Short communication

Title: *In Vitro* and *In Vivo* Effects of the phytohormone inhibitor fluridone against *Neospora caninum* infection

Rochelle Haidee D. Ybañez[a, b, #], Arpron Leesombun[a, #], Maki Nishimura[a], Ryuma Matsubara[c,d], Mikiko Kojima[e], Hitoshi Sakakibara[e], Kisaburo Nagamune[c,f] and Yoshifumi Nishikawa[a]*

Author affiliations:

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan*

[b] *Biology and Environmental Studies Program, University of the Philippines Cebu, Gorordo Avenue, Lahug, Cebu City 6000, Philippines*

[c] *Department of Parasitology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

[d] *Graduate School of Life and Environmental Studies, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*

[e] *RIKEN Center for Sustainable Resource Science, 1-7-22, Suehiro, Tsurumi, Yokohama, 230-0045, Japan*

[f] *Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*

*Corresponding author: Yoshifumi Nishikawa

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

Tel.: +81-155-49-5886

Fax: +81-155-49-5643

E-mail: nisikawa@obihiro.ac.jp

[#] Both authors contributed equally to this work.

Abstract

Neospora caninum causes abortion and stillbirth in cattle. Identification of effective drugs against this parasite remains a challenge. Previous studies have suggested that disruption of abscisic acid (ABA)-mediated signaling in apicomplexan parasites such as *Toxoplasma gondii* offers a new drug target. In this study, the ABA inhibitor, fluridone (FLU), was evaluated for its action against *N. caninum*. Production of endogenous ABA within *N. caninum* was confirmed by ultra-performance liquid chromatography-tandem quadruple mass spectrometry. Subsequently, FLU treatment efficacy was assessed using *in vitro*. Results revealed that FLU inhibited the growth of *N. caninum* and *T. gondii in vitro* (IC_{50} 143.1 ± 43.96 μM and 330.6 ± 52.38 μM, respectively). However, FLU did not affect parasite replication at 24 h post-infection, but inhibited egress of *N. caninum* thereafter. To evaluate the effect of FLU *in vivo*, *N. caninum*-infected mice were treated with FLU for 15 days. FLU treatment appeared to ameliorate acute neosporosis induced by lethal parasite challenge. Together, our data shows that ABA might control egress in *N. caninum*. Therefore, FLU has potential as a candidate drug for the treatment of acute neosporosis.

Keywords: *Neospora caninum*; fluridone; abscisic acid

Neospora caninum, the causative agent of bovine neosporosis, is an obligate, intracellular, apicomplexan parasite that is genetically different from, but structurally similar to, *Toxoplasma gondii* [1]. The disease it causes is closely associated with abortion and stillbirth in the dairy industry [1]. Recently, assessment of the global economic impact of *N. caninum* revealed annual losses ranging from 1.1 million USD in New Zealand to more than 1 billion USD in the USA, with more losses occurring in the dairy industry than in the beef industry [2]. Control and treatment of this parasitic disease is important, but efforts have thus far been limited. Drugs such as sulfonamides, clindamycin, pyrimethamine, and ponazuril are available for treatment of canine neosporosis [3]. Nevertheless, development of new drug treatment options for *N. caninum* remains a challenge.

It was recently discovered that the closely related protozoan parasite *T. gondii* produces and uses the plant hormone, abscisic acid (ABA) [4]. ABA is an important phytohormone that regulates plant growth, development, dormancy, and stress responses [5]. In a recent study using fluridone (FLU), which is an inhibitor of ABA synthesis, ABA was shown to control the calcium-dependent egress and development of *T. gondii* [4]. Calcium signaling is a very important pathway that regulates diverse cellular processes [6]. In apicomplexan parasites, this signaling pathway directs motility, cell invasion, and egress [7]. Increase in the concentration of ABA causes calcium ion influx, thereby triggering exit of the parasite from infected cells. This process occurs in the apicoplast, a plant-like derivative organelle that is a plastid homolog in plants [4]. *N. caninum* invades a host cell during its tachyzoite stage, replicates within a parasitophorous vacuole (PV), and lyses the host cell during egress to start another cycle of infection. Although the role of calcium signaling in *N. caninum* is unknown, addition of the calcium ionophore A23187 was effective at releasing the parasite from its host cells [8]. In common with all apicomplexan parasites, *N. caninum* contains an apicoplast [9]. Thus, these results suggest that calcium signaling may play a

crucial role in *N. caninum* egress.

FLU is generally used as an herbicide, but because it has an effect on the apicoplast, it could be a potential drug against *N. caninum*. Thus, studying the action of FLU against *N. caninum* is worthwhile. The present study aimed to evaluate the *in vitro* and *in vivo* actions of FLU against *N. caninum* infection.

N. caninum-expressing green-fluorescent protein (Nc-GFP) [10], *N. caninum* (Nc-1 strain), GFP-expressing *T. gondii* (RH-GFP strain) [11] and *T. gondii* (RH strain) were propagated and maintained using monolayers of African green monkey kidney (Vero) cells in Eagle's minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum. Purification of the tachyzoites was done by washing the parasites with the medium to remove host-cell debris. The parasite pellet was resuspended in the same medium and passed through a 27-gauge needle and a MF-Millipore 5.0-μm-pore membrane filter (Millipore, Bedford, MA, USA).

To confirm that ABA is produced in *N. caninum* (Nc-1 strain) and to compare it with *T. gondii* (RH strain), the hormone was extracted from purified tachyzoites of both parasites using ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (AQUITY UPLC System/XEVO-TQS; Waters, Milford, MA, USA) with an ODS column (AQUITY UPLC BEH C18, 1.7 μm, 2.1 × 100 mm; Waters) as described previously [12].

Monolayers of human foreskin fibroblast (HFF) cells were grown in 96-well plates (cell suspensions 1 x10^5 cells/ml in DMEM supplemented with 10% FBS). To examine the effects of FLU on the intracellular parasites, RH-GFP (5x10^4 tachyzoites per well) was added to the wells for 4 h and the extracellular parasites were washed away. Then, FLU (Sigma-Aldrich) at the indicated concentrations (100 µl/well of medium) were added for 72 h. The fluorescence intensity of GFP was measured using a microplate reader (SH-900, Corona Electric Co., Ltd, Ibaraki, Japan). The correlation coefficient between the fluorescence

intensity of GFP and the number of parasites (a two-time serial dilution ranging from 1000,000 to 7812.5 parasites) was calculated using the Pearson correlation coefficient and a positive correlation was confirmed. The growth inhibition of GFP-expressing parasite (%) was expressed as follows: [(average fluorescence intensity of GFP with medium alone) – (the fluorescence intensity of GFP treated with FLU) / (average fluorescence intensity of GFP with medium alone)] × 100.

To measure *N. caninum* replication in HFF cells, Nc-GFP parasites grown in HFF cells were cultured on glass coverslips. Three hours after infection, the cells were treated with either FLU at 100 μM or DMSO (control). Following treatment at time intervals of 24, 48, and 72 h post-infection (hpi), parasite replication was evaluated by counting the number of intracellular parasites based on GFP signal per PV (a total of 100 randomly selected vacuoles) using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan). To confirm the parasite egress, the number of extracellular tachyzoites at 72 hpi was counted by a hemocytometer. To evaluate an impact of FLU on host cell invasion of *N. caninum*, Nc-GFP parasites were pretreated with FLU for 1 h at 37°C. Then, the pretreated parasites were added to Vero cells (parasites per host cell ratio =2:1) and the extracellular parasites were washed away at 2–3 hpi. The infection rates at 24 hpi were calculated by counting the number of infected cells based on GFP signal in the Vero cells (a total of 100 randomly selected Vero cells labelled with Hoechst 33342) using the Fluorescence Microscope.

Female BALB/c mice were obtained from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions in the animal facility at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan). This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Obihiro University of Agriculture and Veterinary Medicine. The protocol was approved by the

Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 24-17, 25-66). *In vivo* infections in female BALB/c mice (7 weeks old) with the Nc-1 strain of *N. caninum* tachyzoites were performed. Parasite infection (day 0) and FLU treatment (days 1–15) were administered intraperitoneally. Body weight, morbidity, and mortality were monitored within a 30-day period post-infection (See Table S1).

The mass spectrometry determination confirmed that ABA was produced in *N. caninum* and *T. gondii* (Fig. 1A). The amount of ABA produced in *N. caninum* tended to be lower than that in *T. gondii*. Moreover, the *in vitro* parasite growth assay revealed that FLU inhibited the growth of *N. caninum* and *T. gondii* (IC_{50} 143.1 ± 43.96 μM and 330.6 ± 52.38 μM, respectively) (Fig. 1B). This result suggested that *N. caninum* was sensitive to FLU treatment than *T. gondii*. Furthermore, FLU concentrations < 1,500 μM are considered to be safe in this study (data not shown). Since FLU inhibited the egress of *T. gondii* [4], the replication of *N. caninum* was examined (Fig. 2A). No notable difference was observed in PV size between the treated and control cultures at 24 hpi. However, at 48 and 72 hpi, parasite cultures treated with FLU had larger PVs, while large numbers of re-infected parasites were observed in the untreated culture. As shown in Fig. 2B, treatment with FLU reduced the number of extracellular tachyzoite at 72 hpi, indicating that FLU inhibited the egress of *N. caninum*. Furthermore, pretreatment of extracellular parasite with FLU reduced the infection rate (Fig. 2B). This result indicated that FLU had an impact on host cell invasion of *N. caninum*. In an *in vivo* analysis, we found that mortality in the mice treated with FLU at 1 mg/kg only started at 23 dpi while that of the untreated control group started at 8 dpi (Fig. 2C). In terms of morbidity, both groups showed similar trends, but the FLU-treated group had a lower morbidity score during the early stages of the infection (Fig. 2C).

Apicomplexan parasites including *Plasmodium* spp., *Babesia* spp., *T. gondii*, *Isospora*

belli, and *Cyclospora cayetanensis* all possess a non-photosynthetic plastid-like organelle called the apicoplast [13]. Two essential metabolic functions operate inside the apicoplast: the mevalonate-independent 1-deoxy-D-xylulose 5-phosphate pathway for isoprenoid synthesis, and type II fatty acid synthesis. ABA synthesis in plants occurs via isoprenoids [14]. Therefore, parasites with apicoplast organelles may have the ability to produce ABA. In the present study, we confirmed ABA production in *N. caninum*, suggesting that this parasite may not only be similar to *T. gondii* in terms of its structure, but also in its biology. Since *N. caninum* possesses an apicoplast [9], this parasite may contain an indirect pathway for ABA production that is similar to *T. gondii* [4]. ABA can activate ADP-ribose cyclase, which results in overproduction of intracellular cyclic ADP-ribose and an increase in intracellular Ca^{2+} concentrations [15]. In *T. gondii*, ABA production is a density-dependent signal that influences egress or bradyzoite conversion via ABA-mediated cyclic ADP-ribose formation and calcium signaling [4]. Our study showed that inhibition of ABA synthesis by FLU prevented egress in *N. caninum*, suggesting that ABA mediates calcium signaling for the parasite egress. Future studies aimed at elucidating the molecular mechanism(s) for ABA-dependent parasite egress will provide further insight into *Neospora* biology.

FLU treatment is able to prevent lethal infection with *T. gondii* in mice [4]. In addition, FLU is effective against malaria parasites, which are also apicoplast-containing parasites [16]. The present study suggests further studies on FLU in combination with other potential drugs against *N. caninum*. Results of the *in vivo* infections in mice showed that FLU ameliorated acute neosporosis by delaying death. As *N. caninum* is one of the leading causes of neuromuscular paralysis in dog [17], there is a need to evaluate potential drug targets in this parasite. In our previous immunohistochemical analysis, the infiltration of macrophages or microglia and the production of inducible nitric oxide synthase were predominantly observed in the necrotic and inflammatory lesions of brain from *N. caninum*-infected mice

[18]. Because an ABA pathway creates inflammation in tissues or cells of animals, FLU can be used as an anti-inflammatory drug [15]. Thus, FLU could be exploited to add to the current anti-*Neospora* drugs as it is a registered herbicide with low toxicity to mammalian cells. The present study also suggests further studies on FLU in combination with other potential drugs against *N. caninum*.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science through the "Funding Program for Next Generation World-Leading Researchers (NEXT Program)," initiated by the Council for Science and Technology Policy (2011/LS003) (Y.N.). This work was also supported in part by Grants-in-Aids for Scientific Research 24390103 (K.N.), and for Scientific Research on Innovative Areas 23117007 (K.N.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was also supported by a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan (K.N.), and a grant for research to promote the development of anti-AIDS pharmaceuticals from the Japan Health Sciences Foundation (K.N.). This work was also supported in part by Cooperative Research Grant (23-joint-6) of National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine (Y.N. and K.N.).

References

[1] J.P. Dubey, D.S. Lindsay. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67 (1996) 1–59.

[2] M.P. Reichel, M. Alejandra Ayanegui-Alcérreca, L.F. Gondim, J.Y. Ellis, What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. Int. J. Parasitol. 43 (2013) 133-142.

[3] M.P. Reichel, J.T. Ellis, J.P. Dubey, Neosporosis and hammondiosis in dogs. J. Small Anim. Pract. 48 (2007) 308-312.

[4] K. Nagamune, L.M. Hicks, B. Fux, F. Brossier, E.N. Chini, L.D. Sibley, Abscisic acid controls calcium-dependent egress and development in *Toxoplasma gondii*. Nature 451 (2008) 207-210.

[5] S.R. Cutler, P.L. Rodriguez, R.R. Finkelstein, S.R. Abrams, Abscisic acid: emergence of a core signaling network. Annu. Rev. Plant Biol. 61 (2010) 651-679.

[6] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell. Biol. 1 (2000) 11-21.

[7] S. Lourido, K. Tang, L.D. Sibley, Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. EMBO J. 31 (2012) 4524-4534.

[8] J.H. Behrendt, A. Taubert, H. Zahner, C. Hermosilla, Studies on synchronous egress of coccidian parasites (*Neospora caninum*, *Toxoplasma gondii*, *Eimeria bovis*) from bovine endothelial host cells mediated by calcium ionophore A23187. Vet. Res. Commun. 32 (2008) 325-332.

[9] C.S. Sohn, T.T. Cheng, M.L. Drummond, E.D. Peng, S.J. Vermont, D. Xia, S.J. Cheng, J.M. Wastling, P.J. Bradley, Identification of novel proteins in *Neospora caninum* using an organelle purification and monoclonal antibody approach. PLoS One. 6 (2011) e18383.

[10] C. Abe, S. Tanaka, M. Nishimura, F. Ihara, X. Xuan, Y. Nishikawa, Role of the chemokine receptor CCR5-dependent host defense system in *Neospora caninum* infections. Parasit. Vectors. 8(2015) 5.

[11] Y. Nishikawa, X. Xuan, L. Makala, O. Vielemeyer, K.A. Joiner, H. Nagasawa, Characterization of *Toxoplasma gondii* engineered to express mouse interferon-gamma. Int. J. Parasitol. 33 (2003) 1525-1535.

[12] M. Kojima, T. Kamada-Nobusada, H. Komatsu, K. Takei, T. Kuroha, M. Mizutani, M. Ashikari, M. Ueguchi-Tanaka, M. Matsuoka, K. Suzuki, H. Sakakibara, Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: an application for hormone profiling in Oryza sativa. Plant Cell Physiol. 50 (2009) 1201-1214.

[13] J. Wiesner, A. Reichenberg, S. Heinrich, M. Schlitzer, H. Jomaa, The plastid-like organelle of apicomplexan parasites as drug target. Curr. Pharm. Des. 14 (2008) 855-871.

[14] S.H. Schwartz, X. Qin, J.A. Zeevaart, Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. Plant Physiol. 131 (2003) 1591-1601.

[15] H.H. Li, R.L. Hao, S.S. Wu, P.C. Guo, C.J. Chen, L.P. Pan, H. Ni, Occurrence, function and potential medicinal applications of the phytohormone abscisic acid in animals and humans. Biochem. Pharmacol. 82 (2011) 701-712.

[16] J.L. Leef, P.S. Carlson, Carotenoid synthesis inhibiting herbicides and fatty acid synthesis oxime herbicides as anti-apicomplexa protozoan parasite agents. Patent 847932 (1999) 1–8 (Potomax Ltd. Prtn).

[17] J.S. Barber, A.J. Trees, Clinical aspects of 27 cases of neosporosis in dogs. Vet. Rec. 139 (1996) 439-443.

[18] M. Nishimura, S. Tanaka, F. Ihara, Y. Muroi, J. Yamagishi, H. Furuoka, Y. Suzuki, Y.

Nishikawa, Transcriptome and histopathological changes in mouse brain infected with *Neospora caninum*. Sci. Rep. 5 (2015): 936.

Figure legends

Fig. 1. (A) Quantification of ABA from *N. caninum* (Nc-1) or *T. gondii* using an ultra-performance liquid chromatography system coupled to a tandem quadrupole mass spectrometer equipped with an electrospray interface. Results are the means ± SD of three independent experiments. Student's t-test; P=0.052. (B) Growth inhibition of *N. caninum* and *T. gondii* in parasite-infected cells treated with fluridone (FLU) at varying concentrations. Results are the means ± SD of three independent experiments performed using quadruplicate samples.

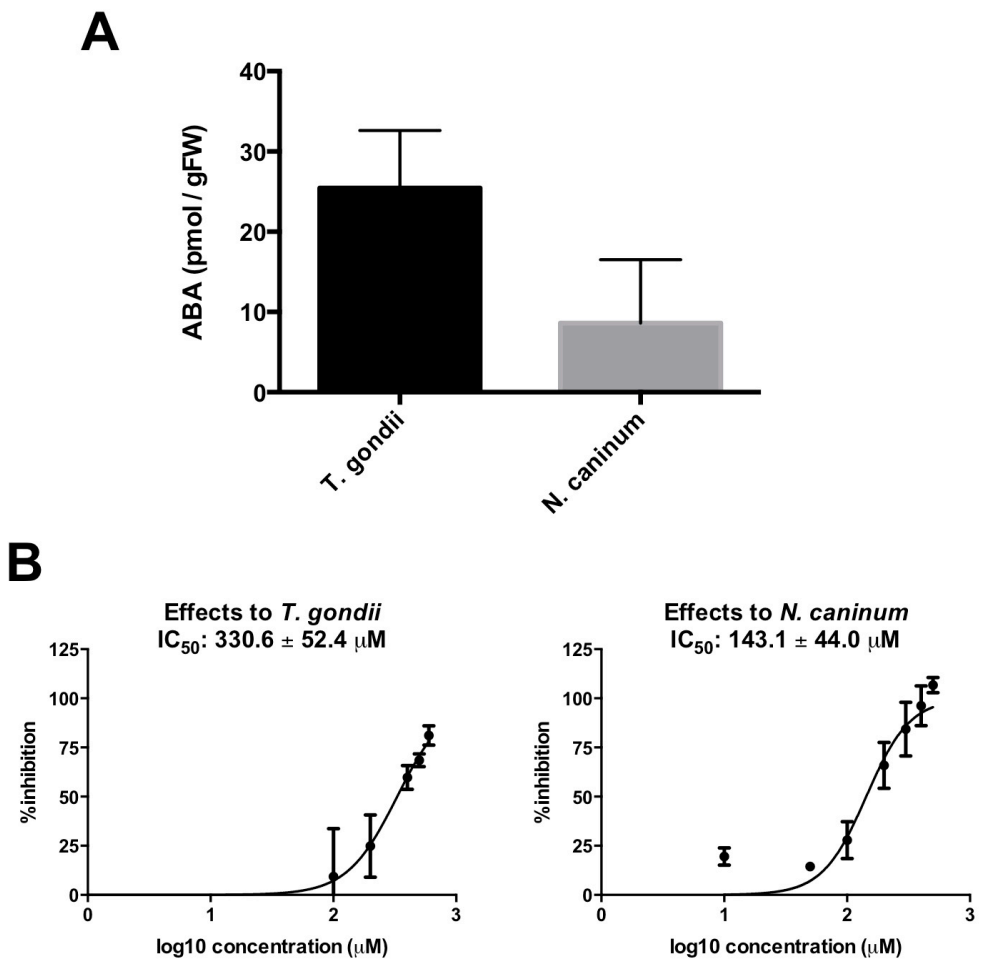

Fig. 1 Ybanez et al.

Fig. 2. (A) Parasite numbers of *N. caninum* per vacuole after treatment with 100 μM of at 24, 48, and 72 h post-infection. The distribution of parasitophorous vacuole (PV) sizes expressed as a percent (%) was determined by 100 randomly selected vacuoles. Values are the means ± SD of triplicate samples. Data is a representative of two independent experiments. Student's t-test; $*P<0.05$. (B) Number of extracellular parasite after treatment with FLU at 72 h post-infection for evaluation of the parasite egress (Left panel, $N = 4$). Infection rate of parasite pretreated with FLU for evaluation of an impact on host cell invasion (Right panel, $N = 3$). Values are the means ± SD. Data is a representative of two independent experiments. One-way analysis of variance (ANOVA) followed by the Tukey–Kramer test; $*P<0.05$. (C) Survival rate (Left panel) and morbidity score (Right panel) of BALB/c mice after lethal challenge with *N. caninum* tachyzoites (1×10^6) and treatment with fluridone (FLU) at 1 mg/kg or PBS (Control) for 15 days. $N = 7$ animals per group.

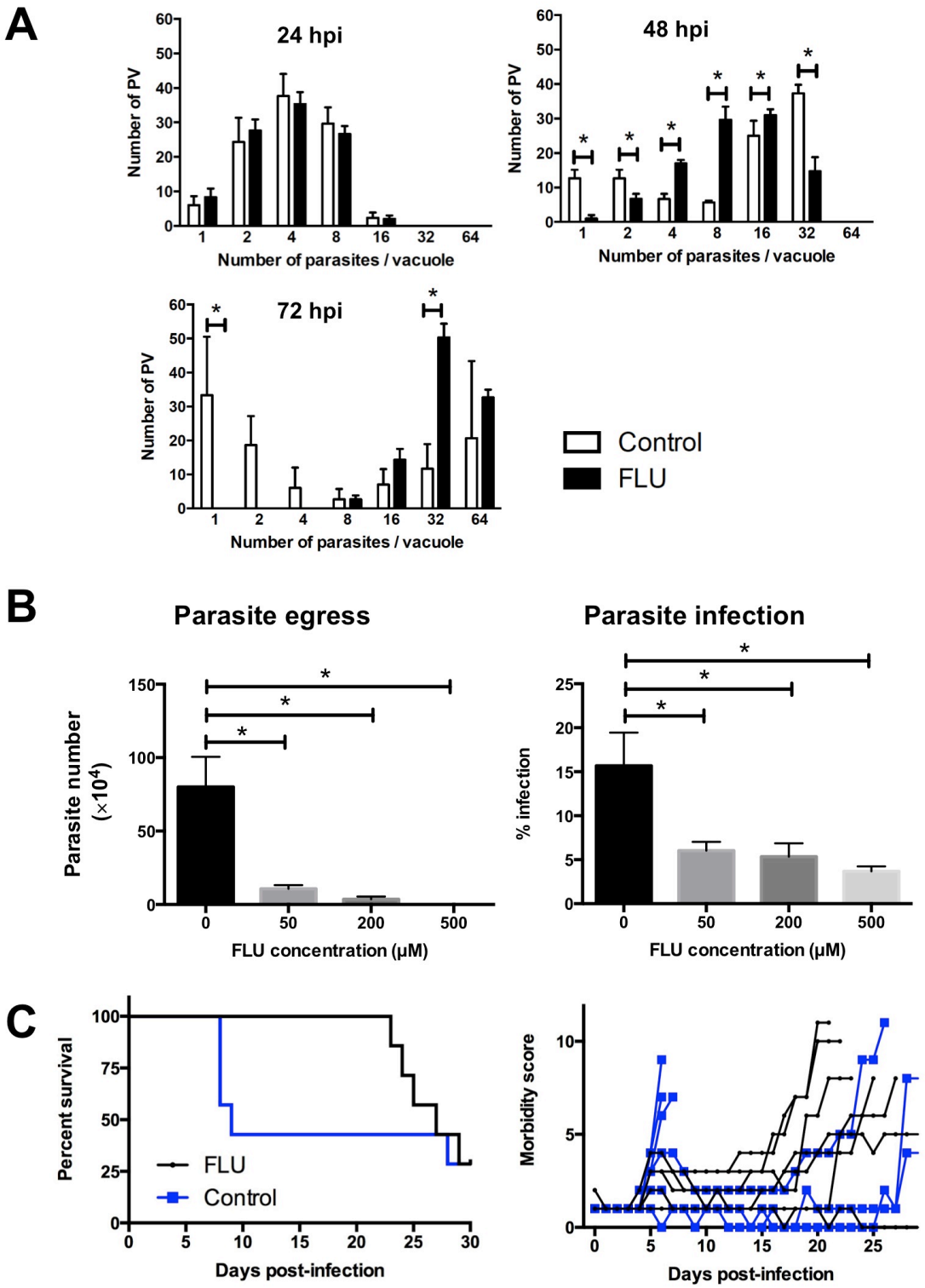

Fig. 2 Ybanez et al.

Supplementary material

Table S1. Morbidity scoring criteria for the assessment of *N. caninum* infection in mice.

Category	Features	Score
A - Febrile response / Physical Appearance	Sleek glossy coat	0
	Ruffled coat	1
	Starry stiff coat	2
	Hunched over / rounded back appearance	2
	Assymetrical eye	2
B - Behavior / Movement (accumulative scoring)	Active	0
	Tottering gait	1
	Reluctance to move	2
C - Neurologic Signs	No apparent signs	0
	legs curl back when held by the tail	1
	Body twists when held by the tail	1
	Leaning to one side when walking	2
	Walks in circles	2
	Drags hind legs to move	3
	(Rapid) Body circling when held by the tail	3
D - Dehydration / Loss of Appetite	Weight maintained/increased from pre-infection level	0
	≤10% weight loss	1
	≤20% weight loss	2
	>20% weight loss	3
E - Other Clinical Signs	Dry, brown feces / light yellow urine	0
	Soft/watery, yellowish feces	1
	Sticky, dark yellow urine	1

Total score = A + B + C + D + E.

Morbidity scoring of BALB/c mice after lethal challenge with *N. caninum* tachyzoites (1×10^6) and treatment with fluridone (FLU) at 1 mg/kg for 15 days. $N = 7$ animals per group. Body weight, morbidity, and mortality were monitored within a 30-day period post-infection. Morbidity scores were assessed using a modified set of criteria adapted by Reichel and Ellis (2009).

M.P. Reichel, J.T. Ellis, *Neospora caninum* – How close are we to development of an efficacious vaccine that prevents abortion in cattle. Int. J. Parasitol. 39 (2009) 1173-1187.