# Differential IFN-γ and TNF-α driven cytokine response distinguishes acute infection of a metatherian host with *Toxoplasma gondii* and *Neospora caninum*

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Running Head: Toxoplasmosis and neosporosis in a metatherian model

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## Abstract

*Toxoplasma gondii* and *Neospora caninum* (both Apicomplexa) are closely related cyst-forming coccidian parasites that differ significantly in their host range and ability to cause disease. Unlike eutherian mammals, Australian marsupials (metatherian mammals) have long been thought to be highly susceptible to toxoplasmosis and neosporosis because of their historical isolation from the parasites. In this study, the carnivorous fat-tailed dunnart (*Sminthopsis crassicaudata*) was used as a disease model to investigate the immune response and susceptibility to infection of an Australian marsupial to *T. gondii* and *N. caninum*. Disease outcome was more severe in *N. caninum* infected dunnarts than in *T. gondii* infected dunnarts, as shown by the severity of clinical and histopathological features of disease and a higher tissue parasite burden in the tissues evaluated. Transcriptome sequencing (RNA-seq) of spleens from infected dunnarts and mitogen-stimulated dunnart splenocytes was used to define the cytokine repertoire. Changes in mRNA expression during the time course of infection was measured using quantitative reverse transcription PCR (qRT-PCR) for key Th1 (IFNγ, TNFα), Th2 (IL-4, IL-6), and Th17 (IL-17A) cytokines. The results show qualitative differences in cytokine responses by the fat-tailed dunnart to infection with *N. caninum* and *T. gondii*. Dunnarts infected with *T. gondii* were capable of mounting a more effective Th1 immune response than those infected with *N. caninum*, indicating the role of the immune response in the outcome scenarios of parasite infection in this marsupial mammal.

## Introduction

*Neospora caninum* and *Toxoplasma gondii* (Apicomplexa: Coccidia) are tissue-cyst forming parasites with a world-wide distribution. Although these closely related parasites share some similar morphological and biological features, they exhibit key differences in host range and pathogenicity (1, 2). *Toxoplasma gondii* is regarded as one of the most successful parasites due to its capacity to infect and cause disease in essentially any mammalian or avian species (3). In humans it is also considered a pathogen of particular significance for pregnant and immunocompromised individuals (3, 4). In contrast, *N. caninum* is capable of infecting many different species but is not zoonotic and mainly causes disease in cattle and dogs (2, 5). In most immunocompetent species, *N. caninum* and *T. gondii* infections are subclinical and severe disease is uncommon (3, 5).

There is increasing interest in defining the role of the host immune response to *T. gondii* and *N. caninum* infection outcome, and in identifying the immune factors that influence control of infection and disease development (2, 3, 6-9). Numerous studies in eutherian models, particularly in mice, show the host immune response is a key determinant of disease outcome following infection with *N. caninum* and *T. gondii* (2, 3, 7, 9-12). Specifically, an efficient cell-mediated Th1 immune response, effected predominately by the pro-inflammatory cytokine IFNγ and, to a lesser extent TNF-α, is critical for controlling disease by restricting parasite replication and inducing chronic latent infections through parasite stage conversion and formation of tissue cysts (11, 13-15). In contrast, a shift in bias towards a humoral Th2 immune response, characterized by increased expression of the anti-inflammatory cytokine IL-4, is associated with

recrudescence of infection, uncontrolled parasite replication and pathological sequelae due to inhibitory effects of Th2 cytokines on Th1 immunity (11, 13, 16-19). More recently, IL-17, the signature cytokine of the Th17 immune response which is promoted by the pro-inflammatory cytokine IL-6, has been implicated in protection against *T. gondii* and *N. caninum* infection and in immunopathology associated with disease (20-23). While there are many advantages to using laboratory mice for *T. gondii* and *N. caninum* investigations, common laboratory mouse strains are highly susceptible to toxoplasmosis and generally resistant to neosporosis (7, 24). Currently nothing is known about the marsupial host immune response to *N. caninum* and *T. gondii* infection.

For millennia, Australian native marsupials evolved in geographic isolation without exposure to *T. gondii* and *N. caninum* until their definitive hosts (cats for *T. gondii* and dogs and dingoes for *N. caninum*) were introduced the continent (3, 25, 26). Current dogma asserts that Australian marsupials are particularly sensitive to developing severe, often fatal toxoplasmosis (3). However, the majority of debilitating marsupial toxoplasmosis cases are described for captive animals and there are many reports of *T. gondii* infection in asymptomatic free-ranging fauna (3, 27-30). Importantly, few experimental investigations to study *T. gondii* infection have been conducted in marsupials (31-33). Even less is known about the effect of *N. caninum* infection in native marsupials. Seroprevalence surveys in Australian cattle and dogs have established that *N. caninum* infection may be common in these species yet prevalence studies in native wildlife are lacking (34-40). As a result, the significance of *N. caninum* as a disease threat for wild populations of marsupials is unknown.

92 The fat-tailed dunnart (*Sminthopsis crassicaudata*) is a shrew-sized arid-zone
93 carnivorous dasyurid marsupial that is closely related to the endangered iconic
94 Tasmanian devil, and is one of the few available laboratory bred marsupial models (41).
95 Recent experimental infections in the fat-tailed dunnart have demonstrated that this
96 species is highly susceptible to *N. caninum* and that resultant disease is associated with
97 severe clinical signs, overwhelming systemic infection, and the production of numerous
98 tissue cysts (25). This outcome was unexpected, given that most immunocompetent
99 animals are largely resistant to neosporosis and tissue cysts are rarely found in infected
100 animals. Accordingly, the fat-tailed dunnart was identified as a valuable experimental
101 model for *Neospora* research, especially for the discovery of the mechanisms
102 contributing to the rapid onset of disease in an immunocompetent species.
103 Defining the immune response to *T. gondii* and *N. caninum* is critical to
104 understanding the pathogenesis of neosporosis and toxoplasmosis in marsupial
105 species. The purpose of this study was to investigate the role of the host immune
106 response in the development of disease subsequent to infection with *N. caninum* and *T.*
107 *gondii.* The unique animal model, the fat-tailed dunnart, was used as a laboratory host
108 due to its known susceptibility to *N. caninum*. The aims of the present study were to
109 characterize the histopathological changes, parasite dissemination, and cytokine
110 expression profiles for IFN-γ, TNF-α, IL-4, IL-17A, and IL-6 at early time points of *N.*
111 *caninum* and *T. gondii* infection. This study presents the first investigation of the
112 marsupial host immune response to *T. gondii* and *N. caninum*, providing valuable
113 information about the potential impact of these parasites on native host species and
114 furthering our knowledge about host-parasite interactions in marsupials.

115

116 **MATERIAL AND METHODS**

117 **Ethics statements and animals.** Animal experiments were approved by the University
118 of Sydney Animal Ethics Committee (project number 551) and complied with the New
119 South Wales Animal Welfare Act and the NHMRC code of practice (2013). Sexually
120 mature outbred fat-tailed dunnarts (*Sminthopsis crassicaudata*), 1-2 years of age,
121 weighing 13.2 – 18.9 g, were sourced from a breeding colony at the University of
122 Sydney. All animals were housed as outlined in Supplementary Material and Methods
123 (Text S1).

124

125 **Preparation and inoculation of *N. caninum* and *T. gondii* tachyzoites.** The NC-
126 Nowra isolate of *N. caninum* and TgAuDg1 isolate of *T. gondii* were used throughout the
127 study (42, 43). Tachyzoites were propagated by serial passages in Vero cells as
128 outlined in Supplementary Material and Methods (Text S1). Each experimental infection
129 included batches of five animals, four were inoculated with the parasite dose and one
130 was inoculated as negative control (Fig. 1). Two independent experiments were done
131 for each parasite species using the previously established minimum dose of tachyzoites
132 required for the development of neosporosis in dunnarts (25). Animals were inoculated
133 intraperitoneally with $10^5$ viable tachyzoites (*T. gondii* or *N. caninum*) suspended in a
134 volume of 0.3 ml sterile phosphate buffered saline (PBS, pH=7.2) or the same volume of
135 PBS alone. Animals were euthanized by $CO_2$ inhalation at 7 days post-infection (p.i.)
136 and 13 or 14 days p.i. for *N. caninum* and *T. gondii*, respectively (Fig. 1 and Table S1).
137 Animal monitoring, clinical evaluation, sample collection, histopathology and

138 immunohistochemistry are detailed in Supplementary Material and Methods (Text S1)
139 and Fig. 1.
140
141 **Quantification of tissue parasite load from *N. caninum* and *T. gondii* infected**
142 **dunnarts.** Genomic DNA was extracted from a variety of tissues (Fig. 1) and parasite
143 tissue burden was assessed by quantitative PCR (qPCR) in a SYBR Green based
144 assay using *T. gondii* primers targeting a 128bp fragment of the single copy SAG1 gene
145 (44) or *N. caninum* primers targeting a 76bp fragment of the multi-copy Nc5 gene (45).
146 Primer sequences and in-house assay optimization conditions are reported in
147 Supplementary Material (Tables S2 and S3). All samples were tested with triplicate
148 qPCR reactions using SsoAdvanced universal SYBR® Green supermix (BioRad,
149 Australia) in the CFX96 Touch™ Real-Time PCR Detection System and the
150 corresponding CFX Manager 3.1 software (BioRad). Standard curves were generated
151 using 10-fold serial dilutions (range 3 to 3 X $10^5$ *T. gondii* or 6 to 6 X $10^5$ *N. caninum*
152 parasites). Data handling and calculations for qPCR data were carried out in qBase$^{PLUS}$
153 Version 3.0 (Biogazelle, Ghent, Belgium). Parasites are expressed as $\log_{10}$ parasites
154 per 100ng of total DNA. The limit of detection for *N. caninum* and *T. gondii* were < 1
155 parasite and 2 parasites in 100ng DNA, respectively (Table S3). DNA extraction and
156 qPCR assay protocols are provided in Supplementary Materials and Methods (Text S1).
157
158 **Quantification of anti-*Neospora* and anti-*Toxoplasma* antibodies.** A competitive
159 ELISA for *N. caninum* (VMRD, Pullman, WA, USA) was used to detect *N. caninum*
160 antibodies following the manufacturer's protocol using undiluted sera and a cut off of ≥

30%. The Toxo-Screen DA modified agglutination test (MAT) kit (bioMérieux, Marcy l'Etoile, France) was used to detect *T. gondii* antibodies using sera diluted at 1:40 following the manufacturer's instructions. These serological assays have been previously shown to be suitable for use in Australian marsupial species (25, 40).

**Transcriptome RNAseq and analysis.** Transcriptomes were generated using total RNA isolated from 1 x $10^6$ mitogen-stimulated splenocytes from an uninfected dunnart (JS2093; see Supplementary Material and Methods Text S1), a spleen from an uninfected dunnart (JS1633), a spleen from a *N. caninum* infected dunnart at 2 weeks p.i. (JS2095), and a spleen from a *T. gondii* infected dunnart at 2 weeks p.i. (JS2097). RNA was isolated using an Isolate II RNA Mini Kit (Bioline) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer, and each had a RIN > 8.0 (Agilent Technologies, Waldbronn, Germany). Purified RNA samples were transferred into tubes containing RNAstable® (Biometrica, San Diego, USA). Illumina HiSeq2000 RNA sequencing was carried out at Macrogen (Seoul, Korea). See Supplementary Material and Methods for *de novo* assembly and bioinformatics analysis (Text S1).

**Quantitative reverse transcription PCR (qRT-PCR) assays to evaluate cytokine expression.** Cytokine transcript sequences for IFN-γ, TNF-α, IL-4, IL-17A, and IL-6 obtained from the dunnart spleen transcriptome were used to design specific primers using Primer3Plus (46) and recommended parameters for designing SYBR® Green primers (47). All assays were designed within exons and amplicon length varied between 91 and 178 base pairs long (Table S2). qPCR assays were carried out in the

CFX96 Touch™ Real-Time PCR Detection System (BioRad). Triplicate technical replicates were run for each cDNA sample with SsoAdvanced universal SYBR® Green supermix (BioRad, Australia). Each run included no template negative controls and no-RT controls. Assay optimization values are reported in Supplementary Material (Table S3).

RNA quantity and quality were evaluated by spectrophotometry and the Agilent 2100 expert chip system and RNA extraction was repeated for any sample with RIN < 7.5. RNA samples were subjected to an additional DNase treatment using the TURBO DNA-*free*™ Kit (ThermoFisher Scientific, Australia). cDNA was synthesized from 2 µg total RNA in a final volume of 20 µl using the Revertaid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Australia). Raw Cq scores, determined by baseline settings in the CFX Manager 3.1 software (BioRad, Australia), were uploaded to qBase$^{PLUS}$ software where the geometric mean of GAPDH and 28S was used to normalize expression level of each target transcript in each sample and 'target specific amplification efficiency' and 'scale to control' parameters were selected to generate CNRQ value of cytokine expression for each sample (48). Details of RNA extraction and qRT-PCR assay protocols are provided in Supplementary Materials and Methods (Text S1).

**Statistical analysis**. Calibrated normalized relative quantity (CNRQ) values for gene expression and tissue parasite load were calculated in qBase $^{PLUS}$ and log transformed to normalize data for statistical analysis. Statistical tests were carried out in GraphPad Prism 7 software (GraphPad, LaJolla, CA USA). A Shapiro-Wilk test was used to

determine the distribution of data and parametric (one-way ANOVA) or nonparametric (Kruskal-Wallis test or Mann-Whitney U) tests were used to compare differences between groups followed by *post hoc* adjustment for multiple comparisons using Tukey-Kramer and Dunn's correction as appropriate (49). Spearman rank correlation tests were used for all correlation tests. Statistical significance for all analyses was established using $P < 0.05$. Results are expressed as the means +/- standard deviation for parametric data and median ± interquartile range for nonparametric data; confidence intervals are provided when appropriate. Experimental qPCR practice and reporting were performed in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (50).

**Accession numbers.** Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFCN00000000.

## RESULTS

**In contrast to *N. caninum* NC-Nowra infected dunnarts, *T. gondii* TgAuDg1 infected dunnarts exhibited only mild disease.** *N. caninum* infected dunnarts demonstrated a significantly higher mean morbidity score than those infected with *T. gondii* ($P<0.0001$). From 11-12 days p.i., *N. caninum* infected dunnarts presented with rapidly progressive clinical signs of disease that necessitated euthanasia at 13 days p.i. Signs included ruffled dull pelage, hunched appearance, aimless diurnal wandering (abnormal for this nocturnal species), reduced awareness of surroundings, obtunded behavior, hindlimb to generalized paresis, and urinary incontinence. These signs were consistent with those previously reported for dunnarts with neosporosis (25) and were not observed in negative control animals or *T. gondii* infected animals, which remained clinically normal throughout the course of the experiment. In *N. caninum* infected dunnarts, daily activity, as measured by distance run and time spent in the exercise wheel, was reduced from days 6-7 onward (Fig. S1C, D, G, H) and reduced daily food intake was observed from 5-7 days p.i. (Fig. S1K, L). In contrast, *T. gondii* infected dunnarts showed a less pronounced reduction in daily activity or were similar to individual baseline activity and control animals (Fig. S1A, B, E, F) and food consumption was not noticeably different from controls (Fig. S1I, J).

Body mass was significantly reduced for *N. caninum* infected dunnarts at 13 days p.i. as compared to controls ($P<0.01$) and *T. gondii* infected animals at 7 and 14 days p.i. ($P<0.01$ and $P<0.001$, respectively). Body mass was statistically similar between other experimental groups (Fig. 2A). *N. caninum* infected animals at 7 and 13 days p.i.

had significantly thinner tails compared to controls and *T. gondii* infected animals at 7 days p.i. ($P<0.01$ and $P<0.05$, respectively) but not 14 days p.i. (Fig. 2B).

**Pathological lesions were more widespread and severe in dunnarts with neosporosis than dunnarts with toxoplasmosis.** At necropsy, macroscopic lesions were not detected in dunnarts at 7 days p.i. In contrast, at 13-14 days p.i. all infected animals had some degree of pulmonary consolidation and congestion, mesenteric lymphadenomegaly, and splenomegaly. *T. gondii* infected dunnarts were in good body condition with no appreciable difference in adipose tissue stores and muscle mass when compared to uninfected animals while *N. caninum* infected dunnarts were in poor body condition with severe atrophy of subcutaneous, visceral, and tail adipose tissue and moderate to marked atrophy of skeletal muscle. In three of four *N. caninum* infected dunnarts, the liver was mildly enlarged and friable with multifocal small poorly delineated red foci scattered throughout the parenchyma. Histopathological changes in *T. gondii* infected dunnarts (7 and 14 days p.i.) and *N. caninum* infected dunnarts (7 and 13 days p.i.) are detailed in Supplementary Materials (Text S2).

To better understand disease development in early *T. gondii* and *N. caninum* infection, severity of histopathological lesions in the brain, tongue, lung, heart, liver, and spleen were semiquantitatively evaluated and ranked with scores reflecting the extent of inflammation and necrosis in different tissues (see Table S4). When histopathological scores of lesion severity in analyzed tissues were compared between the control group and infected groups, dunnarts with toxoplasmosis had significantly more severe lesions only in heart and lung at 14 days p.i. ($P<0.05$) (Fig. 3C-D). In contrast, significantly more

severe lesions were found in tissues examined in *N. caninum* infected animals at 13 days p.i. than the control group (P<0.001 for lung and heart; P<0.01 for brain, tongue, liver; and spleen) and spleen at 7 days p.i. (P<0.05) (Fig. 3A-F). When infected dunnarts were compared at similar time points, significantly more severe lesions were observed in *N. caninum* infected dunnarts at 13 days p.i. than *T. gondii* infected dunnarts at 14 days p.i. in the brain, tongue, liver, and spleen (P<0.05) and approached statistical significance in the lung and heart (P=0.0571) (Fig. 3A-F). Additionally, hepatic lesions were significantly more severe in dunnarts infected with *N. caninum* than *T. gondii* at 7 days p.i. (P<0.05) (Fig. 3E).

**Increased parasite burden was observed in tissues from *N. caninum* NC-Nowra infected dunnarts compared to *T. gondii* TgAuDg1 infected dunnarts**. Parasite tissue load in the brain, tongue, lung, heart, liver, and spleen was quantified by qPCR and this approach was more sensitive in detecting parasites than histopathology and immunohistochemistry (Tables S4 and S5). In *T. gondii* infected dunnarts, parasites were consistently detected in the liver, lung, and heart at 7 days p.i. and liver showed the highest parasite load (Table S5). The tongue and spleen were positive for *T. gondii* in two animals and parasites were undetectable in the brain of all animals at 7 days p.i. (Table S5). With the exception of liver and brain, the median parasite burden continued to increase *in vivo* in evaluated tissues, as demonstrated by a 1.9 to 64.4-fold increase at 14 days p.i. with the highest and lowest increases seen in heart and tongue, respectively (Table S5). At 14 days p.i., the heart and lung were consistently infected and contained the highest numbers of parasites (Table S5). The heart was the only

tissue with a difference in median parasite number of more than one log unit between 7 and 14 days p.i. Parasites were also detected in low numbers in *T. gondii* infected dunnarts at 14 days p.i. in the spleen of three animals, tongue of two animals, and the brain and liver of a single animal (Table S5). In contrast, parasites were consistently detected in all analyzed tissues in *N. caninum* infected dunnarts (Table S5). The heart contained the highest number of parasites at both time points followed by liver and lung (Table S5). Parasite burden increased in all tissues more dramatically than was seen in *T. gondii* infection with all tissues showing a minimum 1 to 2 log unit increase in parasite numbers between 7 and 13 days p.i. The brain had the lowest parasite load at 7 and 13 days p.i. (Table S5). Brain and tongue showed the highest (148.4 and 50.7-fold) increases in median parasite number over time in *N. caninum* infected dunnarts and the liver had the smallest (13.4-fold) increase (Table S5).

Median tissue parasite burden was consistently higher in dunnarts with neosporosis than those with toxoplasmosis (Table S5). At 7 days p.i., *N. caninum* infected dunnarts showed an 8 to 146-fold difference in median parasite load compared to those infected with *T. gondii* with the lowest and highest differences in spleen and heart, respectively. At 13 days p.i., *N. caninum* infected dunnarts showed a 61 to 25,400-fold difference in median parasite load compared to *T. gondii* infected dunnarts at 14 days p.i. with the lowest and highest differences in lung and liver, respectively (Table S5). Significant increases in tissue parasite load were observed in *N. cani*num infected animals at 13 days p.i. compared with *T. gondii* infected animals for the brain ($P<0.01$ and $P<0.05$ for 7 and 14 days p.i., respectively)(Fig. 4A), tongue ($P<0.01$)(Fig. 4B), lung ($P<0.01$)(Fig. 4C), heart ($P<0.01$ and $P<0.05$ for 7 and 14 days p.i.,

312 respectively)(Fig. 4D), liver (P<0.05 and P<0.01 for 7 and 14 days p.i., respectively)(Fig.
313 4E), and spleen (P<0.05)(Fig. 4F). The liver parasite load was also significantly
314 increased in *N. caninum* infected animals at 7 days p.i. compared with *T. gondii* infected
315 animals at 14 days p.i (P<0.05)(Fig. 4E).
316
317 **Parasite tissue load was positively correlated with lesion severity in most tissues**
318 **in *N. caninum* NC-Nowra infected dunnarts, but not *T. gondii* TgAuDg1 infected**
319 **dunnarts.** There was a strong positive correlation between parasite tissue load and
320 overall lesion severity in *N. caninum* infected dunnarts for brain (r=0.9129, P<0.01),
321 tongue (r=0.9452, P<0.01), lung (r=0.8961, P<0.01), heart (r=0.9636, P<0.01), and liver
322 (r=0.9636, P<0.05) but not spleen (data not shown). No significant correlation between
323 tissue parasite load and overall lesion severity in the brain, tongue, lung, heart, liver,
324 and spleen was found in *T. gondii* infected dunnarts. *T. gondii* and *N. caninum* infected
325 dunnarts showed evidence of seroconversion by 14 and 13 days p.i., respectively
326 (Table S1).
327
328 **RNA-seq revealed transcriptional changes in cytokine genes occur in response to**
329 **parasite infection in the dunnart spleen.** In order to obtain dunnart specific cytokine
330 sequences to use for assay development, four transcriptome sequences were
331 generated using RNA purified from uninfected and infected dunnart spleens and
332 mitogen-stimulated dunnart immune cells. Different immune gene transcripts (n=28)
333 were identified (Table 1). The cytokine repertoire of a *T. gondii* and a *N. caninum*

infected dunnart suggested differences in expression of major cytokines compared to an uninfected control (Table 1).

**Splenic cytokine gene mRNA expression from *T. gondii* TgAuDg1 infected dunnarts differed to those from *N. caninum* NC-Nowra infected dunnarts.** To characterize the immune response following infection with *T. gondii* and *N. caninum*, splenic mRNA expression of IFN-γ, TNF-α, IL-4, IL-6, and IL-17A was measured by qRT-PCR. Of the cytokines evaluated, IFN-γ was the most strongly upregulated in dunnarts infected with *T. gondii* and *N. caninum* (median 40 to 284-fold increase compared to the control group) (Table S6). IFN-γ and TNF-α mRNA expression were significantly higher in asymptomatic *T. gondii* infected dunnarts at 14 days p.i. than in the uninfected control group ($P<0.01$)(Fig. 5A, B). A nonsignificant trend of higher median expression of Th1 cytokines with respective increases of 2.8 and 2-fold for IFN-γ and TNF-α was observed in asymptomatic *T. gondii* infected dunnarts at 14 days p.i. compared with clinically diseased *N. caninum* infected dunnarts at 13 days p.i. (Fig. 5A, B). The correlation between TNF-α expression and IFN-γ expression was significant ($r=0.83$, $p<0.0001$) (Fig. 6). Median fold change for IL-4 expression was higher (1.2 to 4-fold increase) in *N. caninum* infected animals at 13 days p.i. compared with other experimental groups but a significant difference was only identified in *N. caninum* infected animals at 7 days p.i. ($P<0.05$) where three of four animals had downregulated IL-4 mRNA expression compared with uninfected controls (Fig. 5C). The IFN-γ:IL-4 gene expression ratio was calculated for each animal based on individual relative-fold cytokine expression. The median IFN-γ:IL-4 gene expression ratio was 2.5 to 118-fold

higher in asymptomatic *T. gondii* infected dunnarts at 14 days p.i. than in other experimental groups; however, this difference was only significant when compared with uninfected controls ($P<0.01$) (Fig. 5F).

IL-17A mRNA did not amplify in two *T. gondii* infected animals at both 7 and 14 days p.i. nor in one uninfected control animal. Of the *T. gondii* infected animals with detectable IL-17A mRNA levels, three of four had downregulated IL-17A mRNA expression compared to uninfected controls. No significant difference in IL-17A and IL-6 mRNA expression was found between infected and uninfected groups (Fig. 5D, E). There was no correlation between the expression of evaluated cytokines and presence of parasite tissue cysts in infected dunnarts (data not shown).

## Discussion

Studies in mice and cattle (eutherian models) show that the host immune response to infection with *T. gondii* and *N. caninum* is a key determinant in infection outcome; however, studies in marsupials (metatherian model) are lacking. In our study, adult outbred fat-tailed dunnarts were infected with the Australian isolates of *N. caninum* NC-Nowra and *T. gondii* TgAuDg1 (type II). Both of these strains are considered to be low virulence strains and produce little to no disease in murine models (42, 43, 51). Despite similarities in parasite inoculum dose and virulence, disease outcome was more severe in dunnarts infected with *N. caninum* than those infected with *T. gondii*. Evidence of differential regulation of Th-related cytokines supports the hypothesis that the immune response plays a role in the outcome of parasite infection in this species.

The development of a cell-mediated Th1 immune response, characterized by increased expression of pro-inflammatory cytokines IFN-γ and TNF-α and decreased expression of the anti-inflammatory Th2 cytokine IL-4, has long been recognized as a critical component of host protection against intracellular pathogens, including *T. gondii* and *N. caninum* (7, 11, 13, 15). Therefore it was hypothesised that Th1 cytokines would be expressed at higher levels in clinically normal dunnarts. Consistent with a Th1 polarized immune response, *T. gondii* infection resulted in significantly upregulated splenic IFN-γ and TNF-α mRNA expression and higher IFN-γ:IL-4 gene expression ratio by 2 weeks p.i. compared to uninfected controls, while *N. caninum* infection did not. These findings suggest that dunnarts with toxoplasmosis were capable of mounting a more effective Th1 type immune response than dunnarts with neosporosis by two

weeks p.i. (52, 53) which may have accounted for milder disease and lower parasite burden observed in *T. gondii* infection.

Of the Th1 cytokines, IFN-γ is generally recognized as the most crucial element of protective immunity in *N. caninum* and *T. gondii* infections (10, 54). IFN-γ is important for restricting intracellular replication due to its role in activating macrophage-mediated mechanisms to kill intracellular pathogens, particularly in the early stages of infection (13-15, 54). TNF-α can work synergistically by activating IFN-γ-primed macrophages to restrict intracellular parasite replication and promote clearance of parasites (54). An inadequate Th1 type immune response and imbalance of Th1:Th2 cytokines may have contributed to disease pathogenesis in dunnarts with neosporosis. The modest elevation in IFN-γ and TNF-α expression induced by *N. caninum* infection was unable to control parasite replication in the dunnart as seen by early widespread parasite dissemination and a markedly high tissue parasite load. In mice, there is some evidence that females produce lower levels of IFN-γ and TNF-α than their male counterparts during the initial stages of *T. gondii* infection which may enhance their susceptibility to disease (55). Although the present *N. caninum* investigations were female-biased, severe fatal neosporosis was previously shown in male dunnarts infected with *N. caninum.* These observations are similar to the disease seen in the females in this current study (25). Therefore, any possible sex-biases in the dunnart immune response are unlikely to influence susceptibility to *N. caninum* infection.

Studies of neosporosis in murine models have shown resistant mouse strains have a mixed immune response characterized by a high IFN-γ:IL-4 ratio while a low IFN-γ:IL-4 ratio is associated with greater disease susceptibility (56). A trend towards

higher IL-4 mRNA expression and a lower IFN-γ:IL-4 ratio was observed in *N. caninum* infected dunnarts compared to those infected with *T. gondii* at two weeks p.i. Despite the absence of statistical significance, these findings suggest that the correct balance of Th1 and Th2 cytokines may play a critical role in limiting disease severity subsequent to infection with *T. gondii* and *N. caninum* in fat-tailed dunnarts (56, 57). While a Th2 biased immune response is generally associated with a detrimental disease outcome, Th2 type cytokines can mitigate immunopathology during acute toxoplasmosis and neosporosis by limiting excessive Th1 cytokine secretion and subsequent pathological effects (11, 17-19, 52, 56-59).

Although significant differences in IL-17A and IL-6 mRNA expression were not identified in this study, a general trend was noted for IL-17A suggestive of some difference between the infected dunnarts. IL-17A was consistently detected in all *N. caninum* infected animals and median mRNA expression levels were elevated compared to other groups. In contrast, IL-17A transcripts were downregulated or undetectable in *T. gondii* infected animals. Studies in *T. gondii* infected IL17R deficient mice have shown that while IL-17 plays a role in reducing parasite burden (22), an exacerbated Th17 response is generally associated with increased tissue damage and greater mortality (21). In koalas, IL-17A is considered a marker for chlamydial pathogenesis with higher levels of expression detected in animals with more severe disease (60).

Parasite burden in host tissue is an important element of disease development in eutherian models (3, 10, 61, 62). Our findings suggest that in the dunnart model, *N. caninum* NC-Nowra disseminates better with a higher capacity of growth and/or better

435 evasion of the host immune response than *T. gondii* TgAuDg1. Both heart and lung
436 were the tissues in which parasites were consistently identified and should therefore be
437 considered as targets for disease surveillance in marsupials in order to increase the
438 likelihood of parasite detection in early infection (63).

439 The severity of acute clinical neosporosis and associated pathological lesions
440 observed in dunnarts in the present study mirrors the findings in a previous report of *N.*
441 *caninum* infection in this species (25). We also found necrosis to be a characteristic
442 feature and muscle, liver, pancreas, and lungs to be severely affected. Involvement of
443 mesometrium and reproductive tissues was seen in *N. caninum* infected dunnarts and
444 may indicate a predilection of *N. caninum* for these tissues. Extensive tissue cyst
445 production appears to be a unique feature of neosporosis in the dunnart model (25).

446 Australian marsupials are generally considered as being highly susceptible to
447 toxoplasmosis (3). The lack of apparent clinical disease in *T. gondii* infected dunnarts in
448 this study was unexpected although not inconsistent with previous reports in
449 marsupials. Infected marsupials can succumb to fulminant disease and die peracutely
450 without evidence of premonitory clinical signs (64). Experimental infections in Tammar
451 wallabies (*Macropus eugenii*) (33) and eastern barred bandicoots (*Perameles gunnii*)
452 (31) given *T. gondii* oocysts *per os* of the low virulence strains ME49 (type II) and VEG
453 (type III), respectively, found infection was fatal within 9-16 days and animals displayed
454 no clinical signs or exhibited only mild behavioral changes prior to death.

455 A unique finding in our study was the rarity of CNS lesions in *T. gondii* infected
456 dunnarts. Necrosis, inflammation, and tissue cysts in the CNS tissues are common
457 features of toxoplasmosis in marsupials (65). This is particularly true for dasyurid

458 species where brain and spinal cord are reported to be the most likely tissues to contain
459 lesions and parasites in infected animals (64, 65). Experimental infections in mice have
460 also shown that brain is parasitized early in infection, irrespective of method of
461 inoculation (61) and encephalitis and tissues cysts commonly appear by the second
462 week of infection (66). It is possible that there is a diminished capacity of the TgAuDg1
463 strain to cross the blood brain barrier in dunnarts or there was irregular distribution of
464 parasites in the brain, such that sampling was not accurately reflective of infection rates.
465 It is also possible that brain infection occurs later in disease in *T. gondii* infected
466 dunnarts than what is reported for murine models.

467 In summary, the data presented herein show that under similar experimental
468 conditions, the fat-tailed dunnarts infected with *T. gondii* TgAuDg1 are capable of
469 mounting a stronger Th1 type immune response by 2 weeks p.i. and have reduced
470 morbidity, pathological lesions, and tissue parasite burden than dunnarts infected with
471 *N. caninum* NC-Nowra. This work illustrates the utility of the fat-tailed dunnart as an
472 experimental model for *Neospora* and *Toxoplasma* research. The suite of optimized and
473 validated cytokine assays designed for this study can be used to evaluate the Th
474 immune response in this species. These assays are useful and reproducible for
475 evaluation of systemic immune responses in dunnart immune tissues and will serve as
476 valuable tools to elucidate host pathogen interactions and be beneficial to future
477 research into additional diseases and efficacy of therapeutic treatments and vaccination
478 in the fat-tailed dunnart.

479

480 **Competing interests**

The authors declare they have no competing interests.

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**Author Contributions.**

Conceived and designed the experiments: SD, DP, MM, JE, JS. Performed the experiments: SD, MM. Analyzed the data: SD, DP, DO, MM, JS. Contributed reagents/materials/analysis tools: BM, DO, MM, JE, JS. Wrote the manuscript: SD, DP, JS. Reviewed manuscript drafts: SD, DP, BM, DO, MM, JE, and JS.

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697 **Figure legends**

698 **FIGURE 1**. Experimental overview explaining numbers of uninfected and infected fat-
699 tailed dunnarts, tissue sampling, and methods. A total of eight dunnarts were infected
700 with either *Toxoplasma gondii* (Tg) or *Neospora caninum* (Nc) and were euthanized at 7
701 days post infection (dpi) or 13-14dpi. Two independent experiments were conducted for
702 both *N. caninum* and *T. gondii* investigations; each experiment contained four parasite
703 infected animals and one uninfected negative control. For the histopathological analysis,
704 multiple tissues were evaluated by light microscopy and lesion severity was assessed in
705 the brain, tongue, lung, heart, liver, and spleen from each infected and uninfected
706 animal. To identify parasites and parasite tissue cysts by light microscopy, *N. caninum*
707 and *T. gondii* specific immunohistochemistry and BAG5 bradyzoite specific
708 immunohistochemistry were used on sections from parasite infected tissue. For the
709 detection of *N. caninum* and *T. gondii*, DNA isolated from the brain, tongue, lung, heart,
710 liver, and spleen of each parasite infected animal was subjected to qPCR targeting the
711 Nc5 (*N. caninum*) and SAG1 (*T. gondii*) genes. For RNA-Seq analysis, RNA was
712 extracted from the following samples to generate four separate dunnart transcriptomes:
713 (1) uninfected dunnart, (2) *T. gondii* infected dunnart at 14dpi, (3) *N. caninum* infected
714 dunnarts at 13dpi, and (4) mitogen-stimulated splenocytes from an uninfected healthy
715 dunnart. Transcript sequences for IFN-γ, TNF-α, IL-4, IL-17A, and IL-6 identified in
716 dunnart transcriptomes were used to design qRT-PCR assays in order to investigate
717 splenic cytokine expression profiles in each uninfected and infected animal.
718

**FIGURE 2.** Change in percent body weight and tail width for *Toxoplasma gondii* and *Neospora caninum* infected fat-tailed dunnarts at 7 and 13-14 days postinfection. The control group represents the uninfected animals. Percent body weight (A) and tail width change (B) were calculated for each animal based on initial and post-mortem measurements. Each bar represents the means ± SEM of the four biological replicates contained in each experimental group. One-way ANOVA was used to compare changes in body weight and tail width between experimental groups followed by Tukey-Kramer's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

**FIGURE 3.** Summary of lesion severity scores observed in brain, tongue, lung, heart, liver, and spleen of *Neospora caninum* and *Toxoplasma gondii* infected fat-tailed dunnarts**.** The control group consists of uninfected dunnarts and representative of background lesions normally found in this species. For the evaluated sectional area of a given tissue, the extent of inflammation and necrosis were scored separately and scoring ranged from no observation (score = 0) to minimal (<5% affected; score = 1), mild (5-10% affected; score = 2), moderate (11-30% affected; score = 3), or severe (>30% affected; score = 4). The final lesion severity score for brain (A), tongue (B), lung (C), heart (D), liver (E), and spleen (F) is based on the sum of scores assigned for inflammation and necrosis. Box represents the 25$^{th}$ to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group, middle line is median and

742 whiskers are the minimum to maximum values. Kruskal-Wallis one-way ANOVA was
743 used to compare parasite infected groups to controls followed by Dunn's *post hoc*
744 adjustment and Mann-Whitney test was used to compare *N. caninum* and *T. gondii*
745 infected animals at 7 days p.i. and 13 and 14 days p.i., respectively. *, $P < 0.05$; **, $P <$
746 0.01; ***, $P < 0.001$. Data shown are representative of two independent *T. gondii*
747 experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N.*
748 *caninum*; Tg, *T. gondii*; dpi, days postinfection.
749
750 **FIGURE 4.** The parasite tissue load in fat-tailed dunnarts experimentally infected with
751 *Toxoplasma gondii* and *Neospora caninum* at 7 and 13-14 days p.i. determined by
752 qPCR assays targeting the SAG1 gene of *T. gondii* and Nc5 gene of *N. caninum*.
753 Results are expressed as total number of parasites in 100ng DNA isolated from the (A)
754 brain, (B) tongue, (C) lung, (D) heart, (E) liver, and (F) spleen. Box represents the 25$^{th}$
755 to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group,
756 middle line is median and whiskers are the minimum to maximum values. Parasite loads
757 for each tissue were compared between different groups by Kruskal-Wallis one-way
758 ANOVA followed by Dunn's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$; ***, $P <$
759 0.001, ****, $P < 0.0001$. Data shown are representative of two independent *T. gondii*
760 experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N.*
761 *caninum*; Tg, *T. gondii*; dpi, days postinfection.
762
763 **FIGURE 5.** Splenic cytokine mRNA expression profiles as determined by RT-qPCR in
764 fat-tailed dunnarts experimentally infected with *Toxoplasma gondii* and *Neospora*

*caninum* at 7 and 13-14 days p.i. The control group represents the uninfected animals. The results are expressed as normalized relative fold changes compared to reference genes GAPDH and 28S and were scaled to control animals. Relative expression of IFN-γ (A), TNF-α (B), IL-4 (C), IL-17A (D), IL-6 (E) and the IFN-γ:IL-4 ratios (F) are shown. Box represents the 25$^{th}$ to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group, middle line is median and whiskers are the minimum to maximum values. Kruskal-Wallis one-way ANOVA was used to compare cytokine expression between experimental groups followed by a Dunn's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

**FIGURE 6.** Correlation between IFN-γ and TNF-α mRNA expression in fat-tailed dunnarts infected with *Toxoplasma gondii* and *Neospora caninum*. Each point represents one animal (uninfected, n=4; *T. gondii* infected, n=4 each for 7 and 14 days p.i.; *N. caninum* infected, n=4 each for 7 and 13 days p.i.) The correlation graph shows a fit line with a confidence curve. r and P values are shown from Spearman rank correlation analysis. r=correlation coefficient; P = significance level.

**TABLE 1.** TMM normalized expression values for gene transcripts identified in transcriptomes generated from (1) uninfected dunnart spleen (JS1633) (2) *T. gondii* infected dunnart spleen at 14 days p.i. (JS2097), (3) *N. caninum* infected dunnart

788 spleen at 13 days p.i. (JS2095), and (4) mitogen-stimulated dunnart splenocytes
789 (50ng/ml PMA, 1µg/ml Ionomycin) (JS2093). Abbreviations: IL, interleukin; IFN,
790 interferon; TSLP, thymic stromal lymphopoietin; LT, lymphotoxin; TNF, tumor necrosis
791 factor.

**TABLE 1** Trimmed mean of M-values (TMM) normalized expression values of immune gene transcripts identified in fat-tailed dunnart (*Sminthopsis crassicaudata*) transcriptomes

| Trinity transcript ID | Gene name | Uninfected dunnart (JS1633) | *T. gondii* infected dunnart (JS2097) | *N. caninum* infected dunnart (JS2095) | Mitogen-stimulated dunnart splenocytes (JS2093) |
|---|---|---|---|---|---|
| TRINITY_DN82803_c0_g1_i1 | IL-1α | 0 | 0.022 | 0.074 | 11.775 |
| TRINITY_DN84816_c0_g1_i1 | IL-1β | 3.214 | 12.775 | 15.454 | 1364.71 |
| TRINITY_DN153847_c0_g1_i1 | IL-2 | 0 | 0.15 | 0.143 | 467.473 |
| TRINITY_DN5397_c0_g1_i1 | IL-4 | 0 | 0 | 0.083 | 7.088 |
| TRINITY_DN53045_c0_g1_i1 | IL-5 | 0.039 | 0.08 | 0.254 | 3.159 |
| TRINITY_DN45695_c0_g1_i2 | IL-6 | 0 | 0.236 | 1.302 | 31.46 |
| TRINITY_DN82539_c0_g1_i2 | IL-10 | 0.055 | 0.762 | 3.472 | 10.282 |
| TRINITY_DN72236_c0_g1_i4 | IL-11 | 0.105 | 7.661 | 31.564 | 0.675 |
| TRINITY_DN101701_c0_g1_i1 | IL-12a | 0 | 0 | 0.147 | 0.778 |
| TRINITY_DN115371_c0_g1_i1 | IL-12b | 0.042 | 0.086 | 0.082 | 4.29 |
| TRINITY_DN80613_c0_g1_i1 | IL-13 | 0.671 | 0.308 | 0.439 | 36.154 |
| TRINITY_DN85376_c0_g1_i1 | IL-15 | 0.115 | 1.013 | 1.73 | 1.245 |
| TRINITY_DN86202_c3_g2_i2 | IL-16 | 6.979 | 22.923 | 17.215 | 5.7 |
| TRINITY_DN53989_c1_g1_i1 | IL-17A | 0.06 | 0.083 | 0 | 406.244 |
| TRINITY_DN92343_c0_g1_i1 | IL-17C | 0 | 0.037 | 0.07 | 1.228 |
| TRINITY_DN50692_c0_g1_i2 | IL-17D | 0.47 | 0.215 | 2.033 | 0.1 |
| TRINITY_DN54141_c0_g1_i1 | IL-17F | 0.084 | 0 | 0.055 | 292.502 |
| TRINITY_DN88373_c2_g2_i1 | IL-21 | 0.079 | 0.776 | 0.528 | 31.338 |
| TRINITY_DN48114_c0_g1_i1 | IL-22 | 0 | 0 | 0.051 | 36.596 |
| TRINITY_DN55354_c0_g1_i1 | IL-23α | 0.085 | 0.177 | 0.643 | 75.463 |
| TRINITY_DN138057_c0_g1_i1 | IL-25 (IL-17E) | 0 | 0 | 0 | 1.547 |
| TRINITY_DN86798_c3_g1_i9 | IL-27 | 1.353 | 1.183 | 1.181 | 0.93 |
| TRINITY_DN60643_c0_g1_i2 | IL-33 | 0 | 0.449 | 0.227 | 0 |
| TRINITY_DN29707_c0_g1_i1 | IFN-γ | 0.041 | 4.094 | 0.483 | 195.057 |
| TRINITY_DN89327_c9_g1_i1 | TNF-α | 2.774 | 6.005 | 3.313 | 251.596 |
| TRINITY_DN66548_c0_g2_i2 | LTα | 1.504 | 1.461 | 0.185 | 14.014 |
| TRINITY_DN91456_c2_g2_i1 | LTβ | 53.669 | 56.419 | 8.468 | 39.825 |
| TRINITY_DN5167_c0_g1_i1 | TSLP | 0.432 | 0.908 | 1.156 | 1.126 |

Note. Transcriptomes included in this table are (1) uninfected dunnart spleen (JS1633), (2) *T. gondii* infected dunnart spleen at 14 days p.i. (JS2097), (3) *N. caninum* infected dunnart spleen at 13 days p.i. (JS2095), and (4) mitogen-stimulated dunnart splenocytes (50ng/ml PMA, 1μg/ml Ionomycin)(JS2093). Abbreviations: IL, interleukin; IFN, interferon; TSLP, thymic stromal lymphopoietin; LT, lymphotoxin; TNF, tumor necrosis factor



Infection and Immunity

**Figure 1**

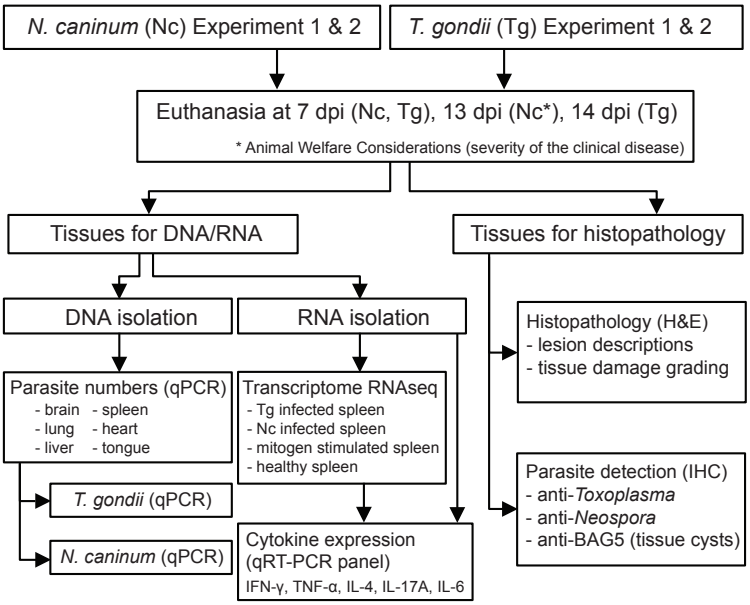

**FIGURE 1**. Flow diagram of experimental overview explaining numbers of uninfected and infected fat-tailed dunnarts (*Sminthopsis crassicaudata*), tissue sampling, and methods. A total of eight dunnarts were infected with either *Toxoplasma gondii* (Tg) or *Neospora caninum* (Nc) and were euthanized at 7 days postinfection (dpi) or 13-14dpi. Two independent experiments were conducted for both *N. caninum* and *T. gondii* investigations; each experiment contained 4 parasite infected animals and one uninfected negative control. For the histopathological analysis, multiple tissues were evaluated by light microscopy and lesion severity was assessed in the brain, tongue, lung, heart, liver, and spleen from each infected and uninfected animal. To identify parasites and parasite tissue cysts by light microscopy, *N. caninum* and *T. gondii* specific immunohistochemistry and BAG5 bradyzoite specific immunohistochemistry were used on sections from parasite infected tissue. For the detection of *N. caninum* and *T. gondii*, DNA isolated from the brain, tongue, lung, heart, liver, and spleen of each parasite infected animal was subjected to qPCR targeting the Nc5 (*N. caninum*) and SAG1 (*T. gondii*) genes. For RNA-Seq analysis, RNA was extracted from the following samples to generate four separate dunnart transcriptomes: (1) uninfected dunnart, (2) *T. gondii* infected dunnart at 14dpi, (3) *N. caninum* infected dunnarts at 13dpi, and (4) mitogen-stimulated splenocytes from an uninfected healthy dunnart. Transcript sequences for IFN-γ, TNF-α, IL-4, IL-17A, and IL-6 identified in dunnart transcriptomes were used to design qRT-PCR assays in order to investigate splenic cytokine expression profiles in each uninfected and infected animal.







Infection and Immunity

## Figure 2

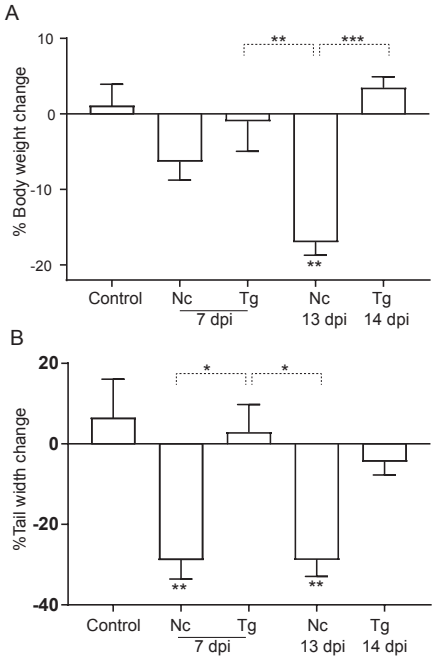

**FIGURE 2.** Percent body weight and tail width change for *Toxoplasma gondii* and *Neospora caninum* infected fat-tailed dunnarts (*Sminthopsis crassicaudata*) at 7 and 13-14 days postinfection. The control group represents the uninfected animals. Percent body weight (A) and tail width change (B) were calculated for each animal based on initial and post-mortem measurements. Each bar represents the means ± SEM of the four biological replicates contained in each experimental group. One-way ANOVA was used to compare changes in body weight and tail width between experimental groups followed by Tukey-Kramer's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

## Figure 3

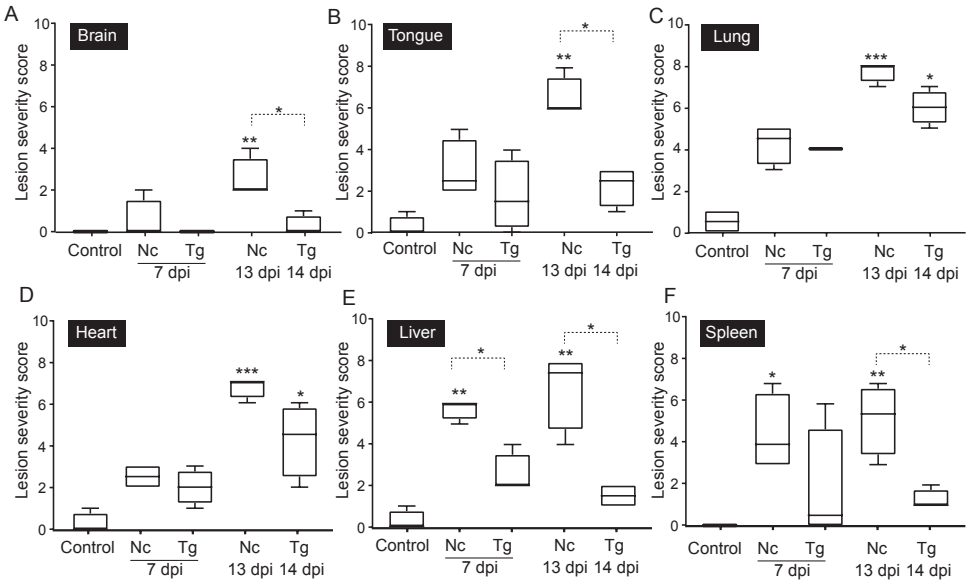

**FIGURE 3.** Summary of lesion severity scores observed in brain, tongue, lung, heart, liver, and spleen of *Neospora caninum* and *Toxoplasma gondii* infected fat-tailed dunnarts (*Sminthopsis crassicaudata*)**.** The control group consists of uninfected dunnarts and representative of background lesions normally found in this species. For the evaluated sectional area of a given tissue, the extent of inflammation and necrosis were scored separately and scoring ranged from no observation (score = 0) to minimal (<5% affected; score = 1), mild (5-10% affected; score = 2), moderate (11-30% affected; score = 3), or severe (>30% affected; score = 4). The final lesion severity score for brain (A), tongue (B), lung (C), heart (D), liver (E), and spleen (F) is based on the sum of scores assigned for inflammation and necrosis. Box represents the 25$^{th}$ to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group, middle line is median and whiskers are the minimum to maximum values. Kruskal-Wallis one-way ANOVA was used to compare parasite infected groups to controls followed by Dunn's *post hoc* adjustment and Mann-Whitney test was used to compare *N. caninum* and *T. gondii* infected animals at 7 days p.i. and 13 and 14 days p.i., respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

## Figure 4

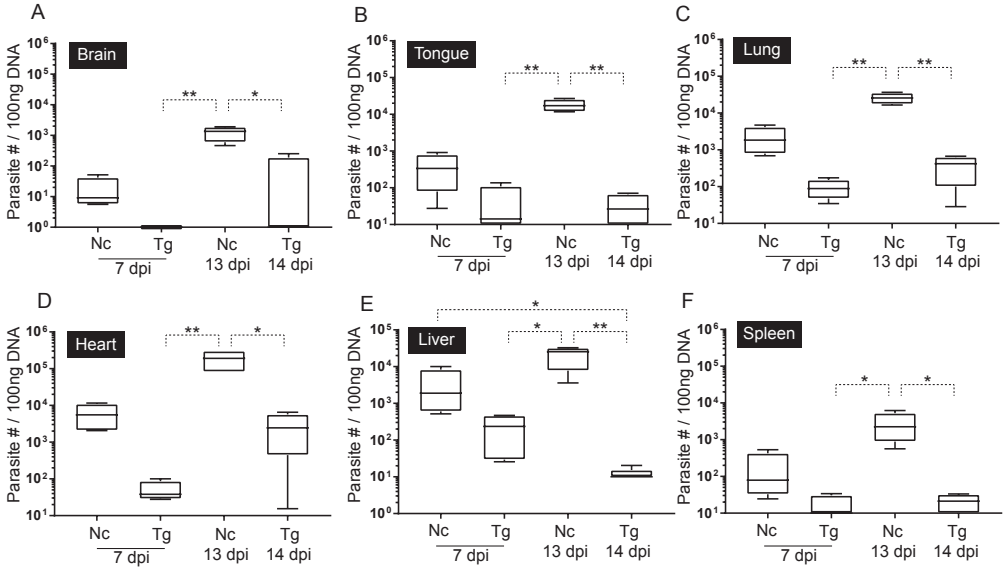

**FIGURE 4.** The parasite tissue load in fat-tailed dunnarts (*Sminthopsis crassicaudata*) experimentally infected with *Toxoplasma gondii* and *Neospora caninum* at 7 and 13-14 days p.i. determined by qPCR assays targeting the SAG1 gene of *T. gondii* and Nc5 gene of *N. caninum*. Results are expressed as total number of parasites in 100ng DNA isolated from the (A) brain, (B) tongue, (C) lung, (D) heart, (E) liver, and (F) spleen. Box represents the 25$^{th}$ to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group, middle line is median and whiskers are the minimum to maximum values. Parasite loads for each tissue were compared between different groups by Kruskal-Wallis one-way ANOVA followed by Dunn's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

## Figure 5

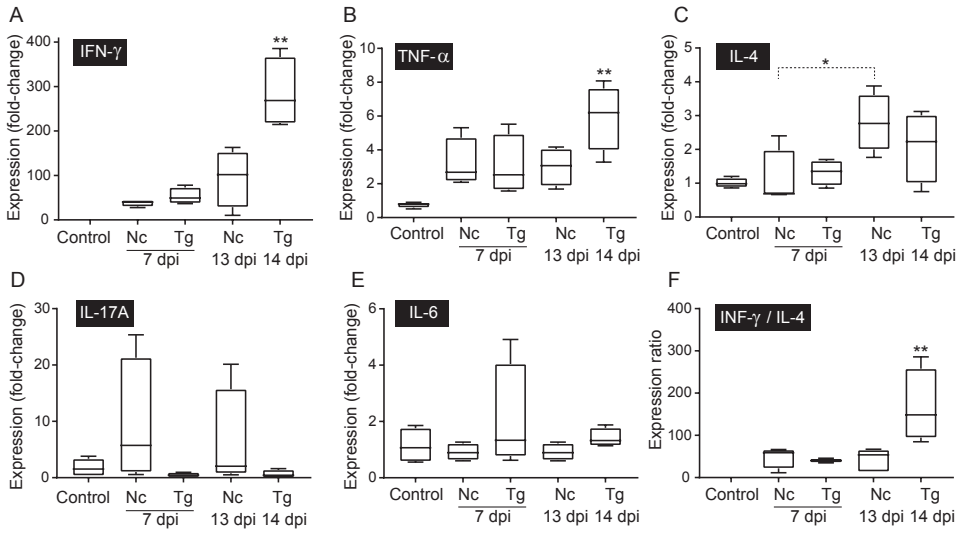

**FIGURE 5.** Splenic cytokine mRNA expression profiles as determined by RT-qPCR in fat-tailed dunnarts (*Sminthopsis crassicaudata*) experimentally infected with *Toxoplasma gondii* and *Neospora caninum* at 7 and 13-14 days p.i. The control group represents the uninfected animals. The results are expressed as normalized relative fold changes compared to reference genes GAPDH and 28S and have been scaled to control animals. Relative expression of IFN-γ (A), TNF-α (B), IL-4 (C), IL-17A (D), IL-6 (E) and the IFN-γ:IL-4 ratios (F) are shown. Box represents the 25$^{th}$ to 75$^{th}$ percentiles of the four biological replicates contained in each experimental group, middle line is median and whiskers are the minimum to maximum values. Kruskal-Wallis one-way ANOVA was used to compare cytokine expression between experimental groups followed by a Dunn's *post hoc* adjustment. *, $P < 0.05$; **, $P < 0.01$. Data shown are representative of two independent *T. gondii* experiments and two independent *N. caninum* experiments. Abbreviations: Nc, *N. caninum*; Tg, *T. gondii*; dpi, days postinfection.

**Figure 6**

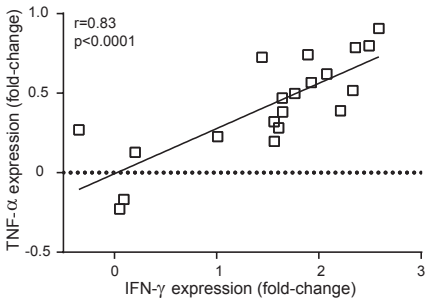

**FIGURE 6.** Correlation between IFN-γ and TNF-α mRNA expression in fat-tailed dunnarts (*Sminthopsis crassicaudata*) infected with *Toxoplasma gondii* and *Neospora caninum*. Each point represents one animal (uninfected, n=4; *T. gondii* infected, n=4 each for 7 and 14 days p.i.; *N. caninum* infected, n=4 each for 7 and 13 days p.i.) The correlation graph shows a fit line with a confidence curve. r and P values are shown from Spearman rank correlation analysis. r=correlation coefficient; P = significance level.