TITLE: Integrative transcriptome and proteome analyses define marked differences between Neospora caninum isolates throughout the tachyzoite lytic cycle

AUTHORS: Horcajo, P., **Xia, D**., Randle, N., Collantes-Fernández, E., Wastling, J., Ortega-Mora, L. M. and Regidor-Cerrillo, J.

JOURNAL: Journal of Proteomics

PUBLISHER: Elsevier

PUBLICATION DATE: 14 November 2017 (online)

DOI: 10.1016/j.jprot.2017.11.007

Integrative transcriptome and proteome analyses define marked differences between *Neospora caninum* isolates throughout the tachyzoite lytic cycle

P. Horcajo[1¥], D. Xia[2¥], N. Randle[3], E. Collantes-Fernández[1], J. Wastling[4], L.M. Ortega-Mora[1], J. Regidor-Cerrillo[1*].

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[2] The Royal Veterinary College, Royal College Street, London, NW1 0TU, United Kingdom.

[3] Department of Infection Biology, Institute of Infection & Global Health, University of Liverpool, Liverpool Science Park IC2, 146 Brownlow Hill, Liverpool, L3 5RF, United Kingdom.

[4] Faculty of Natural Sciences, Keele University, Keele, Staffordshire, ST5 5BG, United Kingdom.

[¥]Authors contributed equally

*Corresponding author: Javier Regidor Cerrillo, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n 28040, Madrid, Spain. E-mail: jregidor@ucm.es

Co-authors e-mail addresses: PH: phorcajo@ucm.es; DX: dxia@rvc.ac.uk; NR: Nadine.Randle@liverpool.ac.uk; ECF: esthercf@ucm.es; JW: j.wastling@keele.ac.uk; LMOM: luis.ortega@ucm.es; JRC: jregidor@ucm.es

25   **Abstract**

26   *Neospora caninum* is one of the main causes of transmissible abortion in cattle. Intraspecific

27   variations in virulence have been widely shown among *N. caninum* isolates. However, the

28   molecular basis governing such variability have not been elucidated to date. In this study label

29   free LC-MS/MS was used to investigate proteome differences between the high virulence isolate

30   Nc-Spain7 and the low virulence isolate Nc-Spain1H throughout the tachyzoite lytic cycle. The

31   results showed greater differences in the abundance of proteins at invasion and egress with 77 and

32   62 proteins, respectively. During parasite replication, only 19 proteins were differentially

33   abundant between isolates. The microneme protein repertoire involved in parasite invasion and

34   egress was more abundant in the Nc-Spain1H isolate, which displays a lower invasion rate.

35   Rhoptry and dense granule proteins, proteins related to metabolism and stress responses also

36   showed differential abundances between isolates. Comparative RNA-seq analyses during

37   tachyzoite egress were also performed, revealing an expression profile of genes associated with

38   the bradyzoite stage in the low virulence Nc-Spain1H isolate. The differences in proteome and

39   RNA expression profiles between these two isolates reveal interesting insights into likely

40   mechanisms involved in specific phenotypic traits and virulence in *N. caninum*.

41

42   **Keywords:** *Neospora caninum,* Low and high virulence isolates, Proteome,

43   Transcriptome

44

1. Introduction

Neospora caninum is a cyst-forming obligate intracellular protozoan parasite that is closely related to *Toxoplasma gondii*, which infects different domestic or wild canids as its definitive host and cattle and other ungulates as intermediate hosts [1]. *N. caninum* has been recognized as one of the main causes of abortion in cattle, resulting in devastating economic losses to the beef and dairy industries [2]. Although various factors are potentially involved in determining the dynamics of *N. caninum* infection, experiments in pregnant cattle have shown the key role of different isolates of *N. caninum* in the severity of disease and its capacity to cause foetal mortality in cattle [3-6]. Host tissue damage occurs as a consequence of the tachyzoite lytic cycle, a process that enables parasite propagation and involves the following successive steps: parasite invasion, adaptation to new intra-cytoplasmatic conditions, intracellular proliferation and egress from host cells [7,8]. Interestingly, the *in vitro* behaviour of a *N. caninum* population in these processes has demonstrated the potential association of the phenotypic traits such as the invasion rate and tachyzoite yield with pathogenicity observed in animal models [9-11]. Nevertheless, the molecular basis and mechanisms that govern such biological diversity in *N. caninum* remain largely unknown. *N. caninum* appears to be highly conserved genetically [12], although previous proteomic approaches have identified some differences between isolates [13-15]. Differences in secretory elements (rhoptry and dense granule proteins) and protein related to gliding motility and oxidative stress have been described among *N. caninum* isolates showing variations in protein expression, post-translational modifications and protein turnover [15]. Recently, an *in vitro* study comparing host cell modulation by *N. caninum* isolates with high (Nc-Spain7) and low (Nc-Spain1H) virulence has shown a great similarity in host transcriptome modulation by both isolates but marked differences in the parasite transcriptome between isolates [16].

In this study, we used a global approach to examine the changes between the *N. caninum* Nc-Spain7 and Nc-Spain1H isolates throughout the fast replicating tachyzoite lytic cycle. We exploited label free LC-MS/MS technology to investigate in deep proteome differences across the tachyzoite lytic cycle: after tachyzoite invasion and adaptation in the host cell at 12 hours post infection (hpi), during active parasite replication at 36 hpi and at early egress at 56 hpi. Furthermore, we analysed the transcriptome status of Nc-Spain7 and Nc-Spain1H using RNA-seq during tachyzoite egress from the host cell. We determined specific patterns of protein abundance for each isolate in each phase of the lytic cycle studied and differences between gene expression profiles that reveal interesting insights into differences in virulence between these two isolates.

2. Materials and methods

2.1 Parasite culture

Parasites were cultured in confluent Marc-145 cultures as previously described [17]. Briefly, medium from Marc-145 cultures grown for 24 h in DMEM with 10% of heat inactivated FBS and 1% antibiotic-antimycotic solution (Gibco, Gaithersburg, MD, USA) was replaced with DMEM supplemented with 2% FCS and 1% of antibiotic-antimycotic solution. Then, cell monolayers were inoculated with an adjusted multiplicity of infection (MOI) of Nc-Spain1H and Nc-Spain7 tachyzoites for parasite passaging onto a new Marc-145 monolayer each three – four days. All experiments in this study were conducted with tachyzoites from both isolates with a limited number of passages (Nc-Spain1H and Nc-Spain7, passage 13-18). All inoculations in *in vitro* assays were performed within one hour after tachyzoite collection from flasks.

2.2 Experimental design and tachyzoite production for proteome and transcriptome analyses

95   The overall experimental design is shown in Fig. 1. All experiments were carried out with

96   three biological replicates.

97   Confluent 24-h Marc-145 DMEM free of phenol red (Gibco, Gaithersburg, MD, USA)

98   and FBS were inoculated with purified Nc-Spain1H tachyzoites at a MOI of 7 and Nc-

99   Spain7 tachyzoites at a MOI of 4. Cell monolayers were recovered at 12 hpi (after

100   completion of invasion and prior to tachyzoite duplication), at 36 hpi (active proliferation

101   in the parasitophorous vacuole) and at 56 hpi (early egress), from T75 cm^2 flasks by cell

102   scraping in 5 ml of PBS supplemented with protease and phosphatase inhibitor cocktail

103   (Sigma-Aldrich, St. Louis, MO, USA), passaged by 25 G needles for host cell disruption

104   and purified using PD-10 (Sephadex G-25 columns -GE-Healthcare, Barrington, IL,

105   USA). Tachyzoite purification was carried out at 4ºC. The number and viability of

106   tachyzoites was determined by trypan blue exclusion followed by counting in a Neubauer

107   chamber. Tachyzoites were pelleted by centrifugation at 1,350 x g for 10 min. and stored

108   at – 80ºC until tachyzoite proteome (TZP) analysis.

109   Tachyzoite samples for transcriptome analysis were obtained as described above. Cell

110   cultures were recovered at 56 hpi, and tachyzoites were purified using PD10 columns as

111   described above. Tachyzoite pellets were directly resuspended in 300 µl of RNAlater

112   (Invitrogen, Carlsbad, CA, USA) and stored at – 80ºC until RNA extraction.

113   The tachyzoite growth and lytic cycle was monitored daily by microscopy, and

114   photomicrographs for each time-point of sample collection were obtained at 400x on an

115   inverted microscope (Nikon Eclipse E400) connected to a digital camera for checking

116   lytic cycle progression and sample collection in the programmed lytic cycle phases

117   (Fig.1A).

118   2.3 LC-MS/MS analyses

119   Detailed materials and methods for sample preparation, LC-MS/MS, proteome data

120   analysis, and Western blot validation are shown in Supplementary file 1. Briefly, prior to

121   trypsin digestion, tachyzoite pellets were resuspended in 25 mM ammonium bicarbonate

122   and RapiGest[TM] (Waters MS Technologies, Milford, MA, USA) for protein

123   solubilization, reduced with DTT and alkylated with iodocetamide for trypsin digestion.

124   Then, the digests were analysed using an LC-MS/MS system comprising an Ultimate

125   3000 nano system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific,

126   Waltham, MA, USA). Reversed-phase liquid chromatography was performed using the

127   Ultimate 3000 nanosystem by a linear gradient of 5-40% solvent B (80% acetonitrile in

128   0.1% formic acid) in 0.1% formic acid (solvent A). The Q-Exactive was operated in data-

129   dependent mode with survey scans acquired at a resolution of 70,000 at m/z 200. Up to

130   the top 10 most abundant isotope patterns were selected and fragmented by higher energy

131   collisional dissociation with normalized collision energies of 30. The maximum ion

132   injection times for the survey scan and the MS/MS scans were 250 and 100 ms,

133   respectively.

134   For proteome data analyses, the Thermo RAW files were imported into Progenesis QI

135   (version 2.0, Nonlinear Dynamics, Durham, CA, USA). Replicate runs were time-aligned

136   using default settings and an auto-selected run as a reference. Spectral data were

137   transformed into .mgf files with Progenesis QI and exported for peptide identification

138   using the Mascot (version 2.3, Matrix Science, London, UK) search engine and the

139   database ToxoDB-26_N*caninum* LIV_Annotated Proteins (version 26, ToxoDB). The

140   false discovery rates were set at 1% and at least two unique peptides were required for

141   reporting protein identifications. Finally, protein abundance (iBAQ) was calculated as the

142   sum of all the peak intensities (from the Progenesis output) divided by the number of

143   theoretically observable tryptic peptides for a given protein (Fig. 1B).

144   The mass spectrometry proteomics data have been deposited in the ProteomeXchange

145   Consortium via the PRIDE [18] partner repository with the dataset identifier PXD007062.

146   The identified proteins were classified according to their parasite localization and

147   functionality according to the Gene Ontology (GO) terms (annotated and predicted) on

148   the ToxoDB website [19] for the Nc-Liverpool isolate (ToxoDB-

149   26_N*caninum*LIV_AnnotatedProteins), *T. gondii* syntenic homologues (version 26,

150   ToxoDB) and previous reports [20-22].

151   Validation of LC-MS/MS results was performed by measuring the differential abundance

152   of the proteins MIC2 (NCLIV_022970), ROP2 (NCLIV_001970), and NTPase

153   (NCLIV_068400) between isolates by Western blot analyses using the SAG1 protein

154   (NCLIV_033230) as a housekeeping gene as previously described [23,24]. Images from

155   WB membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, Hercules,

156   CA, USA) and were analysed with Quantity One quantification software v. 4.0 (Bio-Rad

157   Laboratories, Hercules, CA, USA) for protein quantification.

158   2.4 RNA extraction, sequencing and detection of differential mRNA expression

159   Total RNA was isolated from purified tachyzoites using an RNeasy Mini kit (Qiagen,

160   Hilden, Germany). RNA-Seq was undertaken at the Centre for Genomic Research,

161   University of Liverpool. Briefly, polyadenylated RNA was purified using the

162   Dynabeads® mRNA purification kit (Invitrogen, Carlsbad, CA, USA) and used to

163   prepare RNA-Seq libraries with the Epicentre ScriptSeq v2 RNA-Seq Library Preparation

164   kit (Illumina, San Diego, CA, USA). Libraries were sequenced on the HiSeq2500

165   (Illumina, San Diego, CA, USA) as 2 x 100-bp paired-end sequencing using rapid-run

166   mode chemistry. Filtered sequencing reads were aligned against the reference genome of

167   *N. caninum* (NcLiv26; ToxoDB.org) by the TopHat2 aligner and processed with the

168   Cufflinks software (version 2.1.1) to assemble transcripts, quantify expression levels and

169   analyse differentially expressed genes (DEGs) (Fig. 1C).

170   Validation of transcript expression was performed on three additional biological

171   replicates of tachyzoite samples at 56 hpi prepared as described above using SYBR green

172   quantitative PCR. More detailed material and methods for RNAseq and validation by

173   quantitative real-time PCR are provided in Supplementary file 1.

174   2.5 *In vitro* invasion analysis

175   The invasion capacity of *N. caninum* isolates was determined in a plaque assay in which

176   100 purified tachyzoites of each isolate per well were added to MARC-145 monolayers

177   in 24-well culture plates as previously described [10]. Briefly, the plates were incubated

178   at 37ºC in a 5% CO2-humidified incubator for 48 h, and cell monolayers were labelled

179   by immunofluorescence using anti-tachyzoite hyperimmune rabbit antiserum (1:4000) as

180   the primary antibody and Alexa 594-conjugated goat anti-rabbit secondary antibody

181   (1:1000) (Molecular Probes, Eugene, Oregon, USA). Plate wells were examined using a

182   fluorescence-inverted microscope Nikon Eclipse E400 (Nikon Instruments Europe) at a

183   magnification of 200x to count the labelled parasitophorous vacuoles and lysis plaques

184   (events) per well after parasite growth. Differences between isolates in invasion rates

185   were determined using the U Mann-Whitney test.

186   2.6 Tachyzoite cell cycle analysis

187   The cell cycle phase of the tachyzoite culture was studied by flow cytometry. Tachyzoite

188   samples were obtained under the same conditions as detailed previously for proteomic

189   and transcriptome analyses. Purified tachyzoites were pelleted for 10 min at 1350×g and

190   resuspended in 70% (v/v) ethanol with constant shaking, pelleted and resuspended in

191   PBS. Fixed tachyzoites were labelled by indirect immunofluorescence (IFI) for the

192   differentiation of individual parasites from those forming aggregates. Tachyzoites were

193   permeabilised with PBS containing 3% BSA and 0.25% Triton X-100 for 30 min at 37ºC

194   and labelled using anti-tachyzoite mouse antiserum (1:50 dilution) as the primary

195   antibody and a FITC-conjugated goat anti-mouse IgG at 1:1,000 dilution (Molecular

196   Probes, Eugene, Oregon, USA) as the secondary antibody. Then, the parasites were

197   treated with 250 U RNase A (Ambion, Austin, TX, USA) in the dark for 30 min,

198   resuspended in BD CellFIX™ (Becton-Dickinson, Erembodegem, Belgium), and stained

199   with propidium iodide (PI) (1 μg/ml final concentration). Tachyzoite populations with

200   lower FITC fluorescence were separated as individual tachyzoites. The nuclear DNA

201   content was measured based on the PI fluorescence using a 488 nm argon laser and a

202   Becton-Dickinson FACSCalibur flow cytometer (Becton-Dickinson, Erembodegem,

203   Belgium). Fluorescence was collected in linear mode (10 000 events), and the results were

204   quantified using CELLQuest™ v3.0 (Becton-Dickinson, Erembodegem, Belgium). The

205   percentages of G1 (1 N), S (1–2 N) and G2+M (2 N) tachyzoites were calculated based

206   on defined gates for each population. Two biological replicates were analysed for each

207   isolate.

208

209   **3. Results and discussion**

210   3.1 Nc-Spain1H and Nc-Spain7 tachyzoite proteomes resemble fast-growing,

211   metabolically active and invasive tachyzoites

212   A total of 1,390 proteins were identified with high confidence (FDR< 1% and containing

213   at least two uniquely identified peptides) and quantified using Progenesis software for the

214   Nc-Spain1H and Nc-Spain7 isolates across the tachyzoite lytic cycle. The identified

215   proteins covered ~19.6% of the predicted *N. caninum* proteins deposited in the UniProt

216   database (7,111 predicted proteins, UniProtKB; UP000007494, *Neospora caninum* strain

217   Liverpool) [25]. Raw data are deposited in ProteomeXchange under identifier

218 PXD007062. Each individual identified and quantified protein was categorized by the

219 cellular component and functional prediction. Of the proteins, 14.5% had an unknown

220 localization. Proteins with cytoplasmic (18.49%), nuclear (16.26%) and mitochondrial

221 (11.15%) localizations were also highly represented, followed by proteins associated with

222 the plasma membrane (glycolipid-anchored SAG1-related sequences [SRS] and other

223 proteins localized in the plasma membrane) (9.78%). Proteins from secretory organelles

224 (including micronemes, rhoptries and dense granules) represented approximately 5%

225 (corresponding to 70 proteins) of all those identified proteins (Supplementary Fig. 1A and

226 supplementary file 2). A total of 66.47% of 1,390 proteins from the TZP were assigned

227 to a functional category (Supplementary Fig. 1B and supplementary file 2). The most

228 represented functional categories included proteins involved in metabolism (12%),

229 cellular transport (10%), protein synthesis (9.57%) and protein fate (9.35%). As expected,

230 protein localization and functional profiles identified in both *N. caninum* isolates

231 corresponded to the proteome for the fast growing, metabolically active and invasive

232 tachyzoites.

233 3.2 Relative quantification demonstrated different proteomes for Nc-Spain1H and Nc-

234 Spain7 throughout the tachyzoite lytic cycle

235 Progenesis analysis detected 351 proteins at 12 hpi, 136 proteins at 36 hpi and 214

236 proteins at 56 hpi, with a significant increase or decrease in relative abundance between

237 Nc-Spain1H and Nc-Spain7 TZPs (q < 0.05) (Supplementary file 3). These results

238 demonstrated marked differences in the TZP throughout the lytic cycle between the Nc-

239 Spain1H and Nc-Spain7 isolates. Focusing on significant changes (q < 0.05) in the

240 relative abundance with a fold change ≥ 2 between the Nc-Spain1H and Nc-Spain7

241 isolates, there were 77, 19 and 62 proteins with differing abundance at 12 , 36 and 56 hpi,

242 respectively (Fig. 2A, B and C, respectively and Supplementary file 3). Most of the

243 proteins that were differentially abundant between isolates were unique to each time-point

244 of the tachyzoite lytic cycle (Fig. 3 and supplementary file 4). There were only 7 out of

245 82 Nc-Spain1H proteins and 2 out of 36 Nc-Spain7 proteins that were consistently more

246 abundant across the tachyzoite lytic cycle.

247 Enriched GO terms or pathways associated with a particular isolate were not found likely

248 due to the high proportion of hypothetical proteins. Nonetheless, differences in the

249 abundance of proteins belonging well-established categories related with invasion

250 machinery, metabolism and response to stress were found between isolates and are

251 detailed below (Fig. 4).

252 The proteome results from LC-MS/MS were confirmed by WB analyses with available

253 antibodies. The WB results provided similar results to the LC-MS/MS quantification with

254 a higher abundance of NcMIC2 at 12 and 56 hpi in the Nc-Spain1H isolate and a similar

255 protein abundance of NcROP2 and NcNTPase in both isolates (Supplementary Fig. 2).

256 3.3 Abundance of proteins involved in host cell attachment and invasion varied between

257 Nc-Spain1H and Nc-Spain7 tachyzoite proteomes

258 *Surface antigens differ in abundance between Nc-Spain1H and Nc-Spain7 tachyzoite*
259 *proteomes.*

260 Members of the SRS protein family were differentially abundant between the Nc-

261 Spain1H and Nc-Spain7 isolates. SRS antigens exert a relevant role in host cell

262 attachment, modulation and evasion of host immunity, and the regulation of virulence

263 [26-28]. An orthologue of, but not syntenic to, TgSAG3 (NCLIV_034740) throughout the

264 tachyzoite lytic cycle and an orthologue of SRS67 (NCLIV_046140) at 56 hpi were

265 significantly more abundant in Nc-Spain1H. More interestingly, NcSAG4

266 (NCLIV_019580) showed larger fold changes at all time-points in Nc-Spain1H, with the

267 greatest differences compared with Nc-Spain7 at 36 and 56 hpi (12.8-fold and 14.6-fold,

268 respectively). NcSAG4 has been described as a *N. caninum* bradyzoite stage-specific

269   marker [29]. Furthermore, SRS6 (NCLIV_010050), which has been found over-

270   expressed in bradyzoites of *T. gondii* [30], was also significantly more abundant in Nc-

271   Spain1H at 56 hpi. Bradyzoite development of *T. gondii* has been correlated with a

272   reduction in the tachyzoite growth rate [31]. Nc-Spain1H has shown a low *in vitro* growth

273   rate [9] which may facilitate the conversion to the bradyzoite, although has been

274   previously shown that Nc-Spain1H produce only intermediate bradyzoites *in vitro* [32].

275   The transcriptome analysis, which is detailed in the next section, could corroborate the

276   hypothesis of a pre-bradyzoite stage in Nc-Spain1H since an important bradyzoite gene

277   profile was found to be over-expressed in this isolate. The only SRS protein that was more

278   abundant in Nc-Spain7 throughout the lytic cycle was the orthologue of SRS39

279   (NCLIV_023620), which is also predominantly expressed during the bradyzoite stage of

280   in the *T. gondii* [30]. It could be interesting to determine the relevance of SRS39 in *N.*

281   *caninum.*

282   *The microneme protein repertoire is more abundant in Nc-Spain1H tachyzoites, but Nc-*

283   *Spain1H displays a lower invasion rate.*

284   Micronemes in Apicomplexan parasites are specialized secretory organelles that are

285   critical for essential cellular processes such as attachment and penetration [33]. A total of

286   9 microneme (MIC) proteins were significantly more abundant in Nc-Spain1H at 56 hpi,

287   and most of them were also more abundant in Nc-Spain1H at 12 hpi, four with FC > 2

288   and the other four with FC very close to 2 (> 1.8). Only MIC8 (NCLIV_062770) had a

289   higher abundance at 12 hpi but not at 56 hpi. At 36 hpi, differences in the abundance of

290   MIC proteins were not found. Among MIC proteins with higher abundance in Nc-

291   Sapin1H, orthologues assembling the main TgMIC complexes (MIC1

292   [NCLIV_043270]/MIC4 [NCLIV_002940] /MIC6; MIC2 [NCLIV_022970]/M2AP

293   [NCLIV_051970]; and MIC8 [NCLIV_062770]/MIC3 [NCLIV_010600]) and others as

294　MIC2-like1 (NCLIV_033690), MIC10 (NCLIV_066250), MIC11 (NCLIV_020720) and

295　MIC17B (NCLIV_038110) were identified. Some of these proteins have been

296　characterized in *N. caninum* and have been associated with invasion processes [34-37].

297　In contrast to these results, in previous work comparing changes in the proteome

298　expression between these two isolates using DIGE, NcMIC1 was found more abundant

299　in the highly virulent isolate Nc-Spain7 [15]. However, these contrasting results are likely

300　due to differences in techniques, considering that DIGE analysis detects variations in

301　protein species that are likely also attributed to post-translational modifications.

302　NcAMA1 (NCLIV_028680), which is also involved in *N. caninum* invasion [38], was

303　also increased in Nc-Spain1H TZP at 12 and 56 hpi.

304　In addition to MIC proteins, proteases involved in micronemal protein processing or that

305　contribute to microneme-dependent processes, such as egress, gliding motility, and

306　parasite invasion of host cells, were also more abundant in Nc-Spain1H. Orthologues of

307　the protease SUB1 (NCLIV_021050), which is involved in micronemal protein

308　processing [39], the metalloproteinase toxolysin 4, TLN4, (NCLIV_044230) and the

309　cathepsin L-like protease CPL (NCLIV_004380), proteinase localized outside the

310　micronemes in the vacuolar component, which in *T. gondii* contributes to the proteolytic

311　maturation of proTgM2AP and proTgMIC3 [40], were also found more abundant in Nc-

312　Spain1H at 12 hpi (SUB1) and at 56 hpi (SUB1, TLN4 and CPL). Similarly, PLP1

313　(NCLIV_020990), a perforin-like protein secreted from micronemes that likely plays a

314　role in parasite egress more than invasion [41], and an orthologue of the chitinase-like

315　protein CLP1 (NCLIV_000740) related to macrophage stimulation to release pro-

316　inflammatory cytokines in *T. gondii* [42], were more abundant in Nc-Spain1H at 12

317　(PLP1) and at 56 hpi (PLP1 and CLP1).

318 All these results seem to indicate that the low virulent isolate Nc-Spain1H has powerful

319 machinery involved in host cell attachment and invasion. In this study, we tested the

320 ability of Nc-Spain1H and Nc-Spain7 to invade host cells. The Nc-Spain1H showed a

321 significant reduction of host invasion (p < 0.005) in comparison to Nc-Spain7 (Fig. 5A),

322 as demonstrated in previous work [9,11]. Considering these results, the Nc-Spain1H

323 isolate may be compromised in other unknown mechanisms that are relevant for

324 attachment/invasion processes and the over-expression of all of these factors could be an

325 attempt to compensate for some other deficiency.

326 *Rhoptry proteins also differ in abundance between isolates.*

327 Rhoptry proteins are recognized as one of the major virulence factors in *T. gondii* [43,44].

328 Three proteins from rhoptries had different abundances between isolates. NcROP1

329 (NCLIV_069110), which is involved in early invasion [45], showed an approximately 2-

330 fold higher abundance in Nc-Spain1H at 12 and 56 hpi. Interestingly, a predicted member

331 of the rhoptry kinase family ROP20 specific for *N. caninum* (NCLIV_068850), which is

332 orthologous, but not syntenic, to the *T. gondii* virulence factor ROP24 had greater

333 abundance in the highly virulent isolate Nc-Spain7 across the lytic cycle. The orthologue

334 of the bradyzoite pseudokinase 1, BPK1 (NCLIV_007770) was also more abundant in the

335 Nc-Spain7 isolate across the lytic cycle. BPK1 plays a crucial role in the *in vivo*

336 development of *Toxoplasma* cysts [46]. The abundances of BPK1 and SRS39 in Nc-

337 Spain7 are inconsistent with those other bradyzoite-related proteins that were more

338 abundant in the Nc-Spain1H. It remains to be determined if there is a functional role for

339 these proteins in the tachyzoite stage of *N. caninum*.

340 3.4 Metabolic processes are differentially regulated between Nc-Spain1H and Nc-Spain7
341 isolates

342 *Gluconeogenesis is up-regulated in the low virulent isolate Nc-Spain1H*

343 Eleven proteins related to carbohydrate metabolism were differentially abundant between

344 isolates, with the greatest differences at 12 hpi. Ten proteins were more abundant in Nc-

345 Spain1H: fructose-1,6-bisphosphatase (NCLIV_050070), fructose-1,6-bisphosphatase

346 class 1 (NCLIV_050080), glycerol-3-phosphate dehydrogenase (NCLIV_001180),

347 phosphoglucomutase (NCLIV_010960), phosphoenolpyruvate carboxykinase

348 (NCLIV_041900), glucosamine:fructose-6-phosphate aminotransferase

349 (NCLIV_031610), cytochrome c (NCLIV_060860) and orthologues of aspartate

350 aminotransferase (NCLIV_064760), glycosyltransferase (NCLIV_004200) and citrate

351 synthase I (NCLIV_037460), which showed a 2-2.7-fold higher abundance in Nc-

352 Spain1H. During exponential growth (36 hpi), only the glucosamine-fructose-6-

353 phosphate aminotransferase (NCLIV_031610) was 2-fold more abundant in Nc-Spain1H.

354 At 56 hpi, there were no differences in these proteins between the isolates. Some of these

355 proteins suggest that Nc-Spain1H fulfils a gluconeogenic function. Co-expression of

356 enzymes involved in glycolysis and gluconeogenesis is a mechanism to rapidly adapt to

357 changing nutrient conditions in their host cells [47] and could be an important glucose

358 regulatory mechanism [48]. Over-expression of the function of gluconeogenesis in Nc-

359 Spain1H may also indicate a failure in glucose salvage from the host cell and thus could

360 be an important limiting factor for parasite growth. In relation to this hypothesis, the

361 orthologue of the transporter/permease protein related to carbohydrate transport

362 (NCLIV_039290) was more abundant in the highly virulent isolate Nc-Spain7 (3.3-fold).

363 This carbohydrate transporter in Nc-Spain7 could facilitate the capture of glucose from

364 the host and consequently the faster growth of this isolate.

365 *Fatty acid biosynthesis in the apicoplast (FAS II system) and the metabolism of*

366 *phospholipids are differentially regulated between isolates.*

367 The apicoplast is indispensable for parasite survival and is the location of several anabolic

368 pathways such as type II fatty acid [49]. Interestingly, orthologues of apicoplast triose

369   phosphate translocator APT1 (NCLIV_026210) and beta-hydroxyacyl-acyl carrier

370   protein dehydratase FABZ (NCLIV_004340), showed 2.8 and 2.4-fold higher abundance,

371   respectively, in Nc-Spain7 at 12 hpi. The APT1 was again more abundant in Nc-Spain7

372   (2.3-fold) at 56 hpi. In *T. gondii,* APT1 is required for fatty acid synthesis in the

373   apicoplast, but it also supplies the apicoplast with carbon skeletons for additional

374   pathways and indirectly with ATP and redox equivalents [50]. In addition, APT1 has been

375   shown to be an essential protein for parasite survival in *Toxoplasma* and *Plasmodium.*

376   Conversely, it is striking that the low virulence isolate Nc-Spain1H exhibited up-

377   regulated recycling of the phospholipids pathway, (LAMP, http://www.llamp.net) at 36

378   and 56 hpi. Lipin protein (NCLIV_031190) and the glycerol-3-phosphate acyltransferase

379   (NCLIV_029980) had 2.3 and 2.2-fold higher abundances, respectively, in Nc-Spain1H

380   at 36 hpi. At 56 hpi, the glycerol-3-phosphate acyltransferase (NCLIV_029980) and the

381   acyl-CoA synthetase (NCLIV_054250) had a 2-fold increased abundance in Nc-Spain1H.

382   Phospholipids are the major lipid components of biological membranes, but lipids also

383   serve as signalling molecules, energy stores, post-translational modifiers, and

384   pathogenesis factors. It might be interesting to assess whether this up-regulation of

385   proteins in Nc-Spain1H may be a strategy to counteract the effect of the lack of an

386   apicoplast contribution.

387   All these metabolic differences between isolates could, at least partially, explain their

388   phenotypic variations in growth if, as in *T. gondii,* strain-specific growth rates and

389   virulence are driven by altered metabolic capacities [51].

390   3.5 Other key biological systems are also differentially represented between isolates.

391   *Antioxidant and stress response systems*

392   In *N. caninum* the importance of the production of IFN-gamma-induced NO in

393   macrophages as a mechanism for killing intracellular *N. caninum* has been demonstrated

394 [52]. To counteract this oxidative stress, parasites are equipped with specific ROS-

395 detoxifying mechanisms that are critical for parasite survival and the establishment of

396 infection [53]. The superoxide dismutase SOD2 (NCLIV_058830) had a 2-fold higher

397 abundance in Nc-Spain1H TZP at 12 hpi. SOD2 in *T. gondii* is dually targeted to both the

398 apicoplast and the mitochondrion of *T. gondii,* two organelles that require protection from

399 oxidative stress [54]. Other proteins with oxidoreductase activity, such as the pyridine

400 nucleotide-disulphide oxidoreductase family protein (NCLIV_029160), also showed a

401 higher abundance in Nc-Spain1H at 12 hpi.

402 By contrast, another well-known antioxidant enzyme, the peroxiredoxin-2E-1

403 (NCLIV_014020), was more abundant in Nc-Spain7 throughout the entire tachyzoite

404 lytic cycle. Peroxiredoxins provide another defence mechanism against oxidative damage

405 and, in addition, some peroxiredoxins have been proposed as important

406 immunomodulators in parasites [55,56]. Additionally, the putative serine/threonine

407 protein phosphatase 5 (NCLIV_066900) and the orthologue of the NAD/NADP

408 octopine/nopaline dehydrogenase (NCLIV_042450) displayed higher abundance in Nc-

409 Spain7 at 12 hpi (2-2.5-fold) and 56 hpi (2.7-fold), respectively.

410 These findings could suggest that different ROS-detoxifying mechanisms are used by the

411 different *N. caninum* isolates, which may contribute to their differing resistances to the

412 host immune response.

413 Heat shock proteins are other stress-inducible proteins that can have important roles in

414 parasite survival, although their functions are still unknown. Stress conditions associated

415 with bradyzoite development induce the expression of HSPs such as HSP70 and HSP90

416 and HSP21 in *T. gondii* [57-59]. In the present work, the orthologue of HSP29

417 (NCLIV_041850), which is associated with the membrane of *T. gondii* [60], was 2-fold

418 more abundant in Nc-Spain1H at 12 hpi.

419   *Ubiquitin-proteasome system.*

420   The ubiquitin-proteasome system plays a role the biological processes of parasites such

421   as differentiation, cell cycle progression, proliferation and encystation [61]. The

422   orthologue of serine carboxypeptidase s28 protein (NCLIV_008320) and peptidase,

423   S9A/B/C family (NCLIV_063570), proteins related to the proteasome demonstrated a

424   higher abundance at 36 (2.6 and 2.4-fold, respectively) and 56 (3.6 and 2.5-fold,

425   respectively) hpi in Nc-Spain1H than in Nc-Spain7. Conversely, the ubiquitin family

426   domain-containing protein (NCLIV_012950) and ubiquitin conjugation factor

427   (NCLIV_019660), which are related to the ubiquitination process, were 2-fold more

428   abundant in Nc-Spain7 at 56 hpi. In *T. gondii,* proteasome inhibitors do not affect host

429   cell invasion but block parasite proliferation, daughter-cell budding, as well as DNA

430   synthesis [61]. Furthermore, a study of the ubiquitin proteome of *T. gondii* has revealed

431   a large number of ubiquitinated proteins localized to the cytoskeleton and inner

432   membrane complex, as well as their roles as critical regulators of cell division and cell

433   cycle transitions [62].

434   3.6 Nc-Spain1H and Nc-Spain7 tachyzoite transcriptome also showed marked variations

435   between isolates but were inconsistent with the proteome results

436   We also investigated the transcriptome of Nc-Spain1H and Nc-Spain7 during early egress

437   (56 hpi). Differential expression analysis revealed 550 DEGs between Nc-Spain1H and

438   Nc-Spain7. Among these genes, 369 were over-expressed in Nc-Spain1H and 181 in Nc-

439   Spain-7 (Supplemental file 5). The qPCR for RNA-Seq validation displayed a profile

440   similar to the RNA-Seq results, with a similar significance and direction of fold change

441   in the nine genes analysed (Supplementary Fig. 3).

442   Furthermore, a comparison of the differentially expressed transcripts and the

443   differentially expressed proteins at the same time-point showed that only 41 genes were

444   differentially expressed at both levels. Among them, only approximately 50% of genes

445   showed a consistent direction of fold change. When we compared DEGs and proteins

446   with different abundances (FC>2), 6 matches were obtained. A few studies have reported

447   a less than perfect correlation between transcript and protein expression, in Apicomplexan

448   [63,64] and mammalian systems [65,66]. The reasons for this phenomena are multiple

449   fold, including variations in protein degradation turn-over rates [67], differences in

450   posttranslational regulation [68], cellular functions [69] and last but not the least,

451   technical variations [70]. Despite the weak correlation found between the transcriptomic

452   and proteomic data in this work, transcriptome and proteome profiles highlight some

453   common distinctions among isolates.

454   Unfortunately, similarly to the proteomics results, a large portion (approximately 50%)

455   of these DEGs are annotated as hypothetical proteins or proteins with unknown function,

456   leading to a loss of interesting information. However, the transcriptome profiles showed

457   differences in tachyzoite-bradyzoite conversion, secretory elements, metabolism and

458   transcriptional regulation between isolates.

459   *Bradyzoite-specific genes were highly expressed in the Nc-Spain1H isolate.*

460   A remarkable result was the large number of bradyzoite-specific genes that were over-

461   expressed in the low virulent isolate Nc-Spain1H (Table 1). The gene encoding the

462   bradyzoite cytoplasmic antigen BAG1 (NCLIV_027470), which facilitates the transition

463   from the tachyzoite to bradyzoite in *T. gondii* [71] had the largest fold change between

464   isolates, demonstrating 140.6-fold higher expression in Nc-Spain1H. Other over-

465   expressed genes were the bradyzoite-specific surface antigens in *N. caninum* BSR4

466   (NCLIV_010030) and SAG4 (NCLIV_019580) [29,72]. Interestingly, as we have

467   previous commented, SAG4 protein was also more abundant in the Nc-Spain1H isolate

468   across the lytic cycle in this study. In addition to these stage-specific genes, the gene

related to early tachyzoite conversion into bradyzoites, PMA1 (NCLIV_022240) [73] also displayed higher expression levels in the Nc-Spain1H isolate (23.6-fold). In addition, some isoenzymes involved in glycolysis (lactate dehydrogenase, enolase, glucose 6-phosphate isomerase) that are stage-specifically expressed in *T. gondii* and *N. caninum* bradyzoites [59,74] such as LDH2 (NCLIV_042910) and ENO1 (NCLIV_037490) were expressed at higher levels in Nc-Spain1H. The *N. caninum* gene orthologue of *T. gondii* deoxyribose phosphate aldolase-like, which is involved in the utilization of deoxyribose as a carbon and energy source in the bradyzoite stage [75], also exhibited higher expression in the Nc-Spain1H isolate. Genes related to the tissue cyst wall, such as NcMCP4 (NCLIV_003250) [76] and CST1 (NCLIV_040495), which play essential roles in the structural integrity and persistence of brain cysts [77], had 12-fold and 40.3-fold higher expression levels in the Nc-Spain1H isolate, respectively. Furthermore, higher expression levels of ribosomal proteins have been associated with strains that readily switch from the tachyzoite to bradyzoite in *T. gondii* [78]. This phenotype was also observed in the low virulence Nc-Spain1H isolate since 23 ribosomal proteins showed higher expression. It has been shown that Nc-Spain1H can start tachyzoite-bradyzoite conversion *in vitro* under induction conditions, although it is not really completed to encysted bradyzoites [32]. The bradyzoite-transcriptome and proteome profile expressed by the Nc-Spain1H isolate could be related to its slower growth rate. Interestingly, *T. gondii*, bradyzoite formation is preceded by, and critically dependent on, a parasite cell cycle shift towards slower growth [31]. These early switching parasites have transcriptomes that are very similar to those of tachyzoites, indicating that these early developing parasites are largely slow-growing tachyzoites that can be considered pre-bradyzoites [79]. This pre-bradyzoite stage in Nc-Spain1H may explain, at least partially, its lower virulence in terms of causing abortion in cattle since tachyzoite growth (lower

494   in strains that tend to form bradyzoites) is a phenotypic trait related to virulence in *N.*

495   *caninum* and *T. gondii* [9,44,80].

496   *Host cell attachment and invasion machinery is also differentially expressed between*

497   *isolates.*

498   Seventeen genes encoding for SRS displayed higher abundances in the Nc-Spain1H

499   isolate, while only four were over-expressed in Nc-Spain7. These proteins are highly

500   immunogenic, and their expression is thought to regulate the virulence of infection [26].

501   In the proteome analyses, only five proteins demonstrated different abundances between

502   isolates, and the only one that matched between the transcriptome and proteome was the

503   bradyzoite antigen SAG4.

504   In contrast to the proteome results, the transcriptome analyses revealed fewer microneme

505   genes than SRS genes with differential expression between isolates. Seven had higher

506   expression in Nc-Spain1H and only 2 in Nc-Spain7. According to the proteome results,

507   MIC17B (NCLIV_038110) had higher expression in the Nc-Spain1H isolate. However,

508   the gene encoding the TLN4 protein, with an increased abundance in the Nc-Spain7

509   proteome at 56 hpi, was expressed at a higher level in the Nc-Spain1H isolate.

510   *Secretory effectors, rhoptry and dense granule proteins, also demonstrated differential*

511   *mRNA expression between isolates.*

512   Similarly to the proteomic results, the non-syntenic orthologue of *T. gondii* ROP24 was

513   more highly expressed in the highly virulent isolate Nc-Spain7, and the non-syntenic

514   orthologue of *T. gondii* ROP1 (NCLIV_069110) was more highly expressed in the low

515   virulent isolate Nc-Spain1H, but at 56 hpi instead of 12 hpi in the proteome. Another

516   rhoptry protein with elevated expression in Nc-Spain1H was the predicted lineage-

517   specific rhoptry kinase, subfamily ROPK-Eten1 (NCLIV_017420), which is present in

518   the *T. gondii* ROP38/29/19 gene locus that has been found to be necessary to establish

519   chronic infection in mice [81]. The only dense granule protein with differential expression

520   (2-fold higher in Nc-Spain7) was GRA17 (NCLIV_005560), which in *T. gondii* leads to

521   more rapid growth [82]. In the proteome studies, fewer differences were also found in

522   these proteins, and no GRA proteins were differentially expressed at 56 hpi.

523   In *T. gondii,* two different sub-transcriptomes have been described, depending on the

524   tachyzoite cell cycle phase. The G1-subtranscriptome characterized by the expression of

525   genes related to biosynthetic and metabolic functions and the S/M-subtranscriptome

526   enriched in specific genes for specialized apicomplexan processes, in which are included

527   SAG, MIC and ROP proteins [83]. Because the observed differences in proteome

528   elements and DNA transcription could be a consequence of a different progression across

529   the tachyzoite cell cycle, we examined the DNA content in asynchronously growing Nc-

530   Spain1H and Nc-Spain7 at 12, 36 and 56 hpi. At all time-points analysed, the flow

531   cytometry results for the tachyzoites profiles showed that most parasites were in G1 and

532   there were no differences between isolates (Fig. 5B), supporting a similar tachyzoite cell

533   cycle status at the studied time-points.

534   Although these results showing elevated expression of host cell attachment and invasion

535   machinery and secretory effectors related to virulence in the isolate with a reduced

536   invasion rate and virulence are quite surprising, a recent study has shown that the genes

537   encoding these functions also exhibited elevated expressions in the Nc-Spain1H isolate

538   at 12 hpi [16].

539   *Different ApiAP2 repertoire may lead to differences in RNA expression and proteomes.*

540   Although the mechanisms underlying gene-specific regulation in Apicomplexan parasites

541   are not completely known, the AP2 transcription factors in *T. gondii* have been associated

542   with important functions including stage-specific gene activation, determination of

543   differences between strains, parasite virulence and host invasion [78,79,84,85]. Most of

544 the identified ApiAP2s in *N. caninum* (54 out of 68) are expressed during the tachyzoite

545 stage, similar to *T. gondii* [27]. In our study, seven AP2 factors displayed higher

546 expression in the highly virulence isolate Nc-Spain7 (AP2VIII-3, AP2VIII-4, AP2IX-8,

547 AP2X-2, AP2X-11, AP2XI-1 and AP2XII-8) and two in the low virulence isolate Nc-

548 Spain1H (AP2III-1 and AP2X-10). In the proteome, by contrast, no AP2 factors exhibited

549 a differential abundance between isolates, which was likely due to lower sensitivity of the

550 technique in comparison to RNA-Seq. Some of these factors have been found to be

551 functional in stage-specific or cell cycle-regulated or even strain-specific expression in *T.*

552 *gondii* [79], and thus a similar characterization in *N. caninum* would be very useful to

553 better understand the regulation of gene expression in this parasite.

554 **4. Concluding remarks**

555 In this study, we performed proteome and transcriptome comparisons between two well-

556 characterized *N. caninum* isolates and established marked differences and revealed the

557 mechanisms potentially associated with phenotypic traits and virulence displayed by

558 these isolates. The invasion machinery, metabolism, response to stress and the tendency

559 to form bradyzoites have emerged as principal key factors for isolate behaviour and likely

560 pathogenesis. However, it should be noted that the majority of DEGs and proteins

561 between isolates were annotated as hypothetical and proteins with unknown function.

562 Enhancing the knowledge of this issue could be the best approach to unravel the

563 mechanisms underlying the molecular basis of the virulence of *N. caninum*.

564 Furthermore, the weak correlation between gene expression and protein abundance

565 highlighted the importance of post-transcriptional regulation in these parasites. High-

566 throughput proteomics techniques such as LC-MS/MS therefore offer a more detailed

567 picture than the transcriptome of mechanisms involved in phenotypic diversity and

568 virulence in *N. caninum*. However, proteome analyses are also limited by additional post-

569 transduction mechanisms involved in protein regulation (activation and protein turn

570 over). Nevertheless, despite the weak correlation between the transcript and protein at the

571 individual gene level, both datasets have identified, on a gene family level, a pre-

572 bradyzoite status of the Nc-Spain1H isolate. This observation is very interesting since it

573 could, at least partially, explain the reduced virulence of this isolate.

574 Altogether, the current study provides a global vision of the transcriptional and

575 translational differences between isolates that display marked virulence differences,

576 shedding light on a subset of proteins that are potentially involved in the pathogenesis of

577 this parasite. New studies with a battery of well-characterized isolates may reveal

578 common mechanism of *N. caninum* virulence. Our results lay the foundations for further

579 investigations characterizing the relevance of such proteins in *N. caninum* pathogenesis

580 and virulence.

581 **Conflict of interest statement**

582 The authors have declared no conflict of interest.

592 **Author contributions**

593　LMOM, JW, JRC, PH and ECF conceived and designed the experiments. JRC and PH

594　carried out the tachyzoite samples collection for analyses. DX and NR performed RNA-

595　Seq and LC/MS-MS analyses. PH performed the validation assays. DX performed

596　bioinformatics analyses. PH, JRC and DX analysed data and wrote the manuscript.

597　LMOM and NR critically revised the manuscript.

598

599　**References**

600　[1] J. Dubey, G. Schares, Neosporosis in animals—the last five years, Vet. Parasitol. 180
601　(2011) 90-108.

602　[2] M. P. Reichel, M. Alejandra Ayanegui-Alcérreca, L. F. P. Gondim, J. T. Ellis, What
603　is the global economic impact of *Neospora caninum* in cattle – The billion dollar question,
604　Int. J. Parasitol. 43 (2013) 133-42.

605　[3] A. L. Chryssafidis, G. Canton, F. Chianini, E. A. Innes, E. H. Madureira, S. M.
606　Gennari, Pathogenicity of Nc-Bahia and Nc-1 strains of *Neospora caninum* in
607　experimentally infected cows and buffaloes in early pregnancy, Parasitol. Res. 113 (2014)
608　1521-8.

609　[4] S. Rojo-Montejo, E. Collantes-Fernández, J. Blanco-Murcia, A. Rodríguez-Bertos, V.
610　Risco-Castillo, L. M. Ortega-Mora, Experimental infection with a low virulence isolate
611　of *Neospora caninum* at 70 days gestation in cattle did not result in foetopathy, Vet. Res.
612　40 (2009) 49.

613　[5] S. G. Caspe, D. P. Moore, M. R. Leunda, D. B. Cano, L. Lischinsky, J. Regidor-
614　Cerrillo, G. Álvarez-García, I. G. Echaide, D. Bacigalupe, L. M. Ortega-Mora, A. C.
615　Odeon, C. M. Campero, The *Neospora caninum*-Spain 7 isolate induces placental
616　damage, fetal death and abortion in cattle when inoculated in early gestation, Vet.
617　Parasitol. 189 (2012) 171-81.

618　[6] J. Regidor-Cerrillo, D. Arranz-Solís, J. Benavides, M. Gómez-Bautista, J. A. Castro-
619　Hermida, M. Mezo, V. Pérez, L. M. Ortega-Mora, M. González-Warleta, *Neospora*
620　*caninum* infection during early pregnancy in cattle: how the isolate influences infection
621　dynamics, clinical outcome and peripheral and local immune responses, Vet. Res. 45
622　(2014) 10,9716-45-10.

623　[7] A. Hemphill, B. Gottstein, H. Kaufmann, Adhesion and invasion of bovine endothelial
624　cells by *Neospora caninum*, Parasitology 112 (Pt 2) (1996) 183-97.

625　[8] A. Hemphill, N. Vonlaufen, A. Naguleswaran, N. Keller, M. Riesen, N. Guetg, S.
626　Srinivasan, F. Alaeddine, Tissue culture and explant approaches to studying and

627 visualizing *Neospora caninum* and its interactions with the host cell, Microscopy and
628 Microanalysis 10 (2004) 602-20.

629 [9] J. Regidor-Cerrillo, M. Gómez-Bautista, I. Sodupe, G. Aduriz, G. Álvarez-García, I.
630 Del Pozo, L. M. Ortega-Mora, In vitro invasion efficiency and intracellular proliferation
631 rate comprise virulence-related phenotypic traits of *Neospora caninum*, Vet. Res. 42
632 (2011) 41.

633 [10] A. Dellarupe, J. Regidor-Cerrillo, E. Jiménez-Ruiz, G. Schares, J. M. Unzaga, M. C.
634 Venturini, L. M. Ortega-Mora, Comparison of host cell invasion and proliferation among
635 *Neospora caninum* isolates obtained from oocysts and from clinical cases of naturally
636 infected dogs, Exp. Parasitol. 145 (2014) 22-8.

637 [11] L. Jiménez-Pelayo, M. García-Sánchez, J. Regidor-Cerrillo, P. Horcajo, E.
638 Collantes-Fernández, M. Gómez-Bautista, N. Hambruch, C. Pfarrer, L. M. Ortega-Mora,
639 Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with
640 high and low virulence isolates of *Neospora caninum*, Parasit. Vectors 10 (2017) 463.

641 [12] H. Beck, D. Blake, M. Dardé, I. Felger, S. Pedraza-Díaz, J. Regidor-Cerrillo, M.
642 Gómez-Bautista, L. M. Ortega-Mora, L. Putignani, B. Shiels, A. Tait, W. Weir, Molecular
643 approaches to diversity of populations of apicomplexan parasites, Int. J. Parasitol. 39
644 (2009) 175-89.

645 [13] E. G. Lee, J. H. Kim, Y. S. Shin, G. W. Shin, Y. R. Kim, K. J. Palaksha, D. Y. Kim,
646 I. Yamane, Y. H. Kim, G. S. Kim, M. D. Suh, T. S. Jung, Application of proteomics for
647 comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites, J.
648 Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 815 (2005) 305-14.

649 [14] Y. S. Shin, G. W. Shin, Y. R. Kim, E. Y. Lee, H. H. Yang, K. J. Palaksha, H. J.
650 Youn, J. H. Kim, D. Y. Kim, A. E. Marsh, J. Lakritz, T. S. Jung, Comparison of proteome
651 and antigenic proteome between two *Neospora caninum* isolates, Vet Parasitol 134
652 (2005) 41-52.

653 [15] J. Regidor-Cerrillo, G. Álvarez-García, I. Pastor-Fernández, V. Marugán-Hernández,
654 M. Gómez-Bautista, L. M. Ortega-Mora, Proteome expression changes among virulent
655 and attenuated *Neospora caninum* isolates, J. Proteomics 75 (2012) 2306-18.

656 [16] P. Horcajo, L. Jiménez-Pelayo, M. García-Sánchez, J. Regidor-Cerrillo, E.
657 Collantes-Fernández, D. Rozas, N. Hambruch, C. Pfarrer, L. M. Ortega-Mora,
658 Transcriptome modulation of bovine trophoblast cells in vitro by *Neospora caninum*, Int.
659 J. Parasitol. 47 (2017) 791-9.

660 [17] J. Regidor-Cerrillo, M. Gómez-Bautista, J. Pereira-Bueno, G. Adúriz, V. Navarro-
661 Lozano, V. Risco-Castillo, A. Fernández-García, S. Pedraza-Díaz, L. M. Ortega-Mora,
662 Isolation and genetic characterization of *Neospora caninum* from asymptomatic calves in
663 Spain, Parasitology 135 (2008) 1651-9.

664 [18] J. A. Vizcaíno, A. Csordas, N. del-Toro, J. A. Dianes, J. Griss, I. Lavidas, G. Mayer,
665 Y. Perez-Riverol, F. Reisinger, T. Ternent, Q. W. Xu, R. Wang, H. Hermjakob, 2016
666 update of the PRIDE database and its related tools, Nucleic Acids Res. 44 (2016) 447-56.

667   [19] B. Gajria, A. Bahl, J. Brestelli, J. Dommer, S. Fischer, X. Gao, M. Heiges, J. Iodice,
668   J. C. Kissinger, A. J. Mackey, D. F. Pinney, D. S. Roos, C. J. Stoeckert Jr, H. Wang, B.
669   P. Brunk, ToxoDB: an integrated *Toxoplasma gondii* database resource, Nucleic Acids
670   Res. 36 (2008) 553-56.

671   [20] D. Xia, S. J. Sanderson, A. R. Jones, J. H. Prieto, J. R. Yates, E. Bromley, F. M.
672   Tomley, K. Lal, R. E. Sinden, B. P. Brunk, D. S. Roos, J. M. Wastling, The proteome of
673   *Toxoplasma gondii*: integration with the genome provides novel insights into gene
674   expression and annotation, Genome Biol. 9 (2008) R116.

675   [21] L. Sheiner, J. L. Demerly, N. Poulsen, W. L. Beatty, O. Lucas, M. S. Behnke, M. W.
676   White, B. Striepen, A systematic screen to discover and analyze apicoplast proteins
677   identifies a conserved and essential protein import factor, PLoS Pathog. 7 (2011)
678   e1002392.

679   [22] L. Pollo-Oliveira, H. Post, M. L. Acencio, N. Lemke, H. van den Toorn, V. Tragante,
680   A. J. Heck, A. F. Altelaar, A. P. Yatsuda, Unravelling the *Neospora caninum* secretome
681   through the secreted fraction (ESA) and quantification of the discharged tachyzoite using
682   high-resolution mass spectrometry-based proteomics, Parasit. Vectors 6 (2013) 335.

683   [23] I. Pastor-Fernández, J. Regidor-Cerrillo, G. Álvarez-Garcia, V. Marugán-Hernández,
684   P. García-Lunar, A. Hemphill, L. M. Ortega-Mora, The tandemly repeated NTPase
685   (NTPDase) from *Neospora caninum* is a canonical dense granule protein whose RNA
686   expression, protein secretion and phosphorylation coincides with the tachyzoite egress,
687   Parasit. Vectors 9 (2016) 352.

688   [24] I. Pastor-Fernández, J. Regidor-Cerrillo, E. Jiménez-Ruiz, G. Álvarez-García, V.
689   Marugán-Hernández, A. Hemphill, L. M. Ortega-Mora, Characterization of the *Neospora*
690   *caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle,
691   Parasitology 143 (2016) 97-113.

692   [25] UniProt Consortium, Activities at the Universal Protein Resource (UniProt), Nucleic
693   Acids Res. 42 (2014) 191-98.

694   [26] C. Lekutis, D. J. Ferguson, M. E. Grigg, M. Camps, J. C. Boothroyd, Surface
695   antigens of *Toxoplasma gondii*: variations on a theme, Int. J. Parasitol. 31 (2001) 1285-
696   92.

697   [27] A. J. Reid, S. J. Vermont, J. A. Cotton, D. Harris, G. A. Hill-Cawthorne, S. Konen-
698   Waisman, S. M. Latham, T. Mourier, R. Norton, M. A. Quail, M. Sanders, D.
699   Shanmugam, A. Sohal, J. D. Wasmuth, B. Brunk, M. E. Grigg, J. C. Howard, J. Parkinson,
700   D. S. Roos, A. J. Trees, M. Berriman, A. Pain, J. M. Wastling, Comparative genomics of
701   the apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*: Coccidia
702   differing in host range and transmission strategy, PLoS Pathog. 8 (2012) e1002567.

703   [28] Y. Adomako-Ankomah, G. M. Wier, A. L. Borges, H. E. Wand, J. P. Boyle,
704   Differential locus expansion distinguishes Toxoplasmatinae species and closely related
705   strains of *Toxoplasma gondii*, MBio 5 (2014) e01003-13.

[29] A. Fernández-García, V. Risco-Castillo, A. Zaballos, G. Álvarez-García, L. M. Ortega-Mora, Identification and molecular cloning of the *Neospora caninum* SAG4 gene specifically expressed at bradyzoite stage, Mol. Biochem. Parasitol. 146 (2006) 89-97.

[30] J. D. Wasmuth, V. Pszenny, S. Haile, E. M. Jansen, A. T. Gast, A. Sher, J. P. Boyle, M. J. Boulanger, J. Parkinson, M. E. Grigg, Integrated bioinformatic and targeted deletion analyses of the SRS gene superfamily identify SRS29C as a negative regulator of *Toxoplasma* virulence, MBio 3 (2012) e00321-12.

[31] M. E. Jerome, J. R. Radke, W. Bohne, D. S. Roos, M. W. White, *Toxoplasma gondii* bradyzoites form spontaneously during sporozoite-initiated development, Infect. Immun. 66 (1998) 4838-44.

[32] S. Rojo-Montejo, E. Collantes-Fernández, J. Regidor-Cerrillo, G. Álvarez-García, V. Marugán-Hernández, S. Pedraza-Díaz, J. Blanco-Murcia, A. Prenafeta, L. M. Ortega-Mora, Isolation and characterization of a bovine isolate of *Neospora caninum* with low virulence, Vet. Parasitol. 159 (2009) 7-16.

[33] F. M. Tomley, D. S. Soldati, Mix and match modules: structure and function of microneme proteins in apicomplexan parasites, Trends Parasitol. 17 (2001) 81-8.

[34] A. Naguleswaran, A. Cannas, N. Keller, N. Vonlaufen, G. Schares, F. J. Conraths, C. Bjorkman, A. Hemphill, *Neospora caninum* microneme protein NcMIC3: secretion, subcellular localization, and functional involvement in host cell interaction, Infect. Immun. 69 (2001) 6483-94.

[35] N. Keller, A. Naguleswaran, A. Cannas, N. Vonlaufen, M. Bienz, C. Bjorkman, W. Bohne, A. Hemphill, Identification of a *Neospora caninum* microneme protein (NcMIC1) which interacts with sulfated host cell surface glycosaminoglycans, Infect. Immun. 70 (2002) 3187-98.

[36] N. Keller, M. Riesen, A. Naguleswaran, N. Vonlaufen, R. Stettler, A. Leepin, J. M. Wastling, A. Hemphill, Identification and characterization of a *Neospora caninum* microneme-associated protein (NcMIC4) that exhibits unique lactose-binding properties, Infect. Immun. 72 (2004) 4791-800.

[37] J. Wang, D. Tang, W. Li, J. Xu, Q. Liu, J. Liu, A new microneme protein of *Neospora caninum*, NcMIC8 is involved in host cell invasion, Exp. Parasitol. 175 (2017) 21-7.

[38] H. Zhang, M. K. Compaore, E. G. Lee, M. Liao, G. Zhang, C. Sugimoto, K. Fujisaki, Y. Nishikawa, X. Xuan, Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii,* and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites, Mol. Biochem. Parasitol. 151 (2007) 205-12.

[39] V. Lagal, E. M. Binder, M. H. Huynh, B. F. Kafsack, P. K. Harris, R. Diez, D. Chen, R. N. Cole, V. B. Carruthers, K. Kim, *Toxoplasma gondii* protease TgSUB1 is required for cell surface processing of micronemal adhesive complexes and efficient adhesion of tachyzoites, Cell Microbiol. 12 (2010) 1792-808.

744 [40] F. Parussini, I. Coppens, P. P. Shah, S. L. Diamond, V. B. Carruthers, Cathepsin L
745 occupies a vacuolar compartment and is a protein maturase within the endo/exocytic
746 system of *Toxoplasma gondii*, Mol. Microbiol. 76 (2010) 1340-57.

747 [41] M. S. Roiko, V. B. Carruthers, Functional dissection of *Toxoplasma gondii* perforin-
748 like protein 1 reveals a dual domain mode of membrane binding for cytolysis and parasite
749 egress, J. Biol. Chem. 288 (2013) 8712-25.

750 [42] F. Almeida, A. Sardinha-Silva, T. A. da Silva, A. M. Pessoni, C. F. Pinzan, A. C.
751 Alegre-Maller, N. T. Cecilio, N. S. Moretti, A. R. Damasio, W. R. Pedersoli, J. R. Mineo,
752 R. N. Silva, M. C. Roque-Barreira, *Toxoplasma gondii* Chitinase induces macrophage
753 activation, PLoS One 10 (2015) e0144507.

754 [43] M. A. Hakimi, P. Olias, L. D. Sibley, *Toxoplasma* effectors targeting host signaling
755 and transcription, Clin. Microbiol. Rev. 30 (2017) 615-45.

756 [44] L. D. Sibley, W. Qiu, S. Fentress, S. J. Taylor, A. Khan, R. Hui, Forward genetics in
757 *Toxoplasma gondii* reveals a family of rhoptry kinases that mediates pathogenesis,
758 Eukaryot. Cell 8 (2009) 1085-93.

759 [45] P. N. Ossorio, J. D. Schwartzman, J. C. Boothroyd, A *Toxoplasma gondii* rhoptry
760 protein associated with host cell penetration has unusual charge asymmetry, Mol.
761 Biochem. Parasitol. 50 (1992) 1-15.

762 [46] K. R. Buchholz, P. W. Bowyer, J. C. Boothroyd, Bradyzoite pseudokinase 1 is crucial
763 for efficient oral infectivity of the *Toxoplasma gondii* tissue cyst, Eukaryot. Cell 12
764 (2013) 399-410.

765 [47] M. Blume, R. Nitzsche, U. Sternberg, M. Gerlic, S. L. Masters, N. Gupta, M. J.
766 McConville, A *Toxoplasma gondii* gluconeogenic enzyme contributes to robust central
767 carbon metabolism and is essential for replication and virulence, Cell Host Microbe 18
768 (2015) 210-20.

769 [48] A. M. Guggisberg, A. R. Odom, Sweet talk: regulating glucose metabolism in
770 *Toxoplasma*, Cell Host Microbe 18 (2015) 142-3.

771 [49] G. I. McFadden, E. Yeh, The apicoplast: now you see it, now you don't, Int. J.
772 Parasitol. 47 (2017) 137-44.

773 [50] C. F. Brooks, H. Johnsen, G. G. van Dooren, M. Muthalagi, S. S. Lin, W. Bohne, K.
774 Fischer, B. Striepen, The *toxoplasma* apicoplast phosphate translocator links cytosolic
775 and apicoplast metabolism and is essential for parasite survival, Cell Host Microbe 7
776 (2010) 62-73.

777 [51] C. Song, M. A. Chiasson, N. Nursimulu, S. S. Hung, J. Wasmuth, M. E. Grigg, J.
778 Parkinson, Metabolic reconstruction identifies strain-specific regulation of virulence in
779 *Toxoplasma gondii*, Mol. Syst. Biol. 9 (2013) 708.

780 [52] T. Tanaka, H. Nagasawa, K. Fujisaki, N. Suzuki, T. Mikami, Growth-inhibitory
781 effects of interferon-gamma on *Neospora caninum* in murine macrophages by a nitric
782 oxide mechanism, Parasitol. Res. 86 (2000) 768-71.

783 [53] S. S. Bosch, T. Kronenberger, K. A. Meissner, F. M. Zimbres, D. Stegehake, N. M.
784 Izui, I. Schettert, E. Liebau, C. Wrenger, Oxidative stress control by apicomplexan
785 parasites, Biomed. Res. Int. 2015 (2015) 351289.

786 [54] P. Pino, B. J. Foth, L. Y. Kwok, L. Sheiner, R. Schepers, T. Soldati, D. Soldati-Favre,
787 Dual targeting of antioxidant and metabolic enzymes to the mitochondrion and the
788 apicoplast of *Toxoplasma gondii*, PLoS Pathog. 3 (2007) e115.

789 [55] M. W. Robinson, A. T. Hutchinson, J. P. Dalton, S. Donnelly, Peroxiredoxin: a
790 central player in immune modulation, Parasite Immunol. 32 (2010) 305-13.

791 [56] R. M. Fereig, Y. Nishikawa, Peroxiredoxin 3 promotes IL-12 production from
792 macrophages and partially protects mice against infection with *Toxoplasma gondii*,
793 Parasitol. Int. 65 (2016) 741-8.

794 [57] L. M. Weiss, Y. F. Ma, P. M. Takvorian, H. B. Tanowitz, M. Wittner, Bradyzoite
795 development in *Toxoplasma gondii* and the hsp70 stress response, Infect. Immun. 66
796 (1998) 3295-302.

797 [58] P. C. Echeverria, M. Matrajt, O. S. Harb, M. P. Zappia, M. A. Costas, D. S. Roos, J.
798 F. Dubremetz, S. O. Angel, *Toxoplasma gondii* Hsp90 is a potential drug target whose
799 expression and subcellular localization are developmentally regulated, J. Mol. Biol. 350
800 (2005) 723-34.

801 [59] V. Marugán-Hernández, G. Álvarez-García, V. Risco-Castillo, J. Regidor-Cerrillo,
802 L. M. Ortega-Mora, Identification of *Neospora caninum* proteins regulated during the
803 differentiation process from tachyzoite to bradyzoite stage by DIGE, Proteomics 10
804 (2010) 1740-50.

805 [60] N. de Miguel, P. C. Echeverria, S. O. Angel, Differential subcellular localization of
806 members of the *Toxoplasma gondii* small heat shock protein family, Eukaryot Cell 4
807 (2005) 1990-7.

808 [61] C. Muñoz, J. San Francisco, B. Gutiérrez, J. González, Role of the ubiquitin-
809 proteasome systems in the biology and virulence of protozoan parasites, Biomed. Res.
810 Int. 2015 (2015) 141526.

811 [62] N. C. Silmon de Monerri, R. R. Yakubu, A. L. Chen, P. J. Bradley, E. Nieves, L. M.
812 Weiss, K. Kim, The Ubiquitin proteome of *Toxoplasma gondii* reveals roles for protein
813 ubiquitination in cell-cycle transitions, Cell Host Microbe 18 (2015) 621-33.

814 [63] T. W. Kooij, C. J. Janse, A. P. Waters, *Plasmodium* post-genomics: better the bug
815 you know? Nat. Rev. Microbiol. 4 (2006) 344-57.

816 [64] J. M. Wastling, D. Xia, A. Sohal, M. Chaussepied, A. Pain, G. Langsley, Proteomes
817 and transcriptomes of the Apicomplexa--where's the message? Int. J. Parasitol. 39 (2009)
818 135-43.

819 [65] T. Maier, M. Guell, L. Serrano, Correlation of mRNA and protein in complex
820 biological samples, FEBS Lett. 583 (2009) 3966-73.

821 [66] R. de Sousa Abreu, L. O. Penalva, E. M. Marcotte, C. Vogel, Global signatures of
822 protein and mRNA expression levels, Mol. Biosyst. 5 (2009) 1512-26.

823 [67] B. Schwanhausser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen,
824 M. Selbach, Global quantification of mammalian gene expression control, Nature 473
825 (2011) 337-42.

826 [68] A. Battle, Z. Khan, S. H. Wang, A. Mitrano, M. J. Ford, J. K. Pritchard, Y. Gilad,
827 Genomic variation. Impact of regulatory variation from RNA to protein, Science 347
828 (2015) 664-7.

829 [69] M. Jovanovic, M. S. Rooney, P. Mertins, D. Przybylski, N. Chevrier, R. Satija, E. H.
830 Rodriguez, A. P. Fields, S. Schwartz, R. Raychowdhury, M. R. Mumbach, T. Eisenhaure,
831 M. Rabani, D. Gennert, D. Lu, T. Delorey, J. S. Weissman, S. A. Carr, N. Hacohen, A.
832 Regev, Immunogenetics. Dynamic profiling of the protein life cycle in response to
833 pathogens, Science 347 (2015) 1259038.

834 [70] J. J. Li, P. J. Bickel, M. D. Biggin, System wide analyses have underestimated protein
835 abundances and the importance of transcription in mammals, PeerJ 2 (2014) e270.

836 [71] Y. W. Zhang, K. Kim, Y. F. Ma, M. Wittner, H. B. Tanowitz, L. M. Weiss,
837 Disruption of the *Toxoplasma gondii* bradyzoite-specific gene BAG1 decreases in vivo
838 cyst formation, Mol. Microbiol. 31 (1999) 691-701.

839 [72] V. Risco-Castillo, A. Fernández-García, A. Zaballos, A. Aguado-Martínez, A.
840 Hemphill, A. Rodríguez-Bertos, G. Álvarez-García, L. M. Ortega-Mora, Molecular
841 characterisation of BSR4, a novel bradyzoite-specific gene from *Neospora caninum*, Int.
842 J. Parasitol. 37 (2007) 887-96.

843 [73] M. Holpert, U. Gross, W. Bohne, Disruption of the bradyzoite-specific P-type (H+)-
844 ATPase PMA1 in *Toxoplasma gondii* leads to decreased bradyzoite differentiation after
845 stress stimuli but does not interfere with mature tissue cyst formation, Mol. Biochem.
846 Parasitol. 146 (2006) 129-33.

847 [74] S. Tomavo, The differential expression of multiple isoenzyme forms during stage
848 conversion of *Toxoplasma gondii*: an adaptive developmental strategy, Int. J. Parasitol.
849 31 (2001) 1023-31.

850 [75] A. Ueno, G. Dautu, B. Munyaka, G. Carmen, Y. Kobayashi, M. Igarashi,
851 *Toxoplasma gondii*: Identification and characterization of bradyzoite-specific
852 deoxyribose phosphate aldolase-like gene (TgDPA), Exp. Parasitol. 121 (2009) 55-63.

853   [76] K. R. Buchholz, H. M. Fritz, X. Chen, B. Durbin-Johnson, D. M. Rocke, D. J.
854   Ferguson, P. A. Conrad, J. C. Boothroyd, Identification of tissue cyst wall components
855   by transcriptome analysis of in vivo and in vitro *Toxoplasma gondii* bradyzoites,
856   Eukaryot. Cell 10 (2011) 1637-47.

857   [77] T. Tomita, D. J. Bzik, Y. F. Ma, B. A. Fox, L. M. Markillie, R. C. Taylor, K. Kim,
858   L. M. Weiss, The *Toxoplasma gondii* cyst wall protein CST1 is critical for cyst wall
859   integrity and promotes bradyzoite persistence, PLoS Pathog. 9 (2013) e1003823.

860   [78] M. M. Croken, Y. Ma, L. M. Markillie, R. C. Taylor, G. Orr, L. M. Weiss, K. Kim,
861   Distinct strains of *Toxoplasma gondii* feature divergent transcriptomes regardless of
862   developmental stage, PLoS One 9 (2014) e111297.

863   [79] D. P. Hong, J. B. Radke, M. W. White, Opposing transcriptional mechanisms
864   regulate *Toxoplasma* development, mSphere 2 (2017) e00347-16.

865   [80] J. P. Saeij, J. P. Boyle, J. C. Boothroyd, Differences among the three major strains
866   of *Toxoplasma gondii* and their specific interactions with the infected host, Trends
867   Parasitol. 21 (2005) 476-81.

868   [81] B. A. Fox, L. M. Rommereim, R. B. Guevara, A. Falla, M. A. Hortua Triana, Y. Sun,
869   D. J. Bzik, The *Toxoplasma gondii* rhoptry kinome is essential for chronic infection,
870   MBio 7 (2016) e00193-16.

871   [82] D. A. Gold, A. D. Kaplan, A. Lis, G. C. Bett, E. E. Rosowski, K. M. Cirelli, A.
872   Bougdour, S. M. Sidik, J. R. Beck, S. Lourido, P. F. Egea, P. J. Bradley, M. A. Hakimi,
873   R. L. Rasmusson, J. P. Saeij, The *Toxoplasma* dense granule proteins GRA17 and GRA23
874   mediate the movement of small molecules between the host and the parasitophorous
875   vacuole, Cell Host Microbe 17 (2015) 642-52.

876   [83] M. S. Behnke, J. C. Wootton, M. M. Lehmann, J. B. Radke, O. Lucas, J. Nawas, L.
877   D. Sibley, M. W. White, Coordinated progression through two subtranscriptomes
878   underlies the tachyzoite cycle of *Toxoplasma gondii*, PLoS One 5 (2010) e12354.

879   [84] R. Walker, M. Gissot, M. M. Croken, L. Huot, D. Hot, K. Kim, S. Tomavo, The
880   *Toxoplasma* nuclear factor TgAP2XI-4 controls bradyzoite gene expression and cyst
881   formation, Mol. Microbiol. 87 (2013) 641-55.

882   [85] R. Walker, M. Gissot, L. Huot, T. D. Alayi, D. Hot, G. Marot, C. Schaeffer-Reiss,
883   A. Van Dorsselaer, K. Kim, S. Tomavo, *Toxoplasma* transcription factor TgAP2XI-5
884   regulates the expression of genes involved in parasite virulence and host invasion, J. Biol.
885   Chem. 288 (2013) 31127-38.

886

887   **Figure legends**

888   **Figure 1. Experimental design of the proteome and transcriptome analyses**. Samples

889   for proteome and transcriptome analyses were assayed in triplicate. (A)

890 Photomicrographs for each time-point of sample collection at 400x. Black arrows indicate

891 parasitophorous vacuoles at 12, 36 and 56 hpi. Red arrows indicate an area of recent

892 egress. (B) Experimental design for proteome analyses. Tachyzoites were collected at 12,

893 36 and 56 hpi and purified using PD-10 columns. Purified tachyzoites were digested and

894 analysed on an Easy-Spray column fused to a silica nanoelectrospray emitter coupled to

895 a Q-Exactive mass spectrometer. For proteome data analyses, Progenesis QI (version 2.0,

896 Nonlinear Dynamics) and Mascot (version 2.3, Matrix Science) for peptide identification

897 were used. (C) Experimental design for transcript analyses. Total RNA was isolated from

898 purified tachyzoites, and then polyadenylated RNA was purified and used to prepare

899 RNA-seq libraries. Libraries were sequenced with HiSeq2500. The TopHat2 aligner was

900 used to align sequencing reads against the reference genome, and the Cufflinks software

901 (version 2.1.1) was applied to assemble transcripts, quantify expression levels and analyse

902 differentially expressed genes.

903 **Figure 2**: **Volcano plot showing the proteome results**. (A) Volcano plot of

904 differentially abundant proteins at 12 hpi. (B) Volcano plot of differentially abundant

905 proteins at 36 hpi. (C) Volcano plot of differentially abundant proteins at 56 hpi. The 'x'

906 axis represents the $\log_2$ fold-change of different protein abundances (Nc-Spain1H vs Nc-

907 Spain7), and 'y' axis represents the negative $\log_{10}$ of the p value. Proteins have been

908 grouped and coloured in the main categories: host attachment and invasion in red,

909 metabolic processes in green, other biological systems in purple and unannotated proteins

910 in grey. The dotted line represents the position of the fold change = 2, from which the

911 differences in abundance are considered significant.

912 **Figure 3. Venn diagrams showing the number of differentially abundant proteins**

913 **between Nc-Spain1H and Nc-Spain7 isolates across tachyzoite lytic cycle.** (A) The

914 total number of proteins increased in abundance (fold change $\geq$ 2) in the Nc-Spain1H

915   isolate each time-point of the lytic cycle (12 hpi, 36 hpi and 56 hpi). (B) The total number

916   of proteins increased in abundance (fold change $\geq$ 2) in the Nc-Spain7 isolate at 12 hpi,

917   36 hpi and 56 hpi. Proteins are listed in Supplemental file 4.

918   **Figure 4. Number of proteins with higher abundance in each isolate grouped in**

919   **functional categories.** (A) Number of proteins with higher abundance in each isolate at

920   12 hpi, (B) number of proteins with higher abundance in each isolate at 36 hpi and (C)

921   number of proteins with higher abundance in each isolate at 56 hpi.

922   **Figure 5:** I**nvasion rates and flow cytometry profiles of Nc-Spain1H and Nc-Spain7**

923   **tachyzoites.** (A) Graphs represent parasite infection rates defined as the percentage of

924   invaded tachyzoites. Bars represent median invasion rates, and each point represents

925   invasion rates determined for 4 replicates from 3 independent assays. (**) indicates P <

926   0.01 (Mann-Whitney U-test). (B) Representative flow cytometric histograms showing the

927   distribution of tachyzoites in each cell cycle phase using a propidium iodide stain. The

928   table shows the average percentages of G1 and G2/M tachyzoites. The green lines show

929   the Nc-Spain7 isolate, and the black lines show the Nc-Spain1H isolate.

930   **Supplemental figure 1.** Predicted subcellular localization and functional classification

931   of identified proteins (A and B, respectively). Proteins were assigned a subcellular

932   localization or functional category combining the results of gene descriptions and Gene

933   Ontology annotation of *N. caninum* provided by ToxoDB. When no information was

934   available, the subcellular localization was predicted using GO annotation in syntenic

935   homologues from the *T. gondii* database and literature searches [20-22]. A detailed list of

936   proteins in each subcellular localization or functional category to accompany this figure

937   is provided in supplemental file 2. The numbers in the pie chart legends correspond to the

938   number of identified proteins in each category.

939 **Supplemental figure 2. Proteome validation by Western blot analyses.** Different

940 abundances of NcMIC2, NcROP2 and NcNTPase in Nc-Spain1H and Nc-Spain7 were

941 analysed by Western blot analyses. Total protein extracts from cultures infected with Nc-

942 Spain1H and Nc-Spain7 collected at 12, 36 and 56 hpi were electrophoresed in bis-

943 acrylamide gels and transferred onto PVDF membranes. For WB analyses rabbit

944 polyclonal anti-serum against NcMIC2, NcROP2, NcNTPase and NcSAG1 and goat anti-

945 rabbit IgG antibody conjugated to peroxidase, as a secondary antibody, were used. Note

946 that SAG1 was used in all cases as a housekeeping gene to normalize the amount of

947 tachyzoite protein in each sample. Expression levels of proteins were assessed by density

948 values (sum of pixel intensities by pixel area) using Quantity One quantification software

949 v. 4.0. The graphics show the protein expression differences that were determined from

950 the intensity ratios obtained for the different protein samples for each isolate.

951 **Supplemental figure 3. Validation of RNA-seq analyses by qPCR**. The graphic shows

952 the fold changes in the expression of *N. caninum* genes in the comparison of Nc-Spain7

953 vs Nc-Spain1H. Red columns show the results of the RNA-seq analyses, and green

954 columns the the qPCR results using SAGI as the housekeeping gene. [1]Predicted member

955 of the rhoptry kinase family (ROPK), subfamily ROP20, [2]orthologue of

956 TGME49_206510 toxolysin TLN4, [3]putative retinitis pigmentosa GTPase regulator, and

957 [4]hypothetical protein.

958 **Supplemental file 1.** Materials and methods. Detailed description of the materials and

959 methods sections: 1) LC-MS/MS analyses, 2) Western blot validation, 4) RNA-seq

960 analyses and 4) transcriptome validation by qPCR.

961 **Supplemental file 2.** Predicted subcellular localization and functional classification of

962 identified proteins from the tachyzoite proteome. Classification according to the gene

963 descriptions and Gene Ontology annotation of *N. caninum* provided by ToxoDB and,

964   when no information was available, from GO annotation in syntenic homologues from

965   the *T. gondii* database and literature searches [20-22].

966   **Supplemental file 3.** Proteins with different abundances between the Nc-Spain1H and

967   Nc-Spain7 proteomes.

968   **Supplemental file 4.** List of proteins belonging to each region of the Venn diagram

969   represented in Figure 3, showing the number of differentially abundant proteins between

970   Nc-Spain1H and Nc-Spain7 isolates across the tachyzoite lytic cycle.

971   **Supplemental file 5.** Transcriptome results.

972   Table 1. Bradyzoite-related genes with higher expression in Nc-Spain1H isolate.

Gene ID[1]	Ortholog in *T. gondii*	Gene Product	Fold change[2]
NCLIV_027470	TGME49_259020	BAG1*	142.65
NCLIV_010030	TGME49_320230	BSR4	3.9
NCLIV_019580	TGME49_280570	SAG4	36.39
NCLIV_022240	TGME49_252640	PMA1*	23.6
NCLIV_042910	TGME49_291040	LDH2*	114.14
NCLIV_037490	TGME49_268860	ENO1*	23.22
NCLIV_010810	TGME49_318750	Deoxyribose-phosphate aldolase	42.61
NCLIV_003250	TGME49_208730	NcMCP4	11.97
NCLIV_040495	TGME49_264670	CST1*	40.29

973   [1]In addition to these genes, 23 ribosomal proteins had increased its expression in Nc-Sapin1H, a higher expression level
974   of ribosomal proteins have been associated with strains that readily switch from tachyzoite to bradyzoite in *T. gondii*
975   [78].
976   *Protein names are assigned from *T. gondii* orthologues because lack of *N. caninum* annotations
977   [2]Fold change of the expression levels in the Nc-Spain1H comparing to Nc-Spain7
978

979

980

A

Nc-Spain1H

Nc-Spain7

12 hours	36 hours	56 hours
Completed invasion	Exponential growth	Early egression

B

12 hours	36 hours	56 hours
Completed invasion	Exponential growth	Early egression

Cell monolayers

Tachyzoite purification

PD-10 column
(GE Healthcare)

Tachyzoite lysis and digestion by trypsin

LC/MS-MS

Q Exactive™
(Thermo Fisher Scientific)

Proteome data analyse

Progenesis QI
(Nonlinear Dynamics)

Differentially abundant proteins between isolates

C

56 hours
Early egression

Cell monolayers

Tachyzoite purification

PD-10 column
(GE Healthcare)

RNA extraction

Rneasy Mini Kit
(Qiagen)

RNA sequencing

HiSeq 2500
System-Illumina

Bioinformatic analysis

TopHat Cufflinks tools

Differentially expressed genes between isolates

Proteome 12 hpi

Proteome 36 hpi

Proteome 56 hpi

Nc-Spain1H vs Nc-Spain7 (log$_2$ FC)

- Host attachment and invasion
- Metabolic processes
- Other biological systems
- Unannotated

A

12 hpi

35

2 12
7
1 3 22

36 hpi   56 hpi

B

12 hpi

16

1 2
2
1 2 12

36 hpi   56 hpi

Figure: Horizontal diverging bar charts showing the number of proteins at three time points (A: 12 hpi, B: 36 hpi, C: 56 hpi) for the following categories — Ubiquitin-proteasome system, Antioxidant and stress response systems, Fatty acid and phospholipids metabolism, Gluconeogenesis, Rhoptry proteins, Microneme proteins, and Surface antigens. Legend: 1H (blue), 7 (orange). X-axis: Number of proteins.

A

% Invasion

Nc-Spain7 Nc-Spain1H

**

B

| 12 hpi | 36 hpi | 56 hpi |

Counts — FL2-A

	G1	G2/M
NcSpain1H	97,22	2,01
NcSpain7	95,7	3,4

	G1	G2/M
NcSpain1H	98,41	1,14
NcSpain7	98,84	0,53

	G1	G2/M
NcSpain1H	85,9	14,1
NcSpain7	88,4	11,1