., Trees, A.J., 2007. Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. Infect. Immun. 75 (3), 1343–1348.
Zhang, H., Nishikawa, Y., Yamagishi, J., Zhou, J., Ikehara, Y., Kojima, N., Yokoyama, N., Xuan, X., 2010. *Neospora caninum*: Application of apical membrane antigen 1 encapsulated in the oligomannose-coated liposomes for reduction of offspring mortality from infection in BALB/c mice. Exp. Parasitol. 125 (2), 130–136.