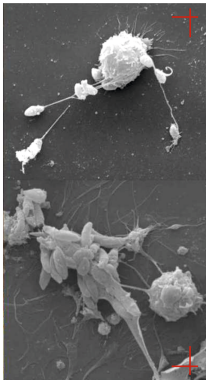
Rodolfo Villagra-Blanco

NETosis-derived effector mechanisms against *Neospora caninum* and new insights of seroprevalances in caprine and canine neosporosis



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zur Erlangung des Grades eines
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Villagra-Blanco, R., Angelova, L., Conze, T., Schares, G., Bärwald, A., Taubert, A., Hermosilla, C., Wehrend, A. (2018). Seroprevalence of *Neospora caninum*-specific antibodies in German breeding bitches. *Parasit Vectors* 11:96. doi: 10.1186/s13071-018-2683-1.

Villagra-Blanco, R., Wagner, H., Dolz, G., Romero-Zúñiga, J.J., Taubert, A., Wehrend, A., Hermosilla, C. (2017). First report on the seroprevalence of *Neospora caninum* in goats from the Federal State of Hesse, Germany. *Berl Munch Tierarztl Wochenschr* 130 (11-12), 10-15. doi: 10.2376/0005-9366-17017.

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Parasitol Parasites Wildl 6 (3): 287-294. doi: https://doi.org/10.1016/j.ijppaw.2017.09.002.

Villagra-Blanco, R., Silva, L. M. R., Muñoz-Caro, T., Yang, Z., Li, J., Gärtner, U., Taubert, A., Zhang, X., Hermosilla, C. (2017). Bovine Polymorphonuclear Neutrophils Cast Neutrophil Extracellular Traps against the Abortive Parasite *Neospora caninum*. *Front Immunol. 8*, 606. http://doi.org/10.3389/fimmu.2017.00606.

Villagra-Blanco R., Silva L.M.R., Gärtner U., Wagner H., Failing K., Wehrend A., Taubert A., Hermosilla C. (2017). Molecular analyses on *Neospora caninum*-triggered NETosis in the caprine system. *Dev Comp Immunol*. 72:119–127. doi:10.1016/j.dci.2017.02.020.

Villagra-Blanco, R., Dolz, G., Montero-Caballero, D., Romero-Zúñiga, J.J. (2015). Seroprevalence of Bluetongue and associated risk factors in Costa Rican sheep flocks. *Br J Virol*. 2(5): 74-79.

Villagra-Blanco, R., Dolz, G., Montero-Caballero, D., Romero-Zúñiga, J.J. (2015). Detection of antibodies against *Chlamydophila abortus* in Costa Rican sheep flocks. *Open Vet J*.5(2): 122-126.

Villagra-Blanco, R., Dolz, G., Solórzano-Morales, A., Alfaro, A., Montero-Caballero, D., Romero-Zúñiga, J.J. (2015). Presence of Maedi Visna in Costa Rican sheep flocks. *Small Rumin Res* 124: 132-136.

Conference contributions

Villagra-Blanco, R., Silva, L. M. R., Muñoz-Caro, T., Yang, Z., Li, J., Gärtner, U., Taubert, A., Zhang, X., Hermosilla, C. (2016). *Neospora caninum*-triggered neutrophil extracellular trap formation is P2Y2-, NOX-, SOCE- and PAD4-dependent. German Veterinary Medical Society (DVG): 2.-4.5.2016, Berlin. p: 57.

Villagra-Blanco, R., Wagner, H., Dolz, G., Romero-Zúñiga, J.J., Taubert, A., Wehrend, A., Hermosilla, C. (2017). Presence of *Neospora caninum*-specific antibodies in goat flocks from the federal state of Hesse. German Veterinary Medical Society (DVG): 12.-16.6.2017, Hannover. p: 21.

Villagra-Blanco R., Silva L.M.R., Gärtner U., Wagner H., Failing K., Wehrend A., Taubert A., Hermosilla C. (2017). *Neospora caninum*-triggered neutrophil extracellular trap formation in goats. German Veterinary Medical Society (DVG): 12.-16.6.2017, Hannover. p: 23.

Villagra-Blanco, R., Wagner, H., Dolz, G., Romero-Zúñiga, J.J., Taubert, A., Wehrend, A., Hermosilla, C. (2017). Nachweis von *Neospora caninum*-spezifischen Antikörpern in hessischen Ziegenbeständen. 42 Leipziger Fortbildungsveranstaltung: Labordiagnostik in der Bestandsbetreuung: 16.6.2017. Leipzig.

Villagra-Blanco, R., Silva, L.M.R., Aguilella-Segura, A., Arcenillas-Hernández, I., Martínez-Carrasco, C., Seipp, A., Gärtner, U., Ruiz de Ybañez, R., Taubert, A., Hermosilla, C. (2017). *Neospora caninum*-induced neutrophil extracellular trap (NET) formation in Bottlenose dolphins (*Tursiops truncatus*). 26th International Conference of

the World Association for the Advancement of Veterinary Parasitology (WAAVP), 4.-8.9.2017, Kuala Lumpur, Malaysia. p: 343.

Villagra-Blanco, R., Esquivel-Suarez, A., Romero-Zúñiga, J.J, Taubert, A., Wehrend, A., Hermosilla, C., Dolz, G. (2017). Detección de anticuerpos contra *Neospora caninum* y *Toxoplasma gondii en* ovinos y caprinos de Costa Rica (in Spanish). XXII Congreso Nacional de Medicina Veterinaria: 15-17.11.2017, San José, Costa Rica.

Villagra-Blanco, R., Angelova, L., Conze, T., Schares, G., Bärwald, A., Taubert, A., Hermosilla, C., Wehrend, A. (2018). Detection of *Neospora caninum*-specific antibodies in breeding bitches. 28th Annual Meeting of the German Society for Parasitology**:** 21 – 24.3 2018, Berlin. p: VPA-P-02.

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ABBREVIATIONS

2-APB - 2- aminoethoxydiphenyl borate

ABAH - Aminobenzoic acid hydrazide

AMPs - Antimicrobial peptides

BPI - Bacterial permeability increasing protein

BUVEC - Bovine umbilical vein endothelial cells

CMK - Methoxysuccinyl Ala-Ala-Pro-Val chloromethylketone

DAMPs - Danger-associated molecular patterns

DNA - Deoxyribonucleic acid

DNase - Deoxyribonuclease

DPI - Diphenylene iodondium

ECs - Endothelial cells

ELISA - Enzyme-linked immunosorbent assay

ERK - Extracellular signal-regulated kinases

ETs - Extracellular traps

GM-CSF - Granulocyte-macrophage colony-stimulating factor

H1 - Histone H1

H2A/H2B - Histone 2A and 2B

H3 - Histone H3

H4 - Histone H4

IFN-γ - Interferon gamma

IgA - Immunoglobuline A

IgG - Immunoglobuline G

IgM - immunoglobuline M

LPS - Lipopolysaccharides

MAPK - Mitogen-activated protein kinases

MARC-145 - Rhesus monkey fetal kidney cells

MPO - Myeloperoxidase

NOX (NADPH-oxidase) - Nicotinamide adenine dinucleotide phosphate-oxidase

NE - Neutrophil elastase

NET - Neutrophil extracellular trap

P2Y2 - ATP-specific G-protein receptor

PAD4 - Peptidylarginine deiminase 4

PAMPS - Pathogen-associated molecular patterns

PGRPs -Peptidoglycan recognition proteins

PMN - Polymorphonuclear neutrophils

PTX - Pentraxin

ROS - Reactive oxygen species

SEM - Scanning electron microscopy

SK3 - Small conductance calcium-activated potassium channel 3

SOCE - Store operated calcium entry

TNF - Tumoral necrosis factor

1. INTRODUCTION

1.1 Apicomplexan parasites

Apicomplexa is the subphylum of single-celled eukaryotic protozoa responsible for diseases of recognizable medical and economic importance in humans, domestic and wild animals (Muller and Hemphill, 2013; Francia and Striepen, 2014). It is estimated that out of a probable 1.2 - 10 million apicomplexan species, approximately 0.1% have been named and described to date (Adl et al., 2007). All apicomplexan protozoa are obligate intracellular parasites infecting different hosts and specific host cell types *in vivo*, in which they evade host immune response and extract their nutrients for further replication (Striepen et al., 2007). Apicomplexan parasites are complex organisms that exhibit a wide variety of morphological shapes depending on their genus and life cycle stages. They inhabit a wide array of terrestrial and marine environments depending on their host niche environments (Šlapeta and Morin-Adeline, 2011). Amongst most relevant apicomplexan genera in domestic and wild animals are included *Babesia* spp. (Gohil et al., 2013), *Besnoitia* spp. (Cortes et al., 2014), *Cryptosporidium* spp. (Šlapeta, 2013), *Eimeria* spp. (Chartier and Paraud, 2012), *Cystoisospora* spp. (Schwarz et al., 2014), *Neospora* spp. (Reichel et al., 2014), *Sarcocystis* spp. (Bahari et al., 2014), *Theileria* spp. (Li et al. 2014) and *Toxoplasma* (Abi Abdallah et al., 2012; Coppens, 2013). Despite the enormous socio-economic impact of these parasites in farm animal production worldwide, until now, *Toxoplasma* and *Plasmodium* have been the best studied parasites (Chapman, 2014), mainly because of their anthropozoonotic potential and high impact on human health worldwide. On the other hand, parasitism in wildlife populations has been recorded as frequently as in domestic populations, but in the wild

setting, parasitism seems to act to reduce vigor and alter behaviour of the host rather than causing significant diseases and losses (Hamilton and Zuk, 1982; Horak et al., 2004; Bouma et al., 2006; Morin-Adeline et al., 2011). How parasite burden and pathology in captive animals translate into the wild is of much interest, especially when large resources are being placed into captive breeding programs of endangered species (Šlapeta and Morin-Adeline, 2011).

1.2 Neospora caninum

Neospora caninum is an obligate apicomplexan intracellular cyst-forming coccidian parasite similar in structure and development to *T. gondii*, which it is also genetically related (Dubey, 2003). However, *N. caninum* causes primarily a reproductive disease mainly in cattle, as well as neuromuscular, cutaneous, visceral and reproductive illness in dogs, but is not considered zoonotic, whereas toxoplasmosis is a serious disease of humans, small ruminants, and many other warm-blooded animals. Although much has been published on the biology of *N. caninum* during the last 26 years since its discovery in 1988 (Dubey et al., 2002), neosporosis continues to be a major problem in cattle and other related ruminant livestock animals, including sheep and goats (Dubey and Schares, 2011). Past and recent surveys clearly indicate that a rather wide range of domestic and wild animals have been exposed to *N. caninum*. Nonetheless, viable *N. caninum* parasites have been isolated from only a few hosts (e. g. cattle, sheep, water buffalo, dog, horse, bison, white-tailed deer) (Dubey et al., 1990; Landmann et al., 2011; Dubey and Schares, 2011).

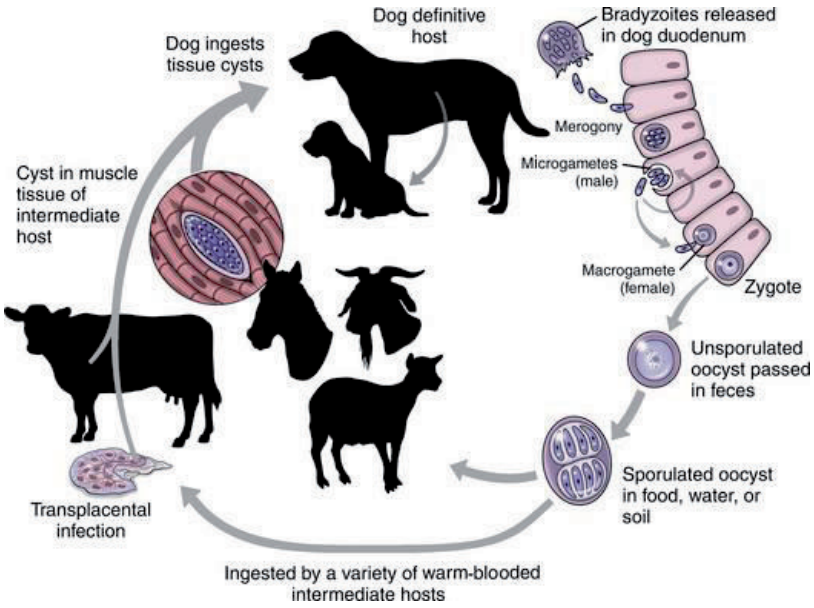

Figure 1. Life cycle of *N. caninum* according to Sykes, J.E. (2013).Neosporosis

1.3 Life cycle stages and host-parasite transmission routes

N. caninum is a coccidian parasite with a wide host range developing a complex life cycle through intermediate as well as a final hosts (Dubey et al., 2007) (Figure 1). Dogs (*Canis familiaris*) and coyotes (*Canis latrans*) are the only recognized final hosts (McAllister et al., 1998; Gondim et al., 2004), whereas a large range of warm-blooded animals can act as intermediate hosts, including cattle (Dubey et al., 1989; Barr et al.,1991), sheep (Dubey et al., 1990), goats (Dubey et al., 1992), horses (Marsh, et al., 1996), deer (Dubey et al., 1996), foxes (Buxton et al., 1997; Schares et al., 2002), water buffaloes (Huong et al.,1998; Rodrigues et al., 2004), raccoons (Lemberger et al.,

2005), camels (Hilali et al., 1998), New World camelids (Wolf et al., 2005), rhinoceros (Williams et al., 2002) and cetaceans (Dubey et al., 2003; Omata et al., 2006).

The parasite life cycle involves also three infection stages: Tachyzoites (invasive rapidly multiplying stages), bradyzoites (tissular encysted stages), micro- and macrogamonts (sexual stages) and sporozoites within oocysts (infective stages). Tachyzoites and bradyzoites are the intracellular stages found in definitive and intermediate hosts (Dubey et al., 2002), meanwhile the environmentally resistant stage of the parasite, the oocysts formed after syngamy of micro- and macrogamonts in intestine of final hosts, is excreted in feces of dogs and coyotes in an unsporulated stage form (McAllister et al., 1998; Lindsay et al., 1999) with a similar environmental resistance to that of *T.gondii* oocysts (Dubey, 2004). Carnivores become affected by ingesting infected tissues containing bradyzoites, and herbivores by the ingestion of contaminated food or drinking water with *N. caninum* sporulated oocysts (Dubey et al., 2006). These events constitute the horizontal (postnatal or lateral) transmission of *N. caninum* (Dubey et al., 2006). On the other hand, the vertical (transplacental or congenital) transmission happens when an infected dam spread tachyzoites to its offspring during pregnancy. Vertical transmission can be described more precisely according to the origin and route of infection of the fetus; so recently, the terms exogenous transplacental transmission and endogenous transplacental transmission have been proposed (Trees and Williams, 2005). Exogenous transplacental transmission takes place after a primary, oocyst-derived, infection of a pregnant dam, while endogenous transplacental transmission occurs in a persistently infected dam after reactivation (recrudescence) of the infection during pregnancy and produces in most of the cases, healthy congenitally infected

offspring (Paré et al., 1996; Anderson et al., 1997; Schares et al., 1998; Davison et al., 1999).

1.4 Economical impact and control of neosporosis

N. caninum represents an important worldwide infectious cause of abortion in cattle and other ruminants, as well as clinical disease in dogs (Dubey and Schares, 2011). Particularly in cattle, fetal death and reproductive failure represent the direct costs (Dubey et al., 2017), meanwhile the indirect losses increase overall costs for farmers, such as expenses for veterinarian assistance and medical diagnosis, the lengthened rebreeding intervals, reduction on replacement of culled cows, decreased value of breeding stock and diminishment of milk yield and weight gain in infected animals (Trees et al., 1999; Dubey and Schares, 2006; Dubey et al., 2007; Ortega-Mora et al., 2006).

According to Reichel et al. (2013), the median losses of *N. caninum*-derived abortion round US $1.298 billion per year, ranging as high as US $ 2.380 billion, which 2/3 incurred in dairy cattle and 1/3 in beef cattle industry. Moreover, the global costs were estimated to US $ 852.4 million in North America, US $ 239.7 in South America, US $ 137.5 million in Australasia and US $ 68.7 million in Europe.

Furthermore, *N. caninum*-induced abortions follow two main patterns: epidemic and endemic (including sporadic abortions) (Reichel et al., 2013). Epidemic abortions occur due to primary infections in previously non-infected dams exposed at the same time to a single source of contagion (McAllister et al., 2000). In this pattern, a large proportion (> 10%) of pregnant animals abort within a short period of time, resulting very costly and

devastating for farmers and therefore it is also called as „storm-like“ abortions (Dubey et al., 2007). Endemic pattern produces abortions intermittently along some months or even years and happens due to presence of persistently infected dams in the flock that transplacentally transmit the parasite to their progeny. This pattern can be also costly (Larson et al; 2004; Hall et al., 2005; Reichel et al., 2013), especially for farmers who wish to sell their animals in herds with high genetic merit (Trees et al., 1999).

Control strategies for *N. caninum* infections have been previously discussed and depend on the country, region, infection rate and associated risk factors (Reichel and Ellis, 2002; Dubey et al., 2007). Also costs of these control options should be also considered (Reichel et al., 2007). In general, national, regional and farm level control programs have been implemented in different areas around the world to effectively control neosporosis (Hall et al., 2005). Control programs should incorporate a cost-benefit calculation comparing expenses of testing and control measures with the benefit of reduced economic losses due to *N. caninum* infection (Larson et al., 2004; Bartels et al., 2006; Häsler et al., 2006; Reichel and Ellis, 2006).

Some control measures have been described in the literature according to prevent:

- Abortions due to exogenous neosporosis:
    - Prevention of waterborne transmission (De Moura et al., 2006; Dubey et al., 2007).
    - Prevention of dogs and other potential definitive hosts from contaminating pastures and feedstuff with feces (Reichel et al., 2013).
    - Prevention of dog access to infected raw ruminant tissues and placentas (Uggla et al., 1998; Reichel et al., 2014).

- Abortions due to endogenous neosporosis:
    - Flock testing, identification of infected animals and culling selectively ("test-culling" strategies): Establish different approaches to slowly reduced abortions by gradual reduction of infected animals and their progeny infarms with predominantly endogenous transplacental transmission and concerning to their economic reality (Larson et al., 2004; Hall et al., 2005; Conraths et al., 2007).
- Abortions due to both endogenous and exogenous neosporosis (standard control measures):
    - Reinforcement of farm biosecurity (Dubey et al., 2007)
    - Quarantine and regular testing of replacement and purchased animals: Minimize vertical transmission and reduce the potential infective risk for definitive hosts (Dubey et al., 2007).
    - Vaccination (Romero et al., 2004; Williams et al., 2007; Weston et al., 2012; Hemphill et al., 2013).
    - Rodent control: Diminishment of dogs infection risk (Dubey et al., 2007)
    - Reproductive management: Embryo transfer (Baillargeon et al., 2001; Landmann et al., 2002) and insemination of seropositive dams with semen from beef bulls (López-Gatius et al., 2005).

1.5 Innate immune responses

Pathogen microorganisms such as virus, bacteria, fungi or parasites disturbing the normal homeostatic mechanisms, are immediately met by cells and molecules of the innate immune system: the physical epithelial/endothelial barriers, leukocytes [e. g.

polymorphonuclear neutrophils (PMN), monocytes, macropahges], dendritic cells, natural killer (NK) cells and circulating plasmatic proteins. Professional phagocytes (i. e. PMN, monocytes, macrophages) detect and react against “pathogen-associated molecular patterns” (PAMPS), for example, some bacterial components (lipopolysaccharides, peptidoglycan, flagellin, microbial DNA or RNA), as well as, endogenous molecules that signal homeostatic danger called “danger-associated molecular patterns” (DAMPs), e. g. shock proteins, high-mobility group box 1 (HMGB1) protein or even host DNA (Janeway et al., 2001; Brinkmann and Zychlinsky, 2012).

Responses of host innate immune cells trigger inflammation, which dilate and increase permeability of blood vessels leading to local blood flow and leakage of fluid. Additionally, adhesive properties of endothelium induce circulating leukocytes to stick to activated endothelial host cells and migrate between them to site of infection, to which they are attracted by chemokines (Janeway et al., 2001; Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012). Within these leukocytes, PMN are the first white cells to be mobilized as they contain potent antimicrobial components, which play an essential role in innate immune mechanisms against multiple kinds of pathogens, including bacteria, fungi and parasites (Urban et al., 2006; Hahn et al., 2013; Hermosilla et al., 2014). Additionally, other activated innate immune cells at the site of infection or injury, e. g. mononuclear phagocytes, remove foreign particles and host debris (a non-phlogistic uptake mechanism denominated, efferocytosis) controlling the natural PMN turnover and apoptosis, which is important on PMN homeostasis in healthy organisms, reducing the antimicrobial and proinflammatory capacities of the granule enzymes, which can be also harmful and toxic to the host (Whyte et al., 1993; Greenlee-Wacker et

al., 2014; Kobayashi et al., 2017). These innate immunological processes contribute also with release of cytokines involved later in lymphocyte-mediated adaptive immune response (Newton and Dixit, 2012).

1.6 Polymorphonuclear neutrophils (PMN) and their role within host innate immune system

The main function of PMN, in early innate immune defense has been classically understood as a variety of potent intracellular microbicidal mechanisms to kill invasive pathogens. PMN are extremely mobile and short-lived leukocytes which are densely packed with secretory granules, being able to respond immediately towards pathogens once migrating from bone marrow. PMN perform several effector mechanisms to combat pathogens, such as phagocytosis, production of oxygen-based antimicrobial radicals known as ROS, excretion of antimicrobial peptides/proteins, and recently described synthesis of neutrophil extracellular traps (NETs) (Brinkmann and Zychlinsky, 2012).

Phagocytosis by professional phagocytes is intensified by prior opsonization of pathogens with complement factors or, in primary exposed host, by specific antibodies recognizing epitopes on pathogen surface. Subsequently phagosomes must fuse with intracellular granules to form the phagolysosome, within which killing of the pathogen will be accomplished by a combination of non-oxidative as well as oxidative mechanisms. Efficient non-oxidative killing mechanisms of PMN include antimicrobial peptides (AMPs) such as cathelicidins, defensins, cathepsins, peptidoglycan recognition proteins and proteases, whereas oxidative killing relies on the production of ROS via the

nicotinamide adenine dinucleotide phosphate-oxidase (NOX) complex (Nathan, 2006; von Kockritz-Blickwede und Nizet, 2009). PMN are the predominant cells of professional phagocyte population, comprising between 50-80 % of total white blood cells (Nathan, 2006; Hahn et al., 2013). Therefore, PMN are considered as a pivotal component of host innate immune system representing first line of defense against invading pathogens and first ones to be recruited to site of infection (Brinkmann et al., 2004; Ermert et al., 2009; Brinkmann and Zychlinsky, 2012; Hahn et al., 2013; Hermosilla et al., 2014).

1.7 Ruminant PMN

Determined species variation in distribution of PMN-derived lysosomal enzymes has been reported, initially in humans and laboratory animals (Rausch and Moore, 1975), but also in ruminants (Buchta, 1990; Singh et al., 1997). These differences could affect the vulnerability and response of these species to specific infections (Sahoo et al., 1998). For example, bovine PMN are the first line defense during clinical mastitis when different pathogens invade mammary glands; these cells migrate from blood into milk and execute different cascades of reactions including both oxidative and non-oxidative mechanisms to destroy these harmful agents (Kobayashi et al., 2005). Along with tissue damage, modulation of apoptosis of both blood and milk PMN is a key feature in mastitis but it depends on the type of mastitis and mastitis causing organism (Burvenich et al., 2007; Kennedy and De Leo, 2009)

A study with different PMN lysates conducted by Sahoo et al. (1998), described higher lysozyme activity of buffalo-derived PMN when compared to cattle and goat, which

could contribute to resistance of these animals against some diseases in tropics (Singh et al., 1997). It is also remarked higher activity of neutrophil elastase (NE) of caprine PMN than in buffalo or cattle PMN and large performance of beta-defensins and myeloperoxidase (MPO) in bovine PMN, especially against parasites (Sahoo et al., 1998; Weiss and Wardrop, 2011; Yildiz et al., 2017). Nevertheless, the release of MPO seems to be more pronounced in caprine PMN than in large ruminants (Sahoo et al., 1998).

1.8 Neutrophil extracellular traps (NETs)

Beginning with the landmark study of Brinkmann et al. (2004), the paradigm of how PMN kill pathogenic bacteria was deeply changed. The discovery of DNA-based antimicrobial NETs, released after PMN death (NETosis) had high implications on our current knowledge concerning not only invasive pathogens but also pathophysiology of infection and inflammatory diseases (Logters et al., 2009; Hahn et al., 2013). Detailed analyses of these novel NET structures revealed that they basically consist of nuclear DNA as a backbone being decorated or adorned with histones, antimicrobial peptides and proteins derived from three PMN granule types (i. e. azurophilic, secondary, and tertiary), such as NE, MPO, pentraxin, lactoferrin, gelatinase, bacterial permeability-increasing protein (BPI), cathepsin G, peptidoglycan recognition proteins (PGRPs) and calprotectin (Bainton et al., 1971; Borregaard and Cowland, 1997; Brinkmann and Zychlinsky, 2007, 2012; von Kockritz-Blickwede and Nizet, 2009, Hahn et al., 2013). By concentrating these highly active components in a small area NETs provide a unique extracellular matrix capable not only to entrap but also to kill invasive pathogens (Fuchs et al., 2007; Ermert et al., 2009; Abi Abdallah and Denkers, 2012; Hahn et al., 2013)

with advantage of minimized damage to surrounding tissues (Logters et al., 2009; Hahn et al., 2013).

NETs have been described so far in a wide range of different species such as humans (Gupta et al., 2005; Baker et al., 2008), mice (Beiter et al., 2006; Buchanan et al., 2006; Wartha et al., 2007; Ermert et al., 2009), horses (Alghamdi and Foster, 2005), cows (Lippolis et al., 2006; Behrendt et al., 2010; Muñoz-Caro et al., 2015a, b), fish (Palić et al, 2007), cats (Wardini et al., 2010), dogs (Wei et al., 2016), chicken (Chuammitri et al., 2009), goats (Silva et al., 2014) and insects (Altincicek et al., 2008). Recently, other leukocytes of host innate immune system, such as eosinophils (Yousefi et al., 2008), mast cells (von Kockritz-Blickwede et al., 2008), macrophages (Aulik et al., 2012; Hellenbrand et al., 2013) and monocytes (Muñoz-Caro et al., 2014; Reichel et al., 2015), have also been reported to extrude NET-like structures which are at present collectively entitled simply as extracellular traps (ETs).

So far, data on NETosis appear to be focused on fungal and bacterial pathogens, such as *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans*, *Escherichia coli, Helicobacter pylori, Histophilus somni*, *Listeria monocytogenes, Mannheimia haemolytica, Mycobacterium tuberculosis, Staphylococcus aureus*, *Streptococcus pyogenes* and feline leukemia virus among others (Brinkmann et al, 2004; Beiter et al., 2006; Urban et al., 2006; Grinberg et al., 2008; Bianchi et al., 2009; Ramos-Kichik et al., 2009; Urban et al., 2009; Aulik et al., 2010; Bruns et al., 2010; Wardini et al., 2010; Hakkim et al., 2011; Guimarães-Costa et al., 2012; Aulik et al., 2012; Hahn et al., 2013; Hellenbrand et al., 2013).

1.9 Investigations on parasite-induced NET formation

While most of NETs-related studies have focused on effects of NET formation on bacterial and fungal pathogens, little attention has been paid to the role of NETs against protozoan and metazoan parasites. By now, NET formation was described to be induced mainly by protozoan parasite species, such as the euglenozoa *Leishmania amazonensis, L. major, L. braziliensis, L. chagasi* and *L. donovani* (Guimarães-Costa et al., 2009; Gabriel et al., 2010; Wang et al., 2011, Guimarães-Costa et al., 2012), the apicomplexan *Plasmodium falciparum, Eimeria bovis* and *T. gondii* (Baker et al., 2008; Behrendt et al., 2010; Abi Abdallah et al., 2012) and on helminth parasites, e. g. *Schistosoma japonicum* (Chuah et al., 2013) and *Haemonchus contortus* (Muñoz-Caro et al., 2015b).

PMN appear to play a pivotal role in *E. bovis* defence since this leukocyte population was identified in parasitized intestine of *E. bovis*-infected calves (Friend and Stockdale, 1980; Muñoz-Caro et al., 2016) and demonstrated to directly interact with *E. bovis* stages and antigen via direct elimination or production of pro-inflammatory cytokines, chemokines and iNOS when encountered (Behrendt et al., 2008). Additionally, PMN were shown to adhere to *E. bovis*-infected endothelial cell layers (Hermosilla et al., 2006) and their phagocytic and oxidative burst activities were demonstrated to be enhanced in response to *E. bovis* sporozoites *in vitro* or in *in vivo* infection (Behrendt et al., 2008). Then, NETs were discovered as an additional effector mechanism of PMN driven by *E. bovis* sporozoite encounter (Behrendt et al., 2010). Scanning electron microscopy (SEM) analyses revealed that sporozoites of *E. bovis* were covered and entrapped within an extracellular network of long drawn out and delicate fibers

originating from dead and disrupted PMN. The DNA nature of *E. bovis*-induced NETs was proven by Sytox Orange staining and DNase treatment. Fluorescence images showing brightly stained fibers and complete loss of fluorescence in DNase-treated samples corroborated classical backbone structure of NETs (Behrendt et al., 2010).

Interestingly, recent analyses doubt a strict species-specificity of *Eimeria*-induced NETosis and rather argue for a general phenomenon, since NETs were induced by *E. bovis* sporozoites in non-adequate caprine and bovine PMN also expelled NETs in response to a non-bovine *Eimeria* spp. (Muñoz-Caro et al., 2015a). Treatment with a NADPH oxidase inhibitor significantly reduced *E. bovis*-triggered NET formation, confirming NADPH oxidase-dependent NETosis (Muñoz-Caro et al., 2015a), which is in agreement with data generated by other authors (Brinkmann et al., 2004; Urban et al., 2006; Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). Further, *E. bovis* sporozoites appeared to be a potent inducer of NETosis since degree of NETs and their kinetics were much higher and faster, respectively, than NET formation induced by generally used positive control, PMA (Behrendt et al., 2010). This observation was in accordance with data on *S. aureus* (Fuchs et al., 2007). Interestingly, the strongest NET formation occurred in response to viable sporozoites of *E. bovis* when compared to heat-inactivated sporozoites or respective lysates (Behrendt et al., 2010; Muñoz-Caro et al., 2015a). Similar findings were recently reported in NETosis response to closely related parasite *T. gondii* (Abi Abdallah et al., 2012) indicating that most probably not only movements of the parasite enhanced NETosis but also certain molecules present at surface or in excretory/secretory material would trigger this effector mechanism.

However, so far no data is available on nature of these molecules, neither in *Eimeria* nor in *Toxoplasma*.

1.10 Suicidal NETosis versus vital NETosis

NETs are released by a novel "suicidal" cell death pathway, called NETosis, different from apoptosis, pyroptosis and necrosis, which allows PMN to kill pathogens far beyond their lifespan (Brinkmann and Zychlinsky, 2007). Interestingly, a current study revealed that some PMN released NETs *in vivo* without undergoing cell death, called vital NETosis, while maintaining their crawling and phagocytic activity (Yousefi et al., 2009, Yipp et al., 2012). Upon stimulation, PMN produce reactive oxygen species (ROS), such as O_2^-, H_2O_2 and HOCl, which function as antimicrobials and are essential for NET formation (Brinkmann and Zychlinsky, 2007; 2012; Fuchs et al., 2007). Consequently, PMN from patients with chronic granulomatous disease (CGD) who lack functional NOX are not capable to form NETs (Fuchs et al., 2007).

1.11 Role of NOX-dependent ROS complex during NETosis

During NETosis several nuclear and cytoplasmic events have to occur in order to initiate complete and proper NETs extrusion. Firstly NOX-dependent ROS production leads to morphological changes such as delobulation of PMN nucleus, disassembly of nuclear envelope and degradation of granule membranes (Fuchs et al., 2007). In addition, peptidylarginine deiminase 4 (PAD4)-mediated histone citrullination, followed by chromatin decondensation seem to be necessary for NETosis (Wang et al., 2009; Abi Abdullah and Denkers, 2012; Hahn et al., 2013). After disassembly of nuclear and granule membranes, mixture of proteins from both nuclear and granular origins, i. e.

histones (H1, H2A/H2B, H3, H4), antimicrobial peptides and proteins, will occur prior to extrusion of protein/histone-decorated NET structures into extracellular space (Hahn et al., 2013). Most studies on NETs formation strengthened on key role of a functional NOX system. However, MPO and NE also seem to be able to regulate proper NET release (Brinkmann and Zychlinsky, 2012). The signaling pathway involved in NETosis was shown to be Raf-MEK-ERK-dependent (Hakkim et al., 2011). In fact, ERK/MAP kinase activation has additionally been described as an essential requirement for NOX-dependent NETosis, whereas Akt and p38 activation pathways are fundamental for both types of NETosis (Douda et al., 2015). Molecules known so far to induce suicidal NETosis include PMA, GM-CFS/LPS, LPS, IL-8, Ca^{2+} ionophore, thapsigarin, chemotactic complement-derived peptide complement factor 5 (C5a), TNF, IFN, *Leishmania* spp. promastigote LPG, *S. epidermidis* δ-toxin, auto-antibodies and LPS-activated platelets (von Köckritz-Blickwede and Nizet, 2009; Cogen et al., 2010; Guimarães-Costa et al., 2012; Abi Abdallah and Denkers, 2012; Brinkmann and Zychlinsky, 2012; Hahn et al., 2013). On the other hand, NOX-dependent NETs formation proved sensitive by blocking with diphenyleneiodonium (DPI), a strong inhibitor of ROS production (Yousefi et al., 2008). DPI has been shown to be not only an effective blocker of NOX but also of other NAD(P)-dependent enzymes (such as glucose 6-phosphate dehydrogenase) and mitochondrial OXPHOS flavoenzymes (Riganti et al., 2015).

1.12 NETosis associated with NOX-independent ROS production

Pathways of NOX-independent ROS release are still unclear, conflicting and incomplete in literature (Stoiber et al., 2015). Both NOX-dependent and -independent NETosis

generate an elevation of cytosolic calcium, a process denominated as calcium influx-mediated NETosis. This calcium influx is mainly performed through regulation of store-operated calcium channels (SOCE) in activated PMN (Burgos et al., 2011; Parker et al., 2012). However, most recent studies evidence a NOX-independent pathway of NETosis that depends on mitochondrial ROS, mediated by small conductance calcium-activated potassium channel 3 (SK3) and working essentially independent from MAPK-ERK activation (Douda et al., 2015).

Moreover, the inability of DPI to full suppress mitochondrial ROS production on NETs formation suggest involvement of ROS from other sources produced during mitochondrial respiration (Bulua et al., 2011; Murphy 2012; Douda et al., 2015). One of these ROS sources to play a particular role in ETosis-mediated inflammation is MPO. Functional MPO is an indispensable requirement for NETosis as demonstrated elsewhere (Metzler et al., 2011; Brinkmann and Zychlinsky, 2012). Extracellular release of MPO by activated PMN during cellular respiratory bursts catalyze oxidation of some anions, i. e. chloride (Cl^-), bromide (Br^-) and thiocyanate (SCN^-), generating oxidants that benefits phagocytes' ability to efficiently kill pathogens (Davies et al., 2011).

1.13 Pathogenesis of bovine neosporosis

Bovine neosporosis is mainly a parasitic disease of the placenta and foetus, usually initiated following a maternal parasitemia, triggered either as the result of a primary (exogenous) maternal infection or following recrudescence of a persistent (endogenous) infection during pregnancy (Dubey et al., 2007; Trees and Williams, 2005; Williams et al., 2009). In cattle, parasitemia appears sporadically between 2 and 41 days when

parasite DNA can be detected in white blood cells depending on route of infection and parasite strain (Maley et al., 2003; Macaldowie et al., 2004). Following a parasitemia, tachyzoites of *N. caninum* are able to establish themselves in maternal caruncular septum before crossing to fetal placental villus. For abortion to occur, the foetus or its placenta has to be so damaged by fast replicating *N. caninum* tachyzoites that it is no longer viable and several factors may interact, to a greater or lesser extent, to influence this inflammatory process (Gibney et al., 2008). Additionally, lesions vary with infection of foetus at different stages of gestation. In first three months, the foetus is vulnerable to infection but around 100 days of gestation it becomes immunocompetent and able to perform early host innate immune responses which may or may not be enough to reach protection against this parasite (Andrianarivo et al., 2001; Innes et al., 2005). Primary parasite-induced placentitis compromises foetal survival directly or causes luteolysis and consequently abortion. Foetal damage may occur due to primary organ/tissue destruction caused by fast multiplication of *N. caninum* tachyzoites or due to insufficient oxygen/nutrition, secondary to placentitis as well as endometritis. Aborted foetus presents lymphoid inflammation, widespread mononuclear infiltrations and multifocal necrosis in many infected tissues which generate hemorrhages, focal leucomalacia (especially in cerebrum) and destruction of hepatic, cardiac, muscular, neural or pulmonary cells (Barr et al., 1990; Ogino et al., 1992). To sum up, *N. caninum* is able to produce abortion using three possible pathological pathways: placental inflammation, fetal damage and maternal/fetal placental necrosis (Dubey et al., 2017).

1.14 Transmission routes and clinical signs of bovine neosporosis

Exogenous *N. caninum*-oocysts are considered key stages in epidemiology of cattle neosporosis as they are environmentally resistant such as oocysts of other related coccidian parasites (i. e. *Eimeria, Toxoplasma, Cystoisopora*) (Uzeda et al., 2007; Neto et al., 2011). Transplacental (vertical or congenital) transmission from cow to foetus and post-natal (horizontal or lateral) transmission via ingestion of sporulated oocysts are the only demonstrated modes of infection in bovine species.

The rate of vertical transmission may vary among herds. In selected Dutch dairy herds vertical transmission rates were estimated > 61 % (Bartels et al., 2007) in one study, and 58% in another one (Dijkstra et al., 2008). Vertical transmission rate was 37.1% in a dairy herd in Argentina; cows with high titers had more infected calves than cows with lower titers (More et al., 2009).

Ingestion of sporulated *N. caninum*-oocysts from environment is the only demonstrated natural mode of infection in cattle after birth (McCann et al., 2007). To date cow to cow transmission has not been observed. Other modes of infection suggested are the lactogenic transmission, but unlikely since under experimental conditions large numbers of tachyzoites were necessary for successful infection (Serrano-Martinez et al., 2007; Ferre et al., 2008). Cows naturally bred with experimentally infected bulls failed to seroconvert (Osoro et al., 2009).

Clinical neosporosis is manifested by abortion in both dairy and beef cattle, and has a worldwide distribution and cows of any age may abort from three months of gestation to term with most abortions occurring at five to six month of gestation (Dubey and

Schares, 2011). Fetuses may die *in utero*, be reabsorbed, mummified, autolyzed, still-born, born alive with clinical signs, or born clinically normal but persistently infected. Moreover, abortions caused by *N. caninum* in cattle generally follow three main patterns: sporadic, endemic and epidemic abortions (Hall et al., 2005: Dubey et al., 2007; Reichel et al., 2013). Neosporosis-derived abortions occur year-round and in some epidemic herd outbreaks as many as 57% of dairy cows have been reported to abort over just a few weeks up to months (Dubey and Schares, 2011). Abortion outbreaks have been classified as epidemic if more than 10% or 12.5% of cows at risk abort within six to eight weeks. A small proportion (<5%) of cows may have repeated abortion due to neosporosis (Dubey et al., 2007; Pabon et al., 2007). A previous *N. caninum*-associated abortion is not considered a cause of sterility (Santolaria et al., 2009).

Clinical signs, other than abortion, which have been only reported in calves under the age of two months, can include neurological signs, an inability to rise and below average birth weight. The hind limbs and/or forelimbs might be flexed or hyperextended and neurological examination might reveal severe ataxia, decreased patellar reflexes, and loss of proprioception. Exophthalmia, nystagmus, hydrocephalus and a narrowing of the spinal cord may also occur in affected calves (Dubey et al., 2007; 2017).

1.15 Bovine neosporosis in Germany

Bovine neosporosis and its associated risk factors in Germany had largely been described (Conraths et al., 1996; Schares et al., 1997, 2003, 2004; Bartels et al., 2006). Several serological studies using different diagnostic techniques, such as ELISA and

IFAT, have confirmed epidemic and endemic abortions in dairy cattle from many regions in Germany (Schares et al., 1999, 2000, 2002; Sörgel et al., 2009) proving that *N. caninum* is involved in approximately 10% of bovine abortion in Northern Bavaria. Particularly in Hesse, obtained seroprevalence in cattle was 4.1% in 10 analyzed farms (Conraths et al., 1996).

Further, clinical manifested bovine neosporosis were detected in two German neonatal calves with metencephalon glial scars and myositis in tongue and gastrointestinal muscles (Peters et al., 2001). Furthermore, brain and liver lesions caused by *N. caninum* in fetuses from North Rhine Westphalia were diagnosed by PCR- (Schares et al., 1997) and immunblot-assays (Söndgen et al., 2001) confirming a rather wide distribution of disease in this Federal State.

On the other hand, familial analyses of asymptomatic vertical transmission of *N. caninum* in German dairy farms with no previous exposure of dams during or after pregnancy showed statistically significant serological differences between herds exhibiting epidemic and endemic *N. caninum*-associated abortions (Schares et al., 1999; 2002).

1.16 Bovine neosporosis in Costa Rica

Presence of *N. caninum* in Costa Rican cattle was reported for first time in 1996 (Perez et al., 1998). Since then, many studies have been conducted to describe important features of neosporosis in Costa Rican dairy cattle, associated risk factors affecting reproductive and productive performance and strategies for prevention and control of infection (Romero et al., 2002, 2005; Romero and Frankena, 2003). A serological study

conducted by Romero et al. (2005) reported seroprevalence of 43.3% in dairy cattle of Costa Rica, where 94.7% of analyzed flocks presented at least three positive animals. Productive and reproductive parameters (including abortion) had been also identified in dairy farms through implementation of a competent herd health management-database software called VAAMP, which is still nowadays useful to analyze epidemiological model of transmission of bovine neosporosis and strategies against this abortive parasite in dairy farms in some countries around the world, including Costa Rica (Romero et al., 2002, 2005).

1.17 Caprine neosporosis

Caprine neosporosis is less prevalent when compared to sheep and cattle (Dubey et al., 2017), however natural and experimental infections have demonstrated lethal lesions in goats with serious economical, clinical, productive and reproductive consequences, which have been lowly investigated (Dubey and Schares, 2011).

Caprine and ovine serological investigations have been widely performed using IFAT to detect principally IgM or/and IgG in the past (Lindsay et al., 1995; Dubey et al., 1996 Buxton et al., 1997). However, recently ELISA have been developed as an additional useful diagnostic tool in large and small ruminants using soluble extracts of sonicated *N. caninum* tachyzoites (Pare et al., 1995; Osawa et al., 1998), recombinant antigens (Lally et al., 1996a), extracted tachyzoite antigen incorporated into an immuno stimulating compound (ISCOM) (Bjorkman et al., 1997) or whole parasites (Williams et al., 1997).

Exact pathogenesis of caprine neosporosis remains largely unknown (Porto et al., 2017) but might resemble those reported for cattle. Caprine abortions with neonatal mortality and neurological tissular cysts compatible with *N. caninum* in Pigmy goats from USA were firstly described by Barr et al. (1992) and Dubey et al. (1992). Since then, abortions, stillbirth and the birth of weak goat kids are the two mainly clinical features of caprine neosporosis reported in natural and experimental infections (Lindsay et al., 1995; Eleni et al., 2004; Moreno et al., 2012; Varaschin et al., 2012; Costa et al., 2014). Principal injuries of fatal caprine neosporosis include hydrocephalus, gliosis, hypocerebellum and meningo-encephalitis (Dubey et al., 1992; 1996). Precisely, histopathological brain examinations in one of these mortal cases were performed in one 3.5 month Costa Rican goat foetus (Please see: Dubey et al., 1996). Moreover, *N. caninum* tachyzoites have been isolated from goat brain, spinal cord, liver and heart (Lindsay et al., 1995; Porto et al., 2016; Nakagaki et al., 2016) where they produce secondary lesions.

Curiously, as far as we know, in Germany there were no previous serological studies neither on presence/absence nor on seroprevalences of *N. caninum*-specific antibodies produced by goats, and in Costa Rica, no additional investigations concerning national seroepidemiolgical status of *N. caninum* in caprine flocks along the different Costa Rican regions have been performed, despite these pathological findings already mentioned and described by Dubey et al. (1996).

1.18 Canine neosporosis

Concerning the definitive host spectrum of *N. caninum*, experimental studies have shown that domestic dogs (*Canis domesticus*), Australian dingos (*C. lupus familiaris*), *coyotes* (*C. latrans*) as well as wolves (*C. lupus*) can act as suitable definitive hosts (McAllister et al., 1998; Gondim et al., 2004; King et al., 2010). Of all these definitive hosts, viable oocysts of *N. caninum* have been demonstrated in faeces of naturally infected dogs (Basso et al., 2009), and in naturally-infected gray wolves (Dubey and Schares, 2011). However no viable oocysts have been detected in faeces of naturally exposed red foxes (*Vulpes vulpes*) in Spain, Ireland and Germany (Wolfe et al., 2001; Almería et al., 2002; Constantin et al., 2011).

The number of oocysts of *N. caninum* shed by domestic dogs is usually rather poor (Dubey and Schares, 2011). Correct identification of these oocysts is epidemiologically important especially because they are structurally similar to another coccidian parasite in dog faeces: *Hammondia heydorni* (Soares et al., 2011). Additional molecular tools for final differentiation of *N. caninum* and *H. heydorni* were in the past reported and applied to rodent tissue (Barratt et al., 2008). The natural mechanisms of *N. caninum* infection in dogs are still not fully understood. Unlike cattle, vertical transmission of *N. caninum* in dogs is considered highly variable and not likely to persist in absence of horizontal transmission. Canine transplacental transmission had been reported in experimental infections (Dubey and Lindsay, 1989), but neonatal neosporosis in dogs is unusual under natural conditions and findings are variable, because not all puppies in a litter become seropositive (Barber and Trees, 1998). Horizontal transmission in dogs is mainly through consumption of meat containing *N. caninum*-cysts with vital bradyzoites

or oral uptake of sporulated oocysts containing sporozoites from environment (Dubey and Schares, 2011).

2. MOLECULAR ANALYSES ON NEOSPORA CANINUM-TRIGGERED NETOSIS IN THE CAPRINE SYSTEM

This chapter is based on the following published paper:

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Molecular analyses on Neospora caninum-triggered NETosis in the caprine system

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ABSTRACT

Neospora caninum is an obligate intracellular protozoan parasite causing serious reproductive disorders in large and small ruminants worldwide. Polymorphonuclear neutrophils (PMN) react against multiple invading pathogens through different mechanisms including the release of neutrophil extracellular traps (NETs). Here, in vitro interactions of caprine PMN and N. caninum tachyzoites were studied. Scanning electron microscopic- and immunofluorescence-analyses demonstrated that caprine PMN undergo NETosis upon contact with tachyzoites of N. caninum, extruding filaments that entrap parasites. Detailed co-localization studies of N. caninum tachyzoite-induced NETs revealed the presence of PMN-derived DNA being decorated with histones (H1, H2A/H2B, H3,H4) and neutrophil elastase (NE) corroborating the molecular characteristics of classical mammalian NETs. As a new result for parasite-induced NETosis, we identified pentraxin and cathepsin B in N. caninum-triggered NETs. Nonetheless, functional inhibition assays revealed that during caprine NET formation triggered by N. caninum different molecular signaling pathways are induced, when compared to other apicomplexan parasites or host species. As such, N. caninum-induced NETosis appears to be influenced by MPO but independent of NADPH oxidase, SOCE, ERK1/2 and p38 MAPK activities. Furthermore, the inhibition of PMN autophagy via blockage of the PI3K-mediated signaling pathway failed to influence tachyzoite-induced NETosis. Since N. caninum-tachyzoites induced caprine NETosis, this effector mechanism should be considered as an early host immune response during acute caprine neosporosis.

Introduction

Neospora caninum is an obligate apicomplexan intracellular parasite similar in structure and development to Toxoplasma gondii (Dubey, 2003). Associated with clinical reproductive and neural infections in dogs, horses, goats, sheep, deer and marine mammals (Dubey and Lindsay, 1996; Anderson et al., 2000; Schares et al., 2001; Dubey, 2003; Omata et al., 2006), it is responsible for reproductive problems mainly in cattle (Hemphill and Gottstein, 2000). Even though protozoan-induced abortions in small ruminants have been commonly related to T. gondii infections (Buxton, 1998; Hurtado et al., 2001; Dubey, 2003; Moreno et al., 2012), N. caninum infections also induce reproductive disorders in sheep and goats with clinical and economic consequences (Dubey and Schares, 2011). Clinical manifestations occurring during caprine neosporosis can include abortion, still birth (Barr et al., 1992; Lindsay et al., 1995; Dubey et al., 1996; Eleni et al., 2004; Costa et al., 2014), and birth of healthy (Koyama et al., 2001) and/or asymptomatic goat kids (Mesquita et al., 2013). Enhanced neonatal mortality being associated with N. caninum infections in goats has been reported from different countries, such as the USA (Barr et al., 1992), Costa Rica (Dubey et al., 1996), Brazil (Corbellini et al., 2001) and Spain (Moreno et al., 2012).

Apicomplexan parasite infections, generally underlie complex adaptive immunological regulations (Gazzinelli et al., 1998; Moore et al., 2005; Boysen et al., 2006) in which after primary infections a

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protective cellular adaptive immunity develops mainly involving T helper cells (Moore et al., 2005; Taubert et al., 2006, 2010; Ruiz et al., 2013, 2015; Hermosilla et al., 2012). In contrast to adaptive immune responses, very little is known on early innate immune reactions against N. caninum, although these immunological reactions are crucial for the establishment of the infection and subsequent disease outcome (Boysen et al., 2006; Muñoz-Caro et al., 2014, 2015a, b; Porto et al., 2016). In particular, polymorphonuclear neutrophils (PMN) play a key role in host innate immunity since they are the most numerous cells in the blood and the first ones to be present at infection sites (Brinkmann et al., 2004; Nathan, 2006; Hahn et al., 2013; Silva et al., 2014). PMN bear different effector mechanisms, such as phagocytosis, production of reactive oxygen species (ROS), release of antimicrobial peptides/proteins and the extrusion of neutrophil extracellular traps (NETs) to combat and eventually kill microorganisms (for review see Brinkmann and Zychlinsky, 2012).

NETs are generally produced following a specific PMN cell death process, known as NETosis, and have been described to occur in vivo and in vitro in different tissues including endothelium (Baker et al., 2008; Maksimov et al., 2016), intestinal mucosa (Muñoz-Caro et al., 2016), and reproductive- and respiratory-tract (Fuchs et al., 2007; Brinkmann and Zychlinsky, 2012; Cheng and Palaniyar, 2013; Muñoz-Caro et al., 2016). So far, NETs were identified in various species, such as humans (Gupta et al., 2005; Baker et al., 2008; Von Köckritz-Blickwede et al., 2009; Brinkmann et al., 2010), mice (Ermert et al., 2009; Abi Abdallah et al., 2012), horses (Alghamdi and Foster, 2005), cattle (Grinberg et al., 2008; Behrendt et al., 2010), fish (Palić et al., 2007; Pijanowski et al., 2013), chickens (Chuammitri et al., 2009), dogs (Jeffery et al., 2015; Wei et al., 2016), insects (Altincicek et al., 2008), crustaceans (Patat et al., 2004; Ng et al., 2013), sheep (Pisanu et al., 2015) and goats (Silva et al., 2014). NETosis is known to mainly rely on NADPH oxidase (NOX)-dependent mechanisms (Fuchs et al., 2007; Von Köckritz-Blickwede et al., 2010; Brinkmann and Zychlinsky, 2012) leading to the extrusion of a mixture of nuclear and cytoplasmic granule contents finally resulting in the formation of nuclear DNA-rich structures being adorned with histones and antimicrobial granular effector molecules, such as pentraxin, lactoferrin, neutrophil elastase (NE), myeloperoxidase (MPO), gelatinase, bacterial permeability-increasing protein (BPI), cathepsin B, peptidoglycan recognition proteins and calprotectin and others (for reviews see Von Köckritz-Blickwede and Nizet, 2009; Brinkmann and Zychlinsky, 2012; Hermosilla et al., 2014; Silva et al., 2016). Additionally, NOX-independent driven NETosis was also described and is accompanied by substantial lower level of ERK1/2 activation and moderate level of PI3K activation, whilst p38 activation appears similar in both, NOX-dependent and -independent NETosis (Douda et al., 2015). Irrespective of NOX-dependency, pathogens may either be immobilized within thin sticky DNA fibers or be killed via local high concentrations of antimicrobial histones, peptides and proteases in vivo (Brinkmann et al., 2004; Von Köckritz-Blickwede and Nizet, 2009; Muñoz-Caro et al., 2016).

By now, NETosis was described to be triggered by different protozoan parasites in vitro and in vivo (Baker et al., 2008; Guimarães-Costa et al., 2009; Wang et al., 2011; Behrendt et al., 2010; Muñoz-Caro et al., 2015a; Silva et al., 2014; Pérez et al., 2016; Abi Abdallah et al., 2012; Reichel et al., 2015; Muñoz-Caro et al., 2014, 2015b; Ávila et al., 2016; Ventura-Juárez et al., 2016). Detailed molecular investigations on apicomplexan-induced NETosis have unveiled its dependency on NOX, NE, MPO, CD11b, ERK1/2, p38 MAPK and SOCE (Behrendt et al., 2010; Abi Abdallah et al., 2012; Muñoz-Caro et al., 2014, 2015a, 2015a; Silva et al., 2014). So far, little is known on NET-mediated immune reactions against N. caninum and to our best knowledge there is only one report on N. caninum-triggered canine NETosis in vitro (Wei et al., 2016). Thus, the aim of the present study was to find in vitro evidence of caprine NETosis in response to N. caninum.

Materials and methods

Ethic statement

All animal procedures were performed according to the Justus Liebig University Giessen Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse (GI 18/14 Nr. A27/2012) and in accordance to the current European Animal Welfare Legislation: ART13TFEU.

Parasites

All NET-related experiments were performed with tachyzoites of N. caninum (strain Nc1) which were cultivated in vitro as described elsewhere (Dubey et al., 1988). In brief, N. caninum tachyzoites were maintained by several passages either in primary bovine umbilical vein endothelial cells (BUVEC) or African green monkey kidney epithelial cells (MARC-145) according to Taubert et al. (2006) and Muñoz-Caro et al. (2014). Vital N. caninum-tachyzoites were collected in supernatants of infected host cells, filtered through 5 μm sterile syringe filters (Sartorius AG) to removed cell debris, washed thrice with sterile PBS (400 × g, 12 min), counted and re-suspended in sterile RPMI 1640 medium (Gibco) until further experimental use.

Host cells

MARC-145 cell monolayers were maintained in DMEM (Sigma-Aldrich) cell culture medium supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich), streptomycin (500 mg/ml; Sigma-Aldrich) and 2% fetal calf serum (FCS; Gibco) and incubated at 37 °C and 5% CO_2 until confluency. Primary BUVEC were isolated according to Taubert et al. (2006) and maintained in modified ECGM [endothelial cell growth medium (PromoCell); 30% (v/v) ECMG and 70% (v/v) M199, supplemented with 1% penicillin and streptomycin and 10% FCS] at 37 °C in 5% CO_2 atmosphere until confluency.

Isolation of caprine PMN

Healthy adult dairy goats (n ¼ 3) kept at the Clinic for Obstetrics, Gynecology and Andrology (Justus Liebig University Giessen) were bled by puncture of the jugular vein. The blood was collected in 12 ml sterile plastic tubes containing heparin as anticoagulant (Li-Heparin Kabe® Labortechnik). Heparinized blood (20 ml) was diluted in 30 ml of sterile PBS containing 0.02% EDTA (Sigma-Aldrich), layered on Biocoll Separating Solution® (Biochrom AG) and centrifuged (800 × g, 45 min). After the removal of plasma, lymphocytes and monocytes, the pellet containing erythrocytes and PMN was re-suspended in 15 ml in RPMI medium 1640 without phenol red (Gibco), treated with Red Blood Cell Lysis® buffer (1 ml, Sigma-Aldrich) to remove erythrocytes and centrifuged (500 × g, 7 min). The pellets were resuspended and PMN were counted in a Neubauer haemocytometer chamber. The percentage of dead PMN was estimated using trypan blue exclusion test (Sigma-Aldrich) as described elsewhere (Behrendt et al., 2010). Finally, caprine PMN were incubated at 37 °C and 5% CO_2 atmosphere for at least 30 min before use.

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Quantification of NETs

Caprine NETs were quantified according to Muñoz-Caro et al. (2014) using Pico Green® (Invitrogen), a specific DNA-binding fluorescent dye. If not stated different, caprine PMN were re-suspended in serum-free medium RPMI 1640 without phenol red and then co-cultured for 60, 90, 120 and 180 min in duplicates with vital N. caninum tachyzoites (37 °C, 1:4 ratio), for time-dependency investigations or co-cultured for 90 min at different ratios (1:1, 1:2, 1:3 and 1:4) for dose-dependency studies. For inhibition experiments, caprine PMN were pre-treated with the respective inhibitor in serum-free medium RPMI 1640 without phenol red for 30 min at 37 °C prior to exposure to N. caninum tachyzoites. The following inhibitors were used: the NOX-inhibitor diphenyleneiodonium chloride [DPI; 10 mM, Sigma-Aldrich, according to O'Donnell et al., 1993], the NE-inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone [CMK; 1 mM, Sigma-Aldrich, according to Scapinello et al. (2011)], the MPO-inhibitor 4-aminobenzoic acid hydrazide [ABAH; 100 mM, Merck, according to Parker et al. (2012)], the SOCE-inhibitor aminoethoxydiphenyl borate [2 APB; 100 mM, Sigma-Aldrich, according to Conejeros et al. (2012)], UO126 as inhibitor of ERK1/2 [50 mM; Sigma-Aldrich, according to Favata et al. (1998)], SB 202190 [10 mM; Sigma-Aldrich, according to Davies et al. (2000)] as an inhibitor of the p38 MAPK signaling pathway and the inhibitor of PI3K-mediated autophagy, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride [LY294; 20 mM, Sigma-Aldrich, according to Tawk et al. (2011)]. For NET quantification, the samples were treated with micrococcal nuclease (0.1 U/ml, New England Biolabs, 15 min, 37 °C) and centrifuged (300xg, 5 min). The supernatants were transferred into a 96-well flat-bottom plate (100 ml per well in duplicates). Then, Pico Green® (50 ml/sample, diluted 1:200 in 10 nM Tris/1 mM EDTA buffer, in the dark) was added. NET formation was determined by spectrofluorometric analysis at an excitation wavelength of 484 nm and an emission wavelength of 520 nm using an automated plate monochrome reader (Varioskan Flash®, Thermo Scientific). NETs were quantified by fluorescence intensity analyses. For negative controls, PMN in plain medium were used. For positive controls, stimulation of PMN with zymosan (1 mg/ml; Invitrogen) was used.

In order to evaluate parasite dose-dependent effects, different PMN: tachyzoites ratios were applied (1:1, 1:2, 1:3, 1:4) and processed as described earlier. For kinetic analyses, PMN and parasites were co-cultured at a ratio of 1:4 for different time periods (60, 90, 120 and 180 min). To dissolve NETs, 90 U of DNase I (Roche Diagnostics) were supplemented 15 min before the end of incubation period.

For comparative reasons, caprine and bovine PMN were simultaneously isolated and analyzed for NET induction in the presence or absence of DPI.

Co-localization of extracellular DNA with histones, NE, pentraxin and cathepsin B in Neospora caninum-induced caprine NETs

After co-culture of caprine PMN with tachyzoites (ratio 1:4, 120 min) on poly-L-lysine-treated coverslips, the fixation of the samples (4% paraformaldehyde, Merck) and three washings in PBS, the samples were blocked with BSA (2%, Sigma-Aldrich, 15 min, RT), incubated in antibody solutions [1 h, RT] and finally mounted in ProLongGold® containing 4^0-6-diamidino-2-phenylindole (DAPI) staining (Invitrogen, 1:1000, 5 min, RT, in the dark) or Sytox Orange® [Invitrogen, 5 mM Sytox Orange®, 10 min, RT, in dark, according to Martinelli et al. (2004)]. For the detection of histones, NE, pentraxin and cathepsin B in NET structures the following antibodies were used: anti-histone (H1, H2A/H2B, H3, H4) monoclonal (mouse clone H11-4, 1:1000, Merck Millipore), anti-NE (AB68672, 1:1000, Abcam), anti-pentraxin (SAB2104614-50UG, 1:1000, Sigma-Aldrich) and anti-cathepsin B (AB58802, 1:1000, Abcam) antibodies. Visualization and illustration was achieved by using an inverted Olympus® IX81 fluorescence microscope being equipped with a digital camera.

Scanning electron microscopy (SEM)

Caprine PMN were co-cultured with vital N. caninum tachyzoites (ratio: 1:4) for 60 and 90 min on poly-L-lysine (Sigma-Aldrich) pre-coated coverslips (Nunc). Cells were fixed in 2.5% glutaraldehyde (Merck), post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold particles. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology (Justus Liebig University Giessen, Germany).

Statistical analysis

Statistical analyses were performed by using Graph Pad Prism® 6 software. One-factorial analyses of variance (ANOVA) with repeated measures were applied to compare co-culture/stimulation conditions using a normal distribution of data. Differences were regarded as significant at a level of $p :; 0.05$ (*); $p :; 0.01$ (**); $p :; 0.001$ (***) and $p :; 0.0001$ (****).

Results

Caprine PMN cast NET-like structures in reaction to Neospora caninum

SEM analyses unveiled that exposure of caprine PMN to N. caninum tachyzoites resulted in the formation of a delicate network of thin strands of PMN-derived fibers being attached to the parasites and seemingly entrapping them (Fig. 1). Kinetic analyses revealed no significant amounts of N. caninum-mediated caprine NETosis. During NETosis, some PMN still exhibited the morphology of intact cells (Fig. 1c and d, black star) whilst others were completely lysed. Furthermore, tachyzoites were observed entrapped in chunky meshworks of PMN-derived filaments (Fig. 1c and d, 90 min).

Neospora caninum-triggered caprine NETs contain histones, NE, cathepsin B and pentraxin

Fluorescence analyses further proved the presence of extracellular DNA within NETs since these structures were labeled by DAPI or Sytox Orange® staining (Fig. 2). N. caninum tachyzoites were located in close proximity to extruded NETs and were often trapped within these structures. Co-localization experiments revealed that NET structures were adorned with classical NETs components, such as histones (Fig. 2a), pentraxin (Fig. 2b), cathepsin B (Fig. 2c), and NE (Fig. 2d).

Neospora caninum-induced caprine NETosis is no time- or dose-dependent

Kinetic studies quantifying NET formation revealed a fast and strong NET induction. N. caninum-triggered NETosis was detected after 60 min of exposure and ongoing, i. e. up to 180 min of exposure. As expected, plain caprine PMN serving as negative controls showed low NET formation when compared to parasite-exposed ones ($p :; 0.05$, Fig. 3a). Overall, no significant differences were

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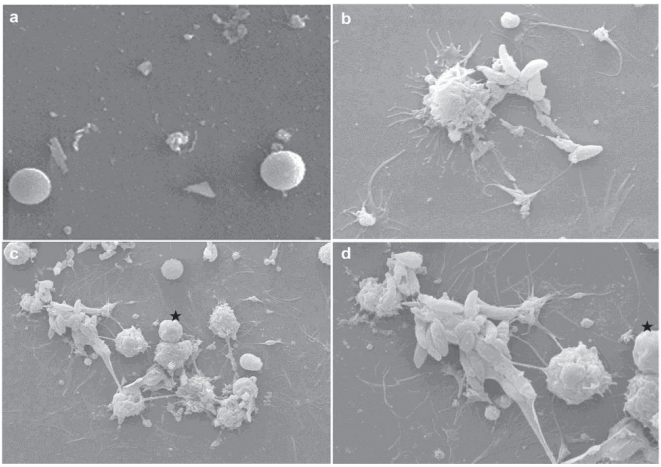

Fig. 1. Caprine NET-like structures in response to Neospora caninum tachyzoite exposure. Scanning electron microscopy analyses revealed NET-like structures being formed by caprine PMN before and after co-culture with N. caninum tachyzoites. (a) Non-activated caprine PMN (negative control). Several tachyzoites entrapped in NET-like filaments (b, 60 min after confrontation) and a rather thicker meshwork of caprine-PMN-released filaments (c and d - magnification of c, 90 min after confrontation) entrapping N. caninum tachyzoites from dead PMN. Black star indicates also a non-activated caprine PMN.

detected between the different time points (60, 90, 120, 180 min).

Furthermore, tachyzoite-induced NET formation was not a dose-independent process, since increasing numbers of N. caninum tachyzoites led to similar results (Fig. 3b). However, significant differences were only observed when PMN-to-tachyzoite ratio of 1:4 was compared with the negative controls were observed at ration 1:4 ($p :::; 0.05$, Fig. 3b).

Distinct antimicrobial proteins involved in Neospora caninum-induced NETs

To further corroborate the characteristics of N. caninum-mediated NETs in goats, we performed inhibition assays with DPI, a potent inhibitor of NOX. Supplementation of DPI throughout the incubation period did not influence tachyzoite-triggered caprine NET formation (Fig. 4a). To test whether this reaction was host-dependent, we additionally performed DPI-related inhibition experiments in the bovine and caprine system within one assay. In agreement, bovine PMN also showed significant NETosis in reaction to tachyzoite exposure ($p :::; 0.01$) and this reaction could not be blocked by DPI (Fig. 4a). Overall, zymosan treatments serving as positive control led to a significant enhancement of NET formation in both host systems ($p :::; 0.001$, Fig. 4a).

Treatments of caprine PMN with the SOCE-inhibitor 2-APB failed to diminish N. caninum-induced NETosis when compared to non-treated but tachyzoites-exposed PMN (Fig. 4b).

As expected, dissolvement of NET-derived extracellular DNA via DNase I treatments resulted in an entire loss of the NET signals (Fig. 5a). In addition, the treatment of PMN with the MPO inhibitor ABAH resulted in a reduction of tachyzoite-triggered NET formation (Fig. 5a). Treatments of caprine PMN with the inhibitor of NE (CMK) also failed to influence parasite-triggered NETosis significantly (Fig. 5a).

Furthermore, we analyzed the actual role of NET-associated molecular signaling pathways in N. caninum-triggered NETosis in the caprine species. Treatments with inhibitors interfering with ERK1/2- (UO126) and p38 (SB202190) MAPK-mediated signaling pathways failed to significantly alter tachyzoite-induced NETs extrusion, showing that N. caninum-derived caprine NETosis appears to function independently of these signaling routes (Fig. 5b).

Finally, we tackled the role of autophagy in N. caninum-mediated caprine NETosis. Therefore, a functional inhibition experiment using LY294, a strong inhibitor of PI3K-mediated autophagy was performed. However, PI3K blockage did not influence N. caninum-triggered NETosis in the caprine system (Fig. 5c).

Discussion

Most studies on pathogen-triggered NETs focused on bacterial, viral and fungal infections (Urban et al., 2006; Fuchs et al., 2007; Aulik et al., 2010; Jenne et al., 2013). Nonetheless, some protozoan parasites have also been identified as potent NET inducers (Baker et al., 2008; Guimarães-Costa et al., 2009; Behrendt et al., 2010; Muñoz-Caro et al., 2015a; Abi Abdallah et al., 2012, Silva et al., 2014; Muñoz-Caro et al., 2014; Reichel et al., 2015; Wei et al., 2016). To the best of our knowledge, we here describe for the first time the release of NETs from caprine PMN in response to the apicomplexan parasite N. caninum, which is known as an important abortive agent in goats worldwide (Dubey and Schares, 2011; Arranz-Solís et al., 2016; Porto et al., 2016).

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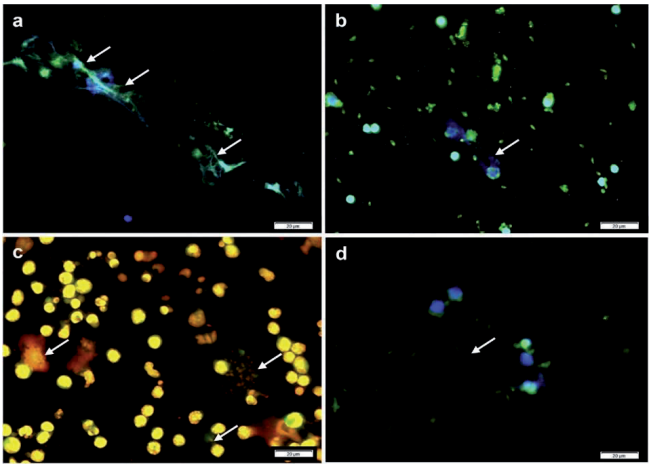

Fig. 2. Co-localization of DNA with histones, pentraxin, cathepsin B and NE in caprine Neospora caninum tachyzoites-induced NET structures. Areas of co-localization (merge, white arrows) of co-cultures of goat PMN and N. caninum tachyzoites that were fixed, permeabilized, stained for DNA using Prolong Gold® DAPI (blue: a, b, d) or Sytox Orange® (orange: c) and probed for different NETs components such as: histones (green, a), pentraxin (green, b), cathepsin (green, c), and NE (green, d).

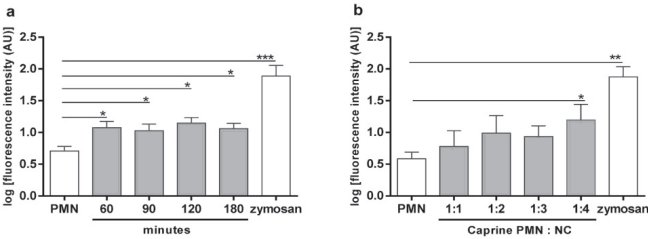

Fig. 3. N. caninum tachyzoite-triggered NET formation is not dose- nor time-dependent. Caprine PMN and N. caninum tachyzoites were co-cultured for different time spans (a: 60, 90, 120 and 180 min, ratio 1:4) or at different ratios (b: PMN:tachyzoites ¼ 1:1, 1:2, 1:3, 1:4, 90 min). Stimulation with zymosan served as positive control, PMN in plain medium as negative control. After incubation, the samples were analyzed for extracellular DNA by quantifying Pico Green®-derived fluorescence intensities. Geometric means of three PMN donors, standard deviation. Differences were regarded as significant at a level of p ::; 0.05(*), p ::; 0.01 (**), p ::; 0.001 (***).

In agreement to observations on other apicomplexan-mediated NETosis (Baker et al., 2008; Behrendt et al., 2010; Muñoz-Caro et al., 2014, 2015a, b; 2016; Silva et al., 2014; Reichel et al., 2015) and to N. caninum-triggered NETosis in the canine system (Wei et al., 2016), we demonstrate extracellular NETs being attached to tachyzoites of N. caninum. In accordance to B. besnoiti-induced NETs (Muñoz-Caro et al., 2014), but in contrast to E. bovis- (Behrendt et al., 2010) and C. parvum-mediated NET induction (Muñoz-Caro et al., 2015b), N. caninum-triggered caprine NETosis was not time-dependent. Overall, a large proportion of tachyzoites appeared to be captured by caprine NETs. The DNA labeling of N. caninum-induced NETs proved the chromatin nature of these PMN-derived extracellular structures. Moreover, the resolution of these network structures by DNase I treatments corroborated the DNA nature of N. caninum-triggered NETs. Alongside to DNA, other classical NET-associated molecules, such as histones and antimicrobial enzymes were detected in N. caninum-triggered NETs. As such, co-localization experiments revealed the simultaneous presence of DNA together with histones and NE, in parasite-triggered NETosis confirming typical molecular characteristics of

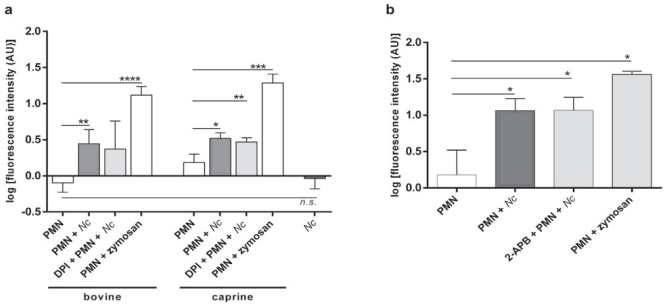

Fig. 4. Caprine N. caninum tachyzoite-triggered NET formation is independent of NOX and SOCE. (a) Comparative analysis of bovine and caprine PMN being confronted to N. caninum in presence or absence of DPI as NOX-inhibitor. (b) Caprine PMN were treated with the SOCE inhibitor 2-APB prior to N. caninum tachyzoite exposure. NET formation was determined by quantifying Pico Green®-derived fluorescence intensities (484 nm excitation/520 nm emission wavelengths). PMN stimulation with zymosan served as positive control and PMN in plain medium were used as negative control. Geometric means of three PMN donors, standard deviation. Differences were regarded as significant at a level of p ::; 0.05 (*), p ::; 0.01 (**), p ::; 0.001 (***) and p ::; 0.0001 (****).

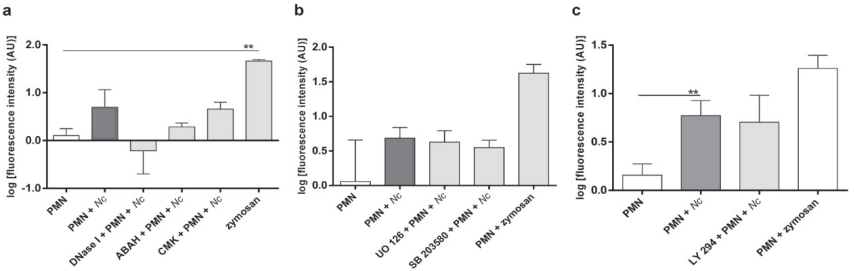

Fig. 5. Caprine N. caninum-triggered NETosis in the presence of inhibitors of NE-, MPO-, ERK1/2-, p38-and PI3K-inhibitors. (a) Caprine PMN cells were pre-treated with the inhibitors of NE (CMK) and MPO (ABAH) prior to N. caninum exposure. Non-treated PMN were processed in parallel. To prove the DNA nature of NETs, the samples were treated with DNase I (15 min). (b) Caprine PMN were pre-treated for 30 min with the inhibitors of ERK1/2 (UO126) and p38 MAPK (SB, 203580) and then co-cultured with N. caninum. (c) Prior to N. caninum tachyzoite confrontation, caprine PMN were exposed to the PI3K inhibitor LY294002. Overall, NET formation was determined by quantifying Pico Green®-derived fluorescence intensities (484 nm excitation/520 nm emission wavelengths). Stimulation of PMN with zymosan was used for positive controls, plain medium served as negative controls. Each condition was performed in duplicates for each PMN donor (n ¼ 3). Differences were regarded as significant at a level of p ::; 0.01 (**).

NETs. These observations are in agreement with former findings in other apicomplexan parasites (Silva et al., 2014; Muñoz-Caro et al., 2014, 2015a, b; Reichel et al., 2015) and with a report on N. caninum-triggered NETosis in the canine system (Wei et al., 2016). Accordingly, the key role of MPO in N. caninum-induced NETosis was proven by functional inhibition experiments, leading to a reduction of tachyzoite-triggered NETosis. MPO plays a key role in ETosis-mediated inflammation and NET formation (Metzler et al., 2011; Stoiber et al., 2015). Moreover, MPO and NE are reported to migrate to the PMN nucleus during NETosis thereby enhancing chromatin decondensation (Papayannopoulos et al., 2010).

Furthermore, we here deliver the first report on pentraxin being involved in apicomplexan-triggered NET formation. Pentraxin is a pivotal component of host innate immune responses, stored in PMN granules and, in common with MPO and proteinase 3, expressed on the apoptotic neutrophil surface (Bottazzi et al., 2009). In NET formation, pentraxin may participate in microbial recognition thereby facilitating the trapping of pathogens as demonstrated for bacteria (Bottazzi et al., 2009). Interestingly, proteomic analyses revealed that pentraxin forms a complex with other NET components in human PMN and appears to boost the actions of the different typical NETs molecules (Daigo and Hamakubo, 2012).

Besides, we here reported for first time on the presence of cathepsin B, a relevant antimicrobial PMN serine protease, in N. caninum-triggered caprine NETs. Cathepsin B and NE were reported to be implicated in protective immune responses against some bacterial infections (Hahn et al., 2011; Walter et al., 2015). In addition, they promote the recruitment of PMN and participate in other cell processes such as embryogenesis, angiogenesis,

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hematopoiesis, tumor growth and metastasis (Vandercappellen et al., 2008; Fortelny et al., 2014; Repnik et al., 2015). Whether cathepsin B actively contributes to tachyzoite damage or killing needs further investigation.

Mammalian-derived NETosis is mainly dependent on NOX activities which result in intracellular ROS production (for review see Brinkmann and Zychlinsky, 2012). However, recent investigations led to the differentiation of two distinct forms of NETosis: i) NOX-dependent NETosis, being associated mainly with cytosolic ROS production (Fuchs et al., 2007; Remijsen et al., 2011), and ii) NOX-independent NETosis showing mitochondrial ROS production via calcium-activated potassium channels (SK channels) (Fay et al., 2006; Douda et al., 2015; Khan and Palaniyar, 2017). Accordingly, Ca^{pp} influx was reported as crucial for PMN activation and NETosis (Conejeros et al., 2011; Burgos et al., 2011; Muñoz-Caro et al., 2015b). In the current study neither NOX- nor SOCE-mediated reactions seemed to be crucial for N. caninum-triggered caprine NETosis since neither DPI nor 2-APB treatments influenced NET formation. This observation clearly differs to findings on closely related parasites, such as E. bovis- (Behrendt et al., 2010; Muñoz-Caro et al., 2015a), E. arloingi (Silva et al., 2014) T. gondii (Reichel et al., 2015), B. besnoiti (Muñoz-Caro et al., 2014) and C. parvum (Muñoz-Caro et al., 2015b). This is also in contrast to the canine system, where 2-APB and DPI significantly inhibited N. caninum-triggered NETosis (Wei et al., 2016).

The importance of Raf-MEK-ERK signaling pathways during NETosis was firstly reported by Hakkim et al. (2011). Whilst in the current study no influence was detected on N. caninum-triggered NETosis by ERK1/2- and p38 MAPK blockage, several other reports indicated a pivotal role of this signaling cascade in the case of T. gondii- (Abi Abdallah et al., 2012), E. bovis- (Muñoz-Caro et al., 2015a) and C. parvum-induced NETosis (Muñoz-Caro et al., 2015b). However, in both, the canine (Wei et al., 2016) and the bovine system (Villagra-Blanco et al., submitted manuscript), blockage of ERK1/2 and p38 MAPK resulted in a significant reduction of N. caninum tachyzoite-triggered NET formation. So far, we do not know whether these differences are specific for the goat and rely on differentially activated signal cascades in the caprine system.

In addition to SOCE, we also investigated the role of PI3K-mediated autophagy in N. caninum-induced NETosis. As previously shown, PI3K/AKT kinase is essential for the activation of both NOX-dependent and NOX-independent NETosis (Douda et al., 2015). However, the current data did not reveal any influence or reaction associated with PI3K-mediated autophagy in N. caninum-induced caprine NETosis. It has to be considered that LY294002 was identified as a rather unspecific inhibitor since it reduces not only the activity of mTOR (mammalian target of rapamycin) and DNA-PK (DNA-dependent protein kinase) (Brunn et al., 1996), but also of other protein kinases, such as CK2 (casein kinase 2) and Pim-1 (proto-oncogen serin kinase 1) (Davies et al., 2000; Jacobs et al., 2005). Thus, the direct inhibitory effects on the autophagy pathway may have been too moderate to directly influence parasite-triggered NETosis. Consequently, a more detailed analysis on autophagy-NETosis-interactions after tachyzoite confrontation is needed.

Overall, the results of this study demonstrate that N. caninum-tachyzoites are competent inducers of NET formation in the caprine system, which is consistent to recent findings in dogs (Wei et al., 2016). Considering the life cycle of N. caninum, which include endogenous obligate intracellular parasite stages, the extracellular immobilization of tachyzoites via NETs might have a significant implication on the outcome of the disease as previously postulated for other coccidian parasites (Muñoz-Caro et al., 2014; Silva et al., 2014, 2016; Hermosilla et al., 2014).

Declaration

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests in the present study.

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References

Abi Abdallah, D.S., Lin, C., Ball, C.J., King, M.R., Duhamel, G.E., Denkers, E.Y., 2012. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect. Immun. 80, 768e777.

Alghamdi, A.S., Foster, D.N., 2005. Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. Biol. Reprod. 73, 1174e1181.

Altincicek, B., Stotzel, S., Wygrecka, M., Preissner, K.T., Vilcinskas, A., 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J. Immunol. 181, 2705e2712.

Anderson, M.L., Andrianarivo, A.G., Conrad, P.A., 2000. Neosporosis in cattle. Anim. Reprod. Sci. 60e61, 417e431.

Arranz-Solís, D., Benavides, J., Regidor-Cerrillo, J., Horcajo, P., Castaño, P., Del Carmen-Ferreras, M., Jiménez-Pelayo, L., Collantes-Fernández, E., Ferre, I., Hemphill, A., Pérez, V., Ortega-Mora, L.M., 2016. Systemic and local immune responses in sheep after Neospora caninum experimental infection at early, mid and late gestation. Vet. Res. 47, 2.

Aulik, N.A., Hellenbrand, K.M., Klos, H., Czuprynski, C.J., 2010. Mannheimia haemolytica and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect. Immun. 78, 4454e4466.

Ávila, E.E., Salaiza, N., Pulido, J., Rodríguez, M.C., Díaz-Godínez, C., Laclette, J.P., Becker, I., Carrero, J.C., 2016. Entamoeba histolytica trophozoites and lipopeptidophosphoglycan trigger human neutrophil extracellular traps. PLoS ONE 11 (7), e0158979.

Baker, V.S., Imade, G.E., Molta, N.B., Tawde, P., Pam, S.D., Obadofin, M.O., Sagay, S.A., Egah, D.Z., Iya, D., Afolabi, B.B., Baker, M., Ford, K., Ford, R., Roux, K.H., Keller 3rd, T.C., 2008. Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malar. J. 7, 41.

Barr, B.C., Anderson, M.L., Woods, L.W., Dubey, J.P., Conrad, P.A., 1992. Neospora-like protozoal infections associated with abortion in goats. J. Vet. Diagn Invest. 4, 365e367.

Behrendt, J.H., Ruiz, A., Zahner, H., Taubert, A., Hermosilla, C., 2010. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite, Eimeria Bovis. Vet. Immunol. Immunopathol. 133, 168.

Bottazzi, B., Garlanda, C., Cotena, A., Moalli, F., Jaillon, S., Deban, L., Montavani, A., 2009. The long pentraxin PTX3 as a prototypic humoral pattern recognition receptor: interplay with cellular innate immunity. Immunol. Rev. 227, 9e18.

Boysen, P., Klevar, S., Olsen, I., Storset, A.K., 2006. The protozoan Neospora caninum directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. Infect. Immun. 74, 953e960.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. Science 303, 1532e1535.

Brinkmann, V., Laube, B., Abu Abed, U., Goosmann, C., Zychlinsky, A., 2010. Neutrophil extracellular traps: how to generate and visualize them. J. Vis. Exp. 36, e1724.

Brinkmann, V., Zychlinsky, A., 2012. Neutrophil extracellular traps: is immunity the

Molecular analyses on *Neospora caninum* triggered NETosis in the caprine system

second function of chromatin? J. Cell Biol. 198, 773e783.
Brunn, G.J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence Jr., J.C., Abraham, R.T., 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J 15, 5256e5267.
Burgos, R.A., Conejeros, I., Hidalgo, M.A., Werling, D., Hermosilla, C., 2011. Calcium influx, a new potential therapeutic target in the control of neutrophil-dependent inflammatory diseases in bovines. Vet. Immunol. Immunopathol. 143, 1e10.
Buxton, D., 1998. Protozoan infections (Toxoplasma gondii Neospora caninum and Sarcocystis spp.) in sheep and goats: recent advances. Vet. Res. 29, 289e310.
Cheng, O.Z., Palaniyar, N., 2013. NET balancing: a problem in inflammatory lung diseases. Front. Immunol. 4, 1.
Chuammitri, P., Ostojic, J., Andreasen, C.B., Redmond, S.B., Lamont, S.J., Palic, D., 2009. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. Vet. Immunol. Immunopathol. 129, 126e131.
Conejeros, I., Patterson, R., Burgos, R.A., Hermosilla, C., Werling, D., 2011. Induction of reactive oxygen species in bovine neutrophils is CD11b, but not dectin-1-dependent. Vet. Immunol. Immunopathol. 139, 308e312.
Conejeros, I., Jara, E., Carretta, M.D., Alarcón, P., Hidalgo, M.A., Burgos, R.A., 2012. 2-Aminoethoxydiphenyl borate (2-APB) reduces respiratory burst, MMP-9 release and CD11b expression, and increases l-selectin shedding in bovine neutrophils. Res. Vet. Sci. 92, 103e110.
Corbellini, L.G., Colodel, E.M., Driemeier, D., 2001. Granulomatous encephalitis in a serologically impaired goat kid associated with degeneration of Neospora caninum tissue cysts. J. Vet. Diagn Invest. 13, 416e419.
Costa, R.C., Orlando, D.R., Abreu, C.C., Nakagaki, K.Y., Mesquita, L.P., Nascimento, L.C., Silva, A.C., Maiorka, P.C., Peconick, A.P., Raymundo, D.L., Varaschin, M.S., 2014. Histological and immunohistochemical characterization of the inflammatory and glial cells in the central nervous system of goat fetuses and adult male goats naturally infected with. Neospora caninum. BMC Veterinary Res. 10, 291.
Daigo, K., Hamakubo, T., 2012. Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. Front. Immun. 3, 378. http://dx.doi.org/10.3389/fimmu.2012.00378.
Davies, S.P., Reddy, H., Caivano, M., Cohen, P., 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. 351, 95e105.
Douda, D.N., Khan, M.A., Grasemann, H., Palaniyar, N., 2015. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. Proc. Natl. Acad. Sci. U. S. A. 112, 2817e2822.
Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal Neospora caninum infection in dogs: isolation of the causative agent and experimental transmission. J. Amer Vet. Med. Assn 193, 1259e1263.
Dubey, J.P., Lindsay, D.S., 1996. A review of Neospora caninum and neosporosis. Vet. Parasitol. 67, 1e59.
Dubey, J.P., Morales, J.A., Villalobos, P., Lindsay, D.S., Blagburn, B.L., Topper, M.L., 1996. Neosporosis-associated abortion in a dairy goat. J. Am. Vet. Med. Assoc. 208, 263e265.
Dubey, J.P., 2003. Review of Neospora caninum and neosporosis in animals. Korean J. Parasitol. 41, 1e16.
Dubey, J.P., Schares, G., 2011. Neosporosis in animals-The last five years. Vet. Parasitol. 180, 90e108.
Eleni, C., Crotti, S., Manuali, E., Costarelli, S., Filippini, G., Moscati, L., Magnito, S., 2004. Detection of Neospora caninum in an aborted goat fetus. Vet. Parasitol. 123, 271e274.
Ermert, D., Urban, C.F., Laube, B., Goosmann, C., Zychlinsky, A., Brinkmann, V., 2009. Mouse neutrophil extracellular traps in microbial infections. J. Innate Immun. 1, 181e193.
Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A., Trzaskos, J.M., 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. 273, 18623e18632.
Fay, A.J., Qian, X., Jan, Y.N., Jan, L.Y., 2006. SK channels mediate NADPH oxidase independent reactive oxygen species production and apoptosis in granulocytes. Proc. Natl. Acad. Sci. U. S. A. 103, 17548e17553.
Fortelny, N., Cox, J.H., Kappelhoff, R., Starr, A.E., Lange, P.F., Pavlidis, P., Overall, C.M., 2014. Network analyses reveal pervasive functional regulation between proteases in the human protease web. PLoS Biol. 12, e1001869.
Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., Zychlinsky, A., 2007. Novel cell death program leads to neutrophil extracellular traps. J. Cell Biol. 176, 231e241.
Gazzinelli, R.T., Talvani, A., Camargo, M.M., Santiago, H.C., Oliveira, M.A., Vieira, L.Q., Martins, G.A., Aliberti, J.C., Silva, J.S., 1998. Induction of cell-mediated immunity during early stages of infection with intracellular protozoa. Braz J. Med. Biol. Res. 31, 89e104.
Grinberg, N., Elazar, S., Rosenshine, I., Shpigel, N.Y., 2008. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic. Escherichia Coli. Infect. Immun. 76, 2802e2807.
Guimarães-Costa, A.B., Nascimento, M.T., Froment, G.S., Soares, R.P., Morgado, F.N., Conceição-Silva, F., Saraiva, E.M., 2009. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc. Natl. Acad. Sci. U. S. A. 106, 6748e6753.
Gupta, A.K., Hasler, P., Holzgreve, W., Gebhardt, S., Hahn, S., 2005. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum. Immunol. 66, 1146e1154.
Hahn, I., Klaus, A., Janze, A.K., Steinwede, K., Ding, N., Bohling, J., Brumshagen, C., Serrano, H., Gauthier, F., Paton, J.C., Welter, T., Maus, U.A., 2011. Cathepsin G and neutrophil elastase play critical and non-redundant roles in lung-protective immunity against Streptococcus pneumoniae in mice. Infect. Immun. 79, 4893e4901.
Hahn, S., Giaglis, S., Chowdhury, C.S., Hösli, I., Hasler, P., 2013. Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin. Immunopathol. 35, 439e453.
Hakkim, A., Fuchs, T.A., Martinez, N.E., Hess, S., Prinz, H., 2011. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat. Chem. Biol. 7, 75e77.
Hemphill, A., Gottstein, B., 2000. A European perspective on Neospora caninum. Int. J. Parasitol. 30 (8), 877e924.
Hermosilla, C., Ruiz, A., Taubert, A., 2012. Eimeria bovis: an update on parasite-host cell interactions. Int. J. Med. Microbiol. 302, 210e215.
Hermosilla, C., Muñoz Caro, T., Silva, L.M.R., Ruiz, A., Taubert, A., 2014. The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology 1, 10. http://dx.doi.org/10.1017/S0031182014000316.
Hurtado, A., Aduriz, G., Moreno, B., Barandita, J., García-Pérez, A.L., 2001. Single tube nested PCR for the detection of Toxoplasma gondii in fetal tissues from naturally aborted ewes. Vet. Parasitol. 102, 17e27.
Jacobs, M.D., Black, J., Futer, O., Swenson, L., Hare, B., Fleming, M., Saxena, K., 2005. PIM-1 ligand-bound structures reveal the mechanism of serine/threonine kinase inhibition by LY294002. J. Biol. Chem. 280, 13728e13734.
Jeffery, U., Kimura, K., Gray, R., Lueth, P., Bellaire, B., LeVine, D., 2015. Dogs cast NETs too: canine neutrophil extracellular traps in health and immune-mediated hemolytic anemia. Vet. Immunol. Immunopathol. 168 (3e4), 262e268.
Jenne, C.N., Wong, C.H., Zemp, F.J., McDonald, B., Rahman, M.M., Forsyth, P.A., McFadden, G., Kubes, P., 2013. Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe 13, 169e180.
Khan, M.A., Palaniyar, N., 2017. Transcriptional firing helps to drive NETosis. Sci. Rep. 7, 41749.
Koyama, T., Kobayashi, Y., Omata, Y., Yamada, M., Furuoka, H., Maeda, R., Matsui, T., Saito, A., Mikami, T., 2001. Isolation of Neospora caninum from the brain of a pregnant sheep. J. Parasitol. 87, 1486e1488.
Lindsay, D.S., Rippey, N.S., Powe, T.A., Sartin, E.A., Dubey, J.P., Blagburn, B.L., 1995. Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of Neospora caninum. Am. J. Vet. Res. 56, 1176e1780.
Maksimov, P., Hermosilla, C., Kleinertz, S., Hirzmann, J., Taubert, A., 2016. Besnoitia besnoiti infections activate primary bovine endothelial cells and promote PMN adhesion and NET formation under physiological flow condition. Parasitol. Res. 115, 1991e2001.
Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P.A., Baumann, C., Fey, M.F., Dummer, R., Simon, H.U., Yousefi, S., 2004. Induction of genes mediating interferon dependent extracellular trap formation during neutrophil differentiation. J. Biol. Chem. 279, 44123e44132. http://dx.doi.org/10.1074/jbc.M405883200.
Mesquita, L.P., Nogueira, C.I., Costa, R.C., Orlando, D.R., Bruhn, F.R.P., Lopes, P.F.R., Nakagaki, K.Y.R., Peconick, A.P., Seixas, J.N., Bezerra Jr., O.S., Raymundo, D.L., Varaschin, M.S., 2013. Antibody kinetics in goats and conceptuses naturally infected with Neospora caninum. Vet. Parasitol. 196, 327e333.
Metzler, K.D., Fuchs, T.A., Nauseef, W.M., Reumaux, D., Roesler, J., Schulze, I., Wahn, V., Papayannopoulos, V., Zychlinsky, A., 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood 117, 953e959.
Moore, D.P., Odeón, A.C., Venturini, M.C., Campero, C.M., 2005. Bovine neosporosis: general concepts, immunity and perspectives for vaccination. Rev. Argent. Microbiol. 37, 217e228.
Moreno, B., Collantes-Fernández, E., Villa, A., Navarro, A., Regidor-Cerrillo, J., Ortega-Mora, L.M., 2012. Occurrence of Neospora caninum and Toxoplasma gondii infections in ovine and caprine abortions. Vet. Parasitol. 187, 312e318.
Muñoz-Caro, T., Hermosilla, C., Silva, L.M.R., Cortes, H., Taubert, A., 2014. Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite Besnoitia besnoiti. PLoS ONE 9 (3), e91415.
Muñoz-Caro, T., Mena Huertas, J.S., Conejeros, I., Alarcon, P., Hidalgo, M.A., Burgos, R.A., Taubert, A., Hermosilla, C., 2015a. CD11b-, ERK/MAP kinase- and SOCE-dependent Eimeria bovis-triggered neutrophil extracellular trap formation. Vet. Res. 46, 23. http://dx.doi.org/10.1186/s13567-015-0155-6.
Muñoz-Caro, T., Lendner, M., Daugschies, A., Hermosilla, C., Taubert, A., 2015b. NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca^{2p} influx are essential for Cryptosporidium parvum-induced NET formation. Dev. Comp. Immunol. 52, 245e254.
Muñoz-Caro, T., Silva, M.R.L., Rentería-Solís, Z., Taubert, A., Hermosilla, C., 2016. Neutrophil extracellular traps in the intestinal mucosa of Eimeria-infected animals. Asian Pac J. Trop. Biomed. 6, 301e307.
Nathan, C., 2006. Neutrophils and immunity: challenges and opportunities. Nat. Rev. Immunol. 6, 173e182.
Ng, T.H., Chang, S.H., Wu, M.H., Wang, H.C., 2013. Shrimp hemocytes release extracellular traps that kill bacteria. Dev. Comp. Immunol. 41, 644e651.
O'Donnell, B.V., Tew, D.G., Jones, O.T., England, P.J., 1993. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem. J. 290, 41e49.

Molecular analyses on *Neospora caninum* triggered NETosis in the caprine system

Omata, Y., Umeshita, Y., Watarai, M., Tachibana, M., Sasaki, M., Murata, K., Yamada, T.K., 2006. Investigation for presence of Neospora caninum, Toxoplasma gondii and Brucella-species infection in killer whales (Orcinus orca) mass-stranded on the coast of Shiretoko, Hokkaido, Jpn. J. Vet. Med. Sci. 68, 523e526.
Palić, D., Ostojic, J., Andreasen, C.B., Roth, J.A., 2007. Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev. Comp. Immunol. 31, 805e816.
Papayannopoulos, V., Metzler, K.D., Hakkim, A., Zychlinsky, A., 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J. Cell Biol. 191, 677e691. http://dx.doi.org/10.1083/jcb.201006052.
Parker, H., Albrett, A.M., Kettle, A.J., Winterbourn, C.C., 2012. Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. J. Leukoc. Biol. 91, 369e376.
Patat, S.A., Carnegie, R.B., Kingsbury, C., Gross, P.S., Chapman, R., Schey, K.L., 2004. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. Eur. J. Biochem. 271, 4825e4833. http://dx.doi.org/10.1111/j.1432-1033.2004.04448.x.
Pijanowski, L., Golbach, L., Kolaczkowska, E., Scheer, M., Verburg-van Kemenade, B.M., Chadzinska, M., 2013. Carp neutrophilic granulocytes form extracellular traps via ROS-dependent and independent pathways. Fish. Shellfish Immunol. 34, 1244e1252. http://dx.doi.org/10.1016/j.fsi.2013.02.010.
Pisanu, S., Cubeddu, T., Pagnozzi, D., Rocca, S., Cacciotto, C., Alberti, A., Marogna, G., Uzzau, S., Addis, M.F., 2015. Neutrophil extracellular traps in sheep mastitis. Vet. Res. 46, 59.
Pérez, D., Muñoz, M.C., Molina, J.M., Muñoz-Caro, T., Silva, L.M.R., Taubert, A., Hermosilla, C., Ruiz, A., 2016. Eimeria ninakohlyakimovae induces NADPH oxidase-dependent monocyte extracellular trap formation and upregulates IL-12 and TNF-α, IL-6 and CCL2 gene transcription. Vet. Parasitol. 227, 143e150.
Porto, W.J., Regidor-Cerrillo, J., de Cássia Peixoto Kim, P., Benavides, J., Dos Santos Silva, A.C., Horcajo, P., da Fonseca-Oliveira, A.A., Ferre, I., Mota, R.A., Ortega-Mora, L.M., 2016. Experimental caprine neosporosis: the influence of gestational stage on the outcome of infection. Vet. Res. 47, 29.
Reichel, M., Muñoz-Caro, T., Sánchez-Contreras, G., Rubio-García, A., Magdowski, G., Gärtner, U., Hermosilla, C., Taubert, A., 2015. Harbour seal (Phoca vitulina) PMN and monocytes release extracellular traps to capture the apicomplexan parasite Toxoplasma gondii. Dev. Comp. Immunol. 50, 106e115.
Remijsen, Q., Berghe, T.V., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., Noppen, S., Delforge, M., Willems, J., Vandenabeele, P., 2011. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell Res. 21, 290e304.
Repnik, U., Starr, A.E., Overall, C.M., Turk, B., 2015. Cysteine cathepsins activate ELR chemokines and inactivate non-ELR chemokines. J. Biol. Chem. 290, 13800e13811.
Ruiz, A., Matos, L., Muñoz, M.C., Hermosilla, C., Molina, J.M., Andrada, M., Rodríguez, F., Pérez, D., López, A., Guedes, A., Taubert, A., 2013. Isolation of an Eimeria ninakohlyakimovae field strain (Canary Islands) and analysis of its infection characteristics in goat kids. Res. Vet. Sci. 94, 277e284.
Ruiz, A., Pérez, D., Muñoz, M.C., Molina, J.M., Taubert, A., Jacobs-Lorena, M., Vega-Rodríguez, J., López, A.M., Hermosilla, C., 2015. Targeting essential Eimeria ninakohlyakimovae sporozoite ligands for caprine host endothelial cell invasion with a phage display peptide library. Parasitol. Res. 114, 4327.
Scapinello, S., Brooks, A.S., MacInnes, J.I., Hammermueller, J., Clark, M.E., Caswell, J.L., 2011. Bactericidal activity of porcine neutrophil secretions. Vet. Immunol. Immunopathol. 139, 113e118.
Schares, G., Heydorn, A.O., Cüppers, A., Conraths, F.J., Mehlhorn, H., 2001. Cyclic transmission of Neospora caninum: serological findings in dogs shedding oocysts. Parasitol. Res. 87, 873e877.
Silva, L.M.R., Muñoz-Caro, T., Rüdiger, G., Vila-Viçosa, M.J.M., Cortes, H.C.E., Hermosilla, C., Taubert, A., 2014. The apicomplexan parasite Eimeria arloingi induces caprine neutrophil extracellular traps. Parasitol. Res. 113, 2797e2807.
Silva, L.M.R., Muñoz-Caro, T., Burgos, R.A., Hidalgo, M.A., Taubert, A., Hermosilla, C., 2016. Far beyond phagocytosis: phagocyte-derived extracellular traps act efficiently against protozoan parasites in vitro and in vivo. Mediat. Inflamm. 2016, 5898074.
Stoiber, W., Obermayer, A., Steinbacher, P., Krautgartner, W.D., 2015. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. Biomolecules 5, 702e723.
Taubert, A., Krüll, M., Zahner, H., Hermosilla, C., 2006. Toxoplasma gondii and Neospora caninum infection of bovine endothelial cells induce endothelial adhesion molecule gene transcription and subsequent PMN adhesion. Vet. Immunol. Immunopathol. 112, 214e222.
Taubert, A., Wimmers, K., Ponksuksili, S., Jimenez, C.A., Zahner, H., Hermosilla, C., 2010. Microarray-based transcriptional profiling of Eimeria bovis-infected bovine endothelial host cells. Vet. Res. 41, 70.
Tawk, L., Dubremetz, J.F., Montcourrier, P., Chicanne, G., Merezegue, F., Richard, V., Payrastre, B., Meissner, M., Vial, H.J., Roy, C., Wengelnik, K., Lebrun, M., 2011. Phosphatidylinositol 3-monophosphate is involved in Toxoplasma apicoplast biogenesis. PLoS Pathog. 7 (2), e1001286 doi: 10.1371.
Urban, C.F., Reichard, U., Brinkmann, V., Zychlinsky, A., 2006. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol. 8, 668e676.
Vandercappellen, J., Van Damme, J., Struyf, S., 2008. The role of CXC chemokines and their receptors in cancer. Cancer 267, 226e244.
Ventura-Juárez, J., Campos-Esparza, M., Pacheco-Yepez, J., López-Blanco, J.A., Adabache-Ortiz, A., Silva-Briano, M., Campos-Rodríguez, R., 2016. Entamoeba histolytica induces human neutrophils to form NETs. Parasite Immunol. 38, 503e509.
Von Köckritz-Blickwede, M., Nizet, V., 2009. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. J. Mol. Med. 87, 775e783.
Von Köckritz-Blickwede, M., Chow, O.A., Nizet, V., 2009. Fetal calf serum contains heat-stable nucleases that degrade neutrophil extracellular traps. Blood 114, 5245e5246. http://doi.org/10.1182/blood-2009-08-240713.
Von Köckritz-Blickwede, M., Chow, O., Ghochani, M., Nizet, V., 2010. Visualization and functional evaluation of phagocyte extracellular traps. Methods Microbiol. 37, 139e160.
Wang, Y., Chen, Y., Xin, L., Beverley, S.M., Carlsen, E.D., Popov, V., Chang, K.P., Wang, M., Soong, L., 2011. Differential microbicidal effects of human histone proteins H2A and H2B on Leishmania promastigotes and amastigotes. Infect. Immun. 79, 1124e1133.
Walter, K., Steinwede, K., Aly, S., Reinheckel, T., Bohling, J., Maus, U.A., Ehlers, S., 2015. Cathepsin g in experimental tuberculosis: relevance for antibacterial protection and potential for immunotherapy. J. Immunol. 195, 3325e3333.
Wei, Z., Hermosilla, C., Taubert, A., He, X., Wang, X., Gong, P., Li, J., Yang, Z., Zhang, X., 2016. Canine neutrophil extracellular traps release induced by the apicomplexan parasite Neospora caninum in vitro. Front. Immunol. 7, 436.

3. BOVINE POLYMORPHONUCLEAR NEUTROPHILS CAST NEUTROPHIL EXTRACELLULAR TRAPS AGAINST THE ABORTIVE PARASITE *NEOSPORA CANINUM*

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Bovine Polymorphonuclear Neutrophils Cast Neutrophil Extracellular Traps against the Abortive Parasite *Neospora caninum*

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Neospora caninum represents a relevant apicomplexan parasite causing severe reproductive disorders in cattle worldwide. Neutrophil extracellular trap (NET) generation was recently described as an efficient defense mechanism of polymorphonuclear neutrophils (PMN) acting against different parasites. *In vitro* interactions of bovine PMN with *N. caninum* were analyzed at different ratios and time spans. Extracellular DNA staining was used to illustrate the typical molecules of NETs [i.e., histones (H3), neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin] *via* antibody-based immunofluorescence analyses. Functional inhibitor treatments were applied to reveal the role of several enzymes [NADPH oxidase (NOX), NE, MPO, PAD4], ATP-dependent P2Y2 receptor, store-operated Ca^{++}entry (SOCE), CD11b receptor, ERK1/2- and p38 MAPK-mediated signaling pathway in tachyzoite-triggered NETosis. *N. caninum* tachyzoites triggered NETosis in a time- and dose-dependent manner. Scanning electron microscopy analyses revealed NET structures being released by bovine PMN and entrapping tachyzoites. *N. caninum*-induced NET formation was found not to be NOX-, NE-, MPO-, PAD4-, ERK1/2-, and p38 MAP kinase-dependent process since inhibition of these enzymes led to a slight decrease of NET formation. CD11b was also identified as a neutrophil receptor being involved in NETosis. Furthermore, *N. caninum*-triggered NETosis depends on Ca^{++} influx as well as neutrophil metabolism since both the inhibition of SOCE and of P2Y2-mediated ATP uptake diminished NET formation. Host cell invasion assays indicated that PMN-derived NETosis hampered tachyzoites from active host cell invasion, thereby inhibiting further intracellular replication. NET formation represents an early and effective mechanism of response of the innate immune system, which might reduce initial infection rates during the acute phase of cattle neosporosis.

Keywords: neutrophil extracellular trap, nicotinamide adenine dinucleotide phosphate-oxidase, protein arginine deiminase 4, g protein-coupled receptor 2, *Neospora caninum*

INTRODUCTION

Neospora caninum is an apicomplexan obligate intracellular parasite with comparable characteristics in structure and development to *Toxoplasma gondii* (1). It has a wide intermediate host range and is responsible for reproductive disorders mainly in cattle but is also associated with clinical reproductive and neural infections in dogs, horses, goats, sheep, and deer (2–4). In general, infections of

Bovine polymorphonuclear neutrophils cast neutrophil extracellular traps against the abortive parasite *Neospora caninum*

apicomplexan parasites, such as *N. caninum*, underlie a complex adaptive immunological regulation (5–8); however, little is known on early host innate immune reactions occurring during primary *N. caninum* infection, despite the fact that early innate host defense reactions should be critical for the actual outcome of infection (7–13). In particular, polymorphonuclear neutrophils (PMN) play a key role in this respect since they are the most abundant innate immune cells in the blood and the first ones to be recruited to the site of infection (14–16). PMN own several effector mechanisms to combat and kill pathogens, such as phagocytosis, production of oxygen-based radicals known as reactive oxygen species (ROS), the excretion of antimicrobial peptides/proteins, and the synthesis of neutrophil extracellular traps (NETs) (17).

NETs are generally released *via* a novel PMN cell death process known as NETosis (17, 18). NETosis is known as a NADPH oxidase (NOX)-dependent mechanism (10, 12, 13, 17, 19), which leads to the extrusion of nuclear and cytoplasmic granule enzymes leading to the formation of DNA-rich networks adorned with different histones (H1, H2A/H2B, H3, H4) and antimicrobial granular effector molecules, such as neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin, lactoferrin, cathepsins, gelatinase, bacterial permeability-increasing protein, peptidoglycan recognition proteins, calprotectin, and other leukocyte proteins (10, 16, 17, 20, 21). Classical NET formation [for review of pathways, see Ref. (17, 22, 23)] was initially proven to be signaled *via* the Raf–MEK–ERK-dependent pathways (24). In contrast to NOX-dependent NETosis, the recently described NOX-independent NETosis is associated with substantial reduced levels of ERK1/2 activation and weak Akt activation, whereas the activation of p38 MAPK is similar in both pathways (25). Irrespective of NOX-dependency, invasive pathogens may either be immobilized within NET-derived sticky DNA fibers or be killed *via* the locally high concentration of antimicrobial histones, peptides, and proteases (14, 21, 26). Moreover, Yipp et al. (27) recently demonstrated that PMN, which undergo NETosis without cell lysis, remain viable and retain their ability to phagocytise bacteria. In agreement with these findings, PMN also seem to be able to release small-sized NETs of mitochondrial origin without suffering cell death (28). By now, NETosis has been described to be triggered by different protozoan parasites *in vitro* and *in vivo*, such as *Plasmodium falciparum* (29), *Leishmania* spp. (30, 31), *Eimeria bovis* (12, 32), *Eimeria arloingi* (33), *T. gondii* (34, 35), *Besnoitia besnoiti* (11), *Cryptosporidium parvum* (13), *Trypanosoma cruzii* (36), and *Entamoeba histolytica* (37). In addition, monocyte-derived extracellular traps have recently been reported in response to tachyzoites of *B. besnoiti* (11) and *T. gondii in vitro* (35). Recent analyses on *Eimeria* spp. and *B. besnoiti*-induced NETosis confirmed their dependency on NOX, NE, MPO, CD11b, ERK1/2, p38 MAPK, and SOCE (12, 13, 32, 33). Moreover, blood vessel analyses of *P. falciparum*-infected patients (29) and intestinal tissue samples of *Eimeria*-infected goats and cattle also proved apicomplexan parasite-triggered NETosis to happen *in vivo* (38).

In contrast to ruminant eimeriosis, nothing is known on NET-based host innate immune reactions against *N. caninum*, although PMN and other leukocytes, such as macrophages and NK cells, seem to play a crucial role in neosporosis *in vivo* (9, 39–41). Thus, the aim of the present study was to analyze the capacity of *N. caninum* tachyzoites to trigger NETs and to unravel effector molecules and pathways being involved in this novel cell death process.

MATERIALS AND METHODS

Ethics statement

This survey was carried out in accordance to the Justus Liebig University Animal Care Committee guidelines. Protocols were approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen) (A9/2012; JLU-No. 521_AZ), in accordance to the prevalent European Animal Welfare Legislation: ART13TFEU and the current applicable German Animal Protection Laws.

Parasites

All NET-related experiments were performed with tachyzoite stages of the apicomplexan parasite *N. caninum* [strain Nc1 (42)], which was cultivated *in vitro* as described elsewhere (7, 11). In brief, *N. caninum* tachyzoites were maintained by serial passages either in primary bovine umbilical vein endothelial cells (BUVEC) or permanent African green monkey kidney epithelial cells (MARC-145). Viable *N. caninum*-tachyzoites were collected from infected host cell layer supernatants, pelleted (400 × g, 12 min), washed thrice in sterile PBS, counted in a Neubauer hemocytometer (Marienfeld-Superior, Germany) and re-suspended in sterile RPMI 1640 medium (Gibco) until further experimental use.

Host cell cultures

MARC-145 cell layers were maintained in cell culture medium DMEM (Sigma-Aldrich) supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich), streptomycin (500 mg/ml; Sigma-Aldrich), and 10% FCS (Gibco) and cultivated at 37°C and 5% CO_2 atmosphere until confluency. Confluent MARC-145 layers were infected with viable *N. caninum* tachyzoites (20 × 10^6 parasites/25 cm^2).

Isolation of primary BUVEC was performed according to the method reported by Taubert et al. (7). In brief, the umbilical cords retrieved from newborn calves were enriched with 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and refrigerated in 0.9% HBSS–HEPES buffer (pH 7.4; Gibco, USA). Endothelial cells were isolated using 0.025% collagenase type II (Worthington Biochemical Corporation, USA), filling the lumen of the ligated umbilical vein and incubating for 20 min at 37°C in 5% CO_2 atmosphere. Then, the umbilical vein was mildly massaged; the collagenase-cell suspension was retrieved and 1 ml FCS (Gibco, USA) was aggregated to inactivate the collagenase type II. After two centrifugations (400 × *g*, 10 min, 4°C), the isolated BUVEC were kept in complete ECGM (endothelial cell growth medium; PromoCell, Heidelberg, Germany), plated in 25 and 75 cm^2 plastic culture flasks (Nunc, Roskilde, Denmark), and incubated at 37°C in 5% CO_2 atmosphere until confluency.

Isolation of Bovine PMN

Healthy adult dairy cows ($n = 3$) were bled by puncture of the jugular vein and 30 ml blood was collected in 50 ml sterile plastic tubes (Greiner), containing 0.1 ml heparin (Sigma-Aldrich) as anticoagulant. Approximately 20 ml heparinized blood was re-suspended in 20 ml PBS with 0.02% EDTA (Sigma-Aldrich), slowly layered on the top of 12 ml Biocoll Separating Solution® (Biochrom AG), and centrifuged (800 × *g*, 45 min). After the extraction of plasma and mononuclear cells, the pellet was washed in 25 ml distilled water and gently shaken during 40 s to lyse erythrocytes. Osmolarity was rapidly normalized using an appropriate volume of Hanks balanced salt solution (4 ml, HBSS 10×, Biochrom AG). To complete the erythrocyte lyses, this step was repeated twice and the PMN were later re-suspended in RPMI medium (Gibco). Calculation and viability of the cells were performed in a Neubauer hemocytometer as described elsewhere (12). Finally, bovine PMN were cultured at 37°C and 5% CO_2 atmosphere for 30 min until further use. As neutrophils have a short lifespan, PMN isolation was performed not exceeding 3 h after blood collection.

Quantification of NETs

Bovine PMN ($n = 3$) were re-suspended in medium RPMI 1640 lacking phenol red and without serum and then confronted in duplicates with vital *N. caninum* tachyzoites (37°C, 4:1 ratio: 1×10^6 *N. caninum* tachyzoites versus 2.5×10^5 bovine PMN/200 µl). For NET blockage, the following inhibitors were used: the NOX-inhibitor DPI [10 µM, Sigma-Aldrich, according to Farley et al. (43)], the leukocyte elastase-inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone [CMK; 1 mM, Sigma-Aldrich, according to Scapinello et al. (44)], the MPO-inhibitor 4-aminobenzoic acid hydrazide [ABAH; 100 µM, Merck, according Parker et al. (45)], the SOCE-inhibitor aminoethoxydiphenyl borate [2-APB; 100 µM, Sigma-Aldrich, according to Conejeros et al. (46)], UO126 as inhibitor of ERK1/2 [50 µM; Sigma-Aldrich, according to Muñoz-Caro et al. (12)], SB 202190 as specific inhibitor of p38 MAPK [10 µM; Sigma-Aldrich, according to Muñoz-Caro et al. (12)], the G-protein-coupled receptor (GPCR) antagonist NF-449 for P2Y2 blockage (GPCR-NF-449, 10 µM; Santa Cruz Biotechnology), and N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-l-Orn amide for PAD4 inhibition (Cl-amidine, 200 µM, Merck). For blocking experiments, PMN were pre-exposed with the corresponding inhibitor in serum-free medium RPMI 1640 without phenol red (RT, GPCR-NF-449, and Cl-amidine: 120 min, all other inhibitors: 30 min) prior to exposure to *N. caninum* tachyzoites. To disrupt NETs and facilitate their DNA quantification, 50 µl of micrococcal nuclease buffer (New England Biolabs) including 0.1 U/µl micrococcal nuclease (New England Biolabs) was supplied to each well and incubated (15 min, 37°C). Next, all the samples were centrifuged (300 × *g*, 5 min). The supernatant of each sample was deposited in duplicate into a 96-well flat-bottom plate (100 µl per well). DNA from NETs was assessed using Pico Green® (Invitrogen), an extracellular DNA-linking fluorescent stain. Fifty microliters of Pico Green® (diluted 1:2,000 in 10 nM Tris buffer with 1 mM EDTA) was added to each well. NET production was quantified according to the fluorescence intensities obtained in the spectrofluorometric analysis (484 nm excitation wavelength and 520 nm emission wavelength) performed by an automated plate monochrome reader (Varioskan Flash®, Thermo Scientific). For negative controls, PMN in normal serum-free medium RPMI 1640 without phenol red were employed. Zymosan (1 mg/ml; Sigma-Aldrich) stimulated PMN served as positive controls according to Muñoz-Caro et al. (12). Diverse PMN-tachyzoites ratios were applied (1:1, 1:2, 1:3, 1:4) for dose-dependency evaluation. For analyses on the role of CD11b in parasite-triggered NETosis, the CD11b receptor was blocked *via* preincubation of bovine PMN in monoclonal mouse anti-bovine CD11b antibodies (MCA1425, diluted 1:5 in PBS; AbDSerotec, 30 min, RT). As antibody control, an irrelevant monoclonal antibody (mouse anti-bovine CD4, AbDSerotec) was used as described elsewhere (12). To resolve NET formation, DNase I (90 U/well, Roche Diagnostics) was supplemented 15 min before the end of incubation period.

Visualization of NETs and detection of histones (H3), NE, MPO, and pentraxin in *N. caninum* tachyzoite-induced NETs

Following PMN: *N. caninum* tachyzoite co-cultivation (ratio 1:4, 120 min, on 15 mm round glass coverslips pretreated with poly-l-lysine), fixation of the samples (4% paraformaldehyde, Merck, 15 min, 37°C), and three washings in PBS, the samples were blocked with BSA (2%, Sigma-Aldrich), incubated in antibody solutions (1 h, RT), and mounted on Prolong Gold® with 4′-6-diamidino-2-phenylindole (DAPI) staining (Invitrogen, 1:1,000, 5 min, RT, in the dark). For the identification of antimicrobial peptides within extracellular DNA structures, the following antibodies were applied: anti-histone (H3) monoclonal [DyLight, ab139848, Abcam (1:1,000)], anti-MPO (Alexa Fluor 488, ABIN906866, https://Antibodies-online.com, 1:1,000), anti-NE (AB68672, Abcam, 1:1,000), and anti-pentraxin (SAB2104614-50UG, Sigma-Aldrich, 1:1,000) antibodies. The immunofluorescence images were taken by a digital camera from an inverted Olympus IX81 fluorescence microscope.

NET-related host cell infection experiments

To analyze the repercussions of parasite-induced NETs on tachyzoite infectivity, three different parallel experimental conditions were chosen: (1) *N. caninum* were cocultured with PMN (1:4 ratio, 2 h, 37°C) allowing for effective NET formation. (2) For infection control, an equal number of non-exposed tachyzoites was incubated in plain medium (2 h, 37°C). (3) The same amount of parasites were incubated with PMN (1:4 ratio, 2 h, 37°C) permitting a competent NET formation and furthermore treated with DNase I (90 U/well, addition of DNase I 15 min before the end of the incubation period) to resolve NET structures and to indirectly measure potential adverse effects of NETs on tachyzoite viability. In a next step, the tachyzoites of setups 1–3 were transferred to confluent BUVEC monolayers

for infection (4 h, 37°C, 5% CO_2 atmosphere). Overall, three different BUVEC isolates were used in this host cell invasion experiment. After incubation, BUVEC layers were washed to remove PMN and free tachyzoites. The infection rates were estimated microscopically (24 h p. i.) in 10 randomly selected vision power fields (400× magnification).

Scanning Electron Microscopy (SEM)

Cattle PMNs were cocultured with viable tachyzoites of *N. caninum* (ratio: 4:1) for 10, 30, 60, and 120 min on 10 mm glass coverslips (Nunc) prepared with poly-l-lysine (Sigma-Aldrich). Then, cells were fixed in 2.5% glutaraldehyde (Merck), post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, dried by CO_2 treatment, and sputtered with gold as described elsewhere (11, 12). SEM samples were analyzed using a Philips XL30 scanning electron microscope (Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany).

Statistical analysis

Statistical analyses were performed by using Graph Pad Prism® 6 software. One- or two-factorial analyses of variance (ANOVA) with repeated measures were applied to compare co-culture/stimulation conditions using a normal distribution of data. Dunnett's multiple comparison tests were performed in dose and kinetic assays as follow-up test to ANOVA. For comparing enzyme activities, Tukey's multiple comparison tests were used. Differences were considered as significant at a level of $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$, and $****p \leq 0.0001$.

RESULTS

N. caninum tachyzoites trigger NET formation

Scanning electron microscopy analyses revealed tachyzoite-triggered generation of a fine network of grosser and slimmer strands of fibers produced by bovine PMN and being solidly adhered to tachyzoites (see **Figure 1**). Kinetic studies reported several stages of NETosis: posterior to 30 min of exposure, smooth PMN-derived filament structures capturing tachyzoites were observed (**Figure 1A**). Here, PMN still presented undamaged cell morphology. Thereafter, parasites were entrapped in an extracellular network of long drawn-out fibers originating from disrupted PMN (**Figures 1B,C**) and conglomerates of *N. caninum* tachyzoites and rather thick and chunky meshworks of PMN-derived filaments (**Figure 1D**, 60 min) were observed.

DAPI-based fluorescence analyses further proved the presence of NET-like structures containing DNA (see **Figure 2**). Furthermore, *N. caninum* tachyzoites were located in close proximity to NETs and presumably were trapped in these extracellular chromatin-rich structures (**Figure 2**). Moreover, co-localization of extracellular chromatin with histones (H3), NE, MPO, and pentraxin in parasite-capturing structures validated the typical nature of NETs (**Figure 2**).

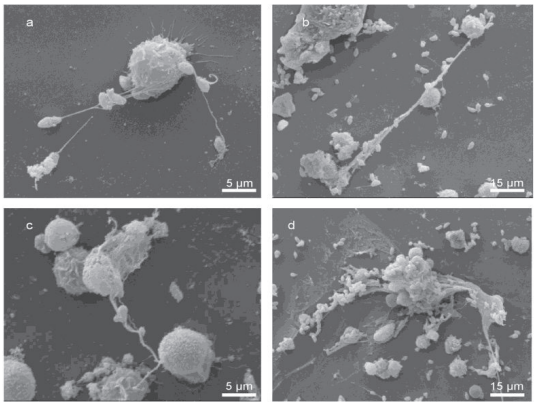

FigUre 1 | Bovine neutrophil extracellular trap (neT) formation in response to *Neospora caninum* tachyzoite exposure. Scanning electron microscopy analyses revealed NETs being formed by bovine polymorphonuclear neutrophils (PMN) after coculture with *N. caninum* tachyzoites for different time spans [**(a–c)** 30 min, **(D)** 60 min]. **(a–c)** Delicate PMN-derived filaroid structures being attached to tachyzoites and **(D)** conglomerates of several tachyzoites and a rather chunky meshwork of PMN-released filaments.

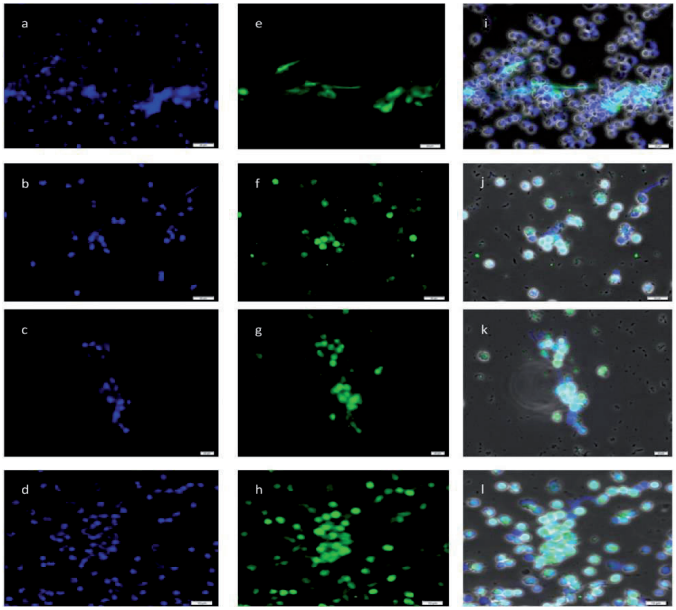

FigUre 2 | co-localization of Dna with histones (h3), neutrophil elastase (ne), myeloperoxidase (MPO), and pentraxin in *Neospora caninum* tachyzoite-induced neutrophil extracellular trap structures. Cocultures of bovine polymorphonuclear neutrophil (PMN) and *N. caninum* tachyzoites (ratio 1:4, 120 min) were fixed, permeabilized, stained for DNA using Prolong Gold® DAPI [blue, **(a–D)**], and probed for histones [green, **(e)**], NE [green, **(F)**], MPO [green, **(g)**], and pentraxin [green, **(h)**] using anti-histone (H3), anti-NE, anti-MPO, and anti-pentraxin antibodies and adequate conjugate systems. Areas of respective co-localization (merges) are illustrated in **(i–l)**.

N. caninum-induced NETosis at different time-and dose-periods

Neutrophil extracellular trap quantification experiments revealed *N. caninum* tachyzoites as strong triggers of NETosis, since these stages induced even stronger reactions than zymosan stimulation of PMN (= positive control, **Figures 3**–**6**). Kinetics on NETosis indicated a significant induction of NETs formation in both incubation time periods (1 and 2 h) compared with the negative control ($p \leq 0.01$, **Figure 3**). As expected, DNase I treatments leading to NET disintegration reduced NETosis under the basal levels of the negative controls (**Figure 3**). Furthermore, increasing amounts of *N. caninum* tachyzoites led to enhanced levels of NET formation as significant differences were observed at a ratio of 1:3 and 1:4 (PMN: tachyzoites) in comparison with the negative controls ($p \leq 0.05$ and $p \leq 0.01$, respectively, **Figure 4**).

N. caninum-induced NETosis is reduced in presence of NOX-, NE-, and MPO inhibitors

To further corroborate the molecular characteristics of *N. caninum*-mediated NETosis, functional blocking experiments with DPI, a potent blocker of NOX, were performed. The PMN treatment with DPI resulted in a clear reduction of tachyzoite-triggered NET formation (**Figure 5A**), despite that this diminution was

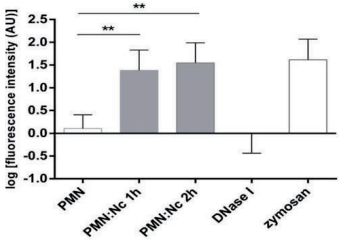

FigUre 3 | Kinetics of *N. caninum* tachyzoites-triggered neutrophil extracellular trap (neT) formation. Bovine polymorphonuclear neutrophils (PMNs) were incubated with tachyzoites (ratio 4:1), zymosan (1 mg/ml, positive control), or plain medium (negative control) for 1 and 2 h. To prove the DNA nature of NETs, the samples were treated with DNase I (15 min). After incubation, all samples were analyzed for extracellular DNA by quantifying Pico Green®-derived fluorescence intensities. Each condition was performed in duplicates. Arithmetic means of three PMN donors, minimum, and maximum. Differences were regarded as significant at a level of $^{**}p \leq 0.01$.

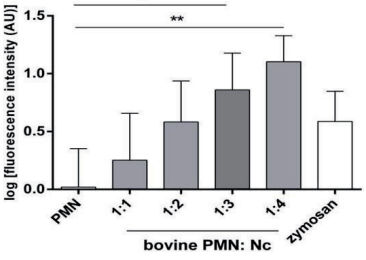

FigUre 4 | Dose assays of *N. caninum* tachyzoites-induced neutrophil extracellular trap formation. Bovine polymorphonuclear neutrophil (PMN) and *N. caninum* tachyzoites were incubated at different ratios (PMN: tachyzoites = 1:1, 1:2, 1:3, 1:4). After incubation (120 min), samples were analyzed for extracellular DNA by quantifying Pico Green®-derived fluorescence intensities. Each condition was performed in duplicates. Arithmetic means of three PMN donors, minimum, and maximum. Differences were regarded as significant at a level of $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

statistically not significant. Additionally, treatments of PMN with MPO and NE inhibitors (ABAH and CMK, respectively) also generate a decrease of tachyzoite-triggered NET formation (**Figure 5A**) underlining the pivotal role of NE and MPO in this process.

ERK1/2-, p38 MAPK-, SOCE-signaling cascades, and bovine CD11b are involved in *N. caninum*-induced NETosis

We here investigated the actual role of NET-associated molecular signaling pathways, receptors, and Ca^{++} influx in *N. caninum*-triggered NETosis. The use of inhibitors affecting ERK1/2-(UO126) and p-38 MAPK-(SB202190) signaling routes in functional NET-derived studies caused a diminishment of tachyzoite-induced NET production (**Figure 5B**), proving a key role of ERK1/2- and p38 MAPK in *N. caninum*-triggered activation of NETosis-related signaling pathways.

Given that NOX-dependent ROS synthesis in bovine neutrophils is being reported as a Ca^{++}-associated process (12, 47, 48), we here furthermore tested whether *N. caninum*-triggered NETosis was influenced by SOCE. Treatments of PMN with 2-ABP produced a decrement of parasite-mediated NETs liberation (**Figure 5B**) proving that intracellular Ca^{++} mobilization is necessary for efficient parasite-induced NETosis.

Until now, no information is available on PMN receptors related to *N. caninum*-triggered NETosis. Therefore, we analyzed whether antibody-mediated blocking of bovine CD11b leads to the diminishment of tachyzoite-triggered NETosis. Indeed, pre-treatment of PMN with anti-CD11b led to a decrease of NET formation, but, however, these reactions were barely no significant.

Inhibition of PAD4 signaling pathway diminished strongly *N. caninum*-triggered NETosis

Since no data are available on the role of histone hypercitrullination in parasite-induced NETosis so far, we here also intended to analyze whether the PMN exposure to Cl-amidine (200 µM), a specific inhibitor of PAD4, might have an impact on PAD4-derived histone hypercitrullination and chromatin decondensation during parasite-triggered NETosis. Cl-amidine pretreatment of bovine PMN resulted in diminished NET production when compared with non-treated but *N. caninum* tachyzoites-exposed PMN (**Figure 5C**). The same experiment was performed with zymosan as positive control (1 mg/ml) and negative controls (PMN cultured in plain medium alone).

Inhibition of the ATP-specific G-Protein receptor P2Y2 reduces *N. caninum*-induced NET Formation

We further intended to determine whether *N. caninum*-triggered NETosis is an energy and ATP-dependent process as seen for other PMN effector mechanisms (49–51). Therefore, the blocker of the ATP-specific G-protein receptor P2Y2 (NF-449) was used here for functional inhibition experiments. In fact, PMN-pretreatment with NF-449 led to a reduction on parasite-triggered NETosis (**Figure 5D**) when compared to non-treated parasite-exposed PMN.

N. caninum-induced NET formation prevents tachyzoites from host cells infection

Host cell penetration is a vital requisite of the parasite *N. caninum* to survive and reproduce successfully within a host. Therefore,

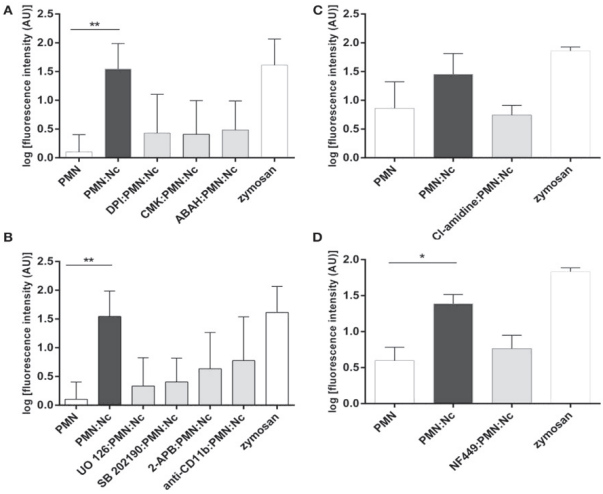

FigUre 5 | Functional neutrophil extracellular trap (neT) inhibition assays. Bovine polymorphonuclear neutrophils (PMN) were treated with the inhibitors of: **(a)** NADPH oxidase (DPI, 20 μM), neutrophil elastase (CMK, 1 mM), myeloperoxidase (ABAH, 100 μM), **(B)** ERK1/2 (UO126, 50 μM), p38 MAPK (SB 203580, 10 μM), SOCE (2-APB, 100 μM), CD11 monoclonal antibodies (diluted 1:5 in PBS), **(c)** PAD4 (Cl-amidine, 200 μM), and **(D)** P2Y2 (NF-449, 120 μM) prior to the exposure to tachyzoites (1:2 ratio). Non-treated PMNs were processed in parallel. Following *N. caninum* tachyzoites exposure, NET formation was determined by quantifying Pico Green®-derived fluorescence intensities (484 nm excitation/520 nm emission wavelengths). Stimulation of PMN with zymosan (1 mg/ml) was used for positive controls, plain medium served as negative control. Each condition was performed in triplicates for each PMN donor ($n = 3$). Differences were regarded as significant at a level of $^*p \leq 0.05$ and $^{**}p \leq 0.01$.

to analyze the effects of NET-mediated parasite entrapment on subsequent tachyzoite host cell infectivity, PMN-pre-exposed tachyzoites were transferred to BUVEC monolayers as suitable specific host cells and infection rates were later calculated. In the same way, an equal amount of tachyzoites, which had not been in contact with PMN before were used to infect BUVEC. The prior confrontation of parasites with PMN and subsequent NET development significantly ($p \leq 0.0001$) prevented *N. caninum* tachyzoites from host cell invasion (**Figure 6**). As such, infection rates decreased from log 60% = 1.778 (resulting from non-exposed tachyzoites = infection controls) to log 20% = 1.3 induced by PMN-pre-exposed tachyzoites. To prove that this impairment was due to NETosis, parallel samples containing the same numbers of tachyzoites and PMN were treated with DNase I treatment (leading to NET disentangle) 165 min after PMN-tachyzoite-exposure (i.e., after a time period, which allowed efficient NET formation) and then used for BUVEC infections. As depicted in **Figure 6**, the infectivity of PMN-pre-exposed *N. caninum* tachyzoite was completely restored by DNase I treatment proving that, first, the ensnarement of tachyzoites within NETs hampered a large proportion of tachyzoites from active host cell invasion, and, second, that NETs had no lethal effects on tachyzoites of *N. caninum* within a period of 3 h.

DISCUSSION

Several protozoan parasites have been identified as potent NET inducers as well (11, 12, 29, 30, 32–35). To the best of our knowledge, we here describe, for the first time, the release of bovine NETs in response to the apicomplexan parasite *N. caninum*, which is known as an important abortive agent affecting not only beef and dairy cattle but also small ruminants worldwide (52–57).

In agreement with observations on other apicomplexan-triggered NETosis (11–13, 29, 32–35, 38, 58), we here report on NETs being attached to tachyzoites of *N. caninum*. The DNA-labeling of *N. caninum*-stimulated NETs confirmed the presence of chromatin structures of these extracellular networks. Moreover, the resolution of these mesh by DNase I treatments corroborated the DNA basis of *N. caninum*-mediated NETosis. NET-associated molecules, such as histones and antimicrobial peptides were

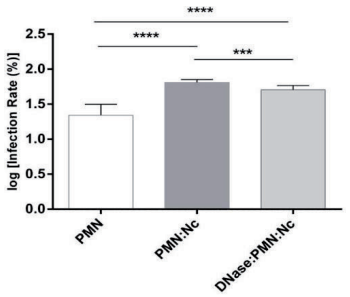

FigUre 6 | invasion assays of *N. caninum* tachyzoites after polymorphonuclear neutrophil (PMn) exposure. *Neospora caninum* tachyzoites were cocultured with bovine PMN for 3 h (=PMN + Nc) allowing for effective neutrophil extracellular trap (NET) formation. In parallel, DNase I was supplemented to the tachyzoite-PMN-suspension 15 min before the end of the incubation period (=PMN + Nc + DNase I) to dissolve potential NET structures. The same number of non-treated tachyzoites in plain medium served as infection controls (=Nc only). Following incubation, the differentially treated tachyzoites were transferred to confluent bovine umbilical vein endothelial cell (BUVEC) monolayers for infection. Twenty-four hours p. i., the BUVEC layers were thoroughly washed and infection rates were estimated. Arithmetic means and SDs of three PMN donors and three BUVEC isolates, minimum, and maximum were calculated. Differences were regarded as significant at a level of ***$p \leq 0.001$ and ****$p \leq 0.0001$.

detected in *N. caninum*-triggered NETs. Consistent to other reports on apicomplexan-induced NETosis (11–13, 33–35, 38, 58), co-localization assays demonstrated the concomitant existence of H3, NE, pentraxin, and MPO in *N. caninum*-caused NETs confirming molecular characteristics of NETs. Consequently, the key action of MPO and NE in *N. caninum*-achieved NETosis was proven through functional inhibition experiments, leading to a reduction of tachyzoite-mediated NETosis in both cases. Furthermore, we here delivered the first report on pentraxin involvement in apicomplexan-triggered NET formation. Pentraxin is a pivotal antimicrobial component of the mammalian host innate immune response, stored in PMN granules and, in common with MPO and proteinase 3, expressed on the apoptotic neutrophil surface while fighting against pathogens (59). During NETosis, pentraxin may participate in microbial recognition, thereby facilitating the trapping of pathogens. Interestingly, proteomic analyses revealed that pentraxin forms a complex with other NET components in human PMN, appearing as a binding molecule that enhances the actions of the different typical NETs molecules (60).

We here demonstrate that NOX participated in *N. caninum* tachyzoite-exposed bovine PMN since DPI treatments resulted in a decrease of parasite-driven NET formation. Similar findings have been reported from *E. bovis*- (12, 32), *T. gondii*- (35), *B. besnoiti*- (11), and *C. parvum*-triggered NETosis (13), emphasizing the importance of NOX-influence in parasite-mediated NETosis (61).

In contrast to *E. bovis*- (32) and *C. parvum*-related NETosis data (13), but according to *B. besnoiti*-induced NETs (11), neither a time- nor a dose-dependency of *N. caninum*-triggered NETosis was demonstrated as significant values were obtained only when each period of incubation (1 and 2 h) and the last two highest infection ratios (1:3 and 1:4) were compared with the negative controls. Furthermore, NET structures were demonstrated being firmly attached to tachyzoites of *N. caninum*, thereby supporting the quantitative data of tachyzoite entrapment showing that parasites were immobilized by extruded NETs. Consequently, *in vitro* host cell invasion experiments involving PMN-pre-exposed *N. caninum* tachyzoites unveiled a significant diminishment of their infectivity (40% reduction) for endothelial host cell. The crucial role of NETosis in this process was proven by the fact that the reduced infectivity could be restored by DNase I treatments. Moreover, this result proved that the tachyzoites were indeed not killed by extruded NETs as also demonstrated for several bacteria (62), protozoan parasites (11–13, 32, 33, 35), as well as metazoan parasites (13, 63, 64).

Taken together, these data confirm the capacity of NETs to hamper *N. caninum* tachyzoites from active host cell invasion *in vitro* by immobilizing them. Taking into account that tachyzoites of *N. caninum* obligatory must infect endothelial host cells *in vivo*, it seems reasonable to speculate that NETosis might represent an efficient defense mechanism during acute cattle neosporosis.

Considering that PMN-derived NOX-activation and subsequent ROS production is known to be Ca^{++}/SOCE-dependent (65), we here employed the SOCE inhibitor 2-APB in NET-related functional studies, as described elsewhere (13, 47, 48). *N. caninum*-triggered NET formation proved to be influenced by SOCE since 2-APB applications limited the tachyzoite-induced NET formation. A Ca^{++} dependency on NET extrusion was also recently published for *E. bovis*- (12) and *C. parvum*-mediated NETosis (13) and for NETs release by human neutrophils in response to other non-parasitic stimulators (66).

The pivotal role of the Raf-MEK-ERK signaling pathways in the process of NETosis was first proven by Hakkim et al. (24). Here, functional inhibition experiments confirmed the importance of ERK1/2- and p38 MAPK-signaling pathways also for *N. caninum*-triggered NET formation. Thus, functional interference of these routes produced a reduction of tachyzoite-mediated NETosis (13). Corresponding findings on ERK1/2 and p38 MAPK have recently been reported on *T. gondii*- (34), *E. bovis*- (12), and *C. parvum*-induced NETosis (13), evidencing a general role of these signaling pathways in apicomplexan-derived NETosis.

Antibody-mediated blockage of CD11b failed to significantly reduce NETosis in the current study, thereby denying an essential role of CD11b in *N. caninum*-triggered NET formation.

PAD4, an enzyme that participates in the citrullination of histones, is known as an essential enzyme of NETosis (67, 68). PAD4-mediated hypercitrullination stimulates decondensation and deployment of chromatin, which allows adequate extrusion of NETs (67, 68). Consistently, functional inhibition experiments using Cl-amidine confirmed the role of PAD4-mediated histone hypercitrunillation for *N. caninum*-triggered NETosis as it resulted in a barely no-significant diminishment of tachyzoite-mediated NETosis.

The stimulation of purinergic receptors (e.g., P2X, P1, P2Y) generally promotes or inhibits cell responses through different signaling events in all kinds of mammalian cells and tissue inflammation (49, 50). Therefore, it requires the local release of extracellular ATP *via* pannexin 1 (PANX1) channels and/or the autocrine feedback regulation of this mechanism involving GPCR, such as P2Y2 (51, 69). These processes may result in the amplification of chemotactic signals through the binding to ATP and triggering PMN polarization/activation (69). In the current work, we, therefore, assayed for the role of P2Y2 in *N. caninum*-induced NETosis. Functional blockage of P2Y2 *via* NF-449 resulted in a decrease of tachyzoite-mediated NETosis, demonstrating for the first time the importance of this energy metabolism-related receptor in parasite-triggered NET formation. Given that P2Y2 also regulates PMN adhesion onto endothelial cells through the binding of ATP and UTP (70, 71) and since NETs were recently found adhered to *B. besnoiti*-infected endothelium (58), further investigation on the interrelationship of P2Y2 and *N. caninum*-induced NETosis will be of interest.

Overall, the current data demonstrated for the first time *N. caninum* tachyzoites as inducers of NET formation in cattle. Considering the life cycle of *N. caninum*, which includes endogenous parasite stages, such as tachyzoites and bradyzoites, exhibiting an obligatory intracellular replication, extracellular immobilization *via* NETosis might have implications in host cell invasion and, therefore, affecting the outcome of acute cattle neosporosis as previously postulated for closely related apicomplexan protozoa (10–12, 33).

CONCLUSION

We identified *N. caninum* tachyzoites as NET inducers in bovine species, involving several molecular mechanisms. These data suggest that NETosis could be an important mechanism during the early host innate immune response against *N. caninum* in cattle.

Availability of Data and Material

All data generated or analyzed during this study are included in this published article.

Ethics statement

This survey was carried out in accordance to the Justus Liebig University Animal Care Committee guidelines. Protocols were approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen) (A9/2012; JLU-No. 521_AZ), in accordance to the prevalent European Animal Welfare Legislation: ART13TFEU and the current applicable German Animal Protection Laws.

Author Contributions

RV-B, LS, TM-C, and ZY performed the NET quantification and inhibition experiments. UG contributed in the performance of scanning electronic microscopy analysis. AT, JL, XZ, and CH cooperated in research design, data analysis, and manuscript's review. All the authors checked and accepted the final manuscript.

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REFERENCES

1. Dubey JP. Review of *Neospora caninum* and neosporosis in animals. *Korean J Parasitol* (2003) 41:1–16. doi:10.3347/kjp.2003.41.1.1
2. Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. *Vet Parasitol* (1996) 67:1–59. doi:10.1016/S0304-4017(96)01035-7
3. Anderson ML, Andrianarivo AG, Conrad PA. Neosporosis in cattle. *Anim Reprod Sci* (2000) 6(0–61):417–31. doi:10.1016/S0378-4320(00)00117-2
4. Schares G, Heydorn AO, Cüppers A, Conraths FJ, Mehlhorn H. Cyclic transmission of *Neospora caninum*: serological findings in dogs shedding oocysts. *Parasitol Res* (2001) 87:873–7. doi:10.1007/s004360100459
5. Gazzinelli RT, Talvani A, Camargo MM, Santiago HC, Oliveira MA, Vieira LQ, et al. Induction of cell-mediated immunity during early stages of infection with intracellular protozoa. *Braz J Med Biol Res* (1998) 31:89–104. doi:10.1590/S0100-879X1998000100012
6. Moore DP, Odeón AC, Venturini MC, Campero CM. Bovine neosporosis: general concepts, immunity and perspectives for vaccination. *Rev Argent Microbiol* (2005) 37:217–28.
7. Taubert A, Krüll M, Zahner H, Hermosilla C. *Toxoplasma gondii* and *Neospora caninum* infection of bovine endothelial cells induce endothelial adhesion molecule gene transcription and subsequent PMN adhesion. *Vet Immunol Immunopathol* (2006) 112:214–22. doi:10.1016/j.vetimm.2006.03.017
8. Taubert A, Wimmers K, Ponksuksili S, Jimenez CA, Zahner H, Hermosilla C. Microarray-based transcriptional profiling of *Eimeria bovis*-infected bovine endothelial host cells. *Vet Res* (2010) 41:70. doi:10.1051/vetres/2010041
9. Boysen P, Klevar S, Olsen I, Storset AK. The protozoan *Neospora caninum* directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. *Infect Immun* (2006) 74:953–60. doi:10.1128/IAI.74.2.953-960.2006
10. Hermosilla C, Muñoz Caro T, Silva LMR, Ruiz A, Taubert A. The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. *Parasitology* (2014) 141:1489–98. doi:10.1017/S0031182014000316
11. Muñoz-Caro T, Hermosilla C, Silva LMR, Cortes H, Taubert A. Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti*. *PLoS One* (2014) 9(3):e91415. doi:10.1371/journal.pone.0091415
12. Muñoz-Caro T, Mena JS, Conejeros I, Alarcón P, Hidalgo MA, Burgos RA, et al. *Eimeria bovis*-triggered neutrophil extracellular trap formation is CD11b-, ERK/MAP kinase- and SOCE-dependent. *Vet Res* (2015) 46:23. doi:10.1186/s13567-015-0155-6
13. Muñoz-Caro T, Lendner M, Daugschies A, Hermosilla C, Taubert A. NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca2+ influx are essential for

Bovine polymorphonuclear neutrophils cast neutrophil extracellular traps against the abortive parasite *Neospora caninum*

Cryptosporidium parvum-induced NET formation. *Dev Comp Immunol* (2015) 52:245–54. doi:10.1016/j.dci.2015.05.007

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* (2004) 303:1532–5. doi:10.1126/science.1092385
2. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* (2006) 6:173–82. doi:10.1038/nri1785
3. Hahn S, Giaglis S, Chowdhury CS, Hösli I, Hasler P. Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. *Semin Immunopathol* (2013) 35:439–53. doi:10.1007/s00281-013-0380-x
4. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol* (2012) 198:773–83. doi:10.1083/jcb.201203170
5. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* (2007) 176:231–41. doi:10.1083/jcb.200606027
6. von Köckritz-Blickwede M, Chow O, Ghochani M, Nizet V. Visualization and functional evaluation of phagocyte extracellular traps. *Methods Microbiol* (2010) 37:139–60. doi:10.1016/S0580-9517(10)37007-3
7. Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* (1971) 134:907–34. doi:10.1084/jem.134.4.907
8. von Köckritz-Blickwede M, Nizet V. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. *J Mol Med* (2009) 87:775–83. doi:10.1007/s00109-009-0481-0
9. Stoiber W, Obermayer A, Steinbacher P, Krautgartner WD. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. *Biomolecules* (2015) 5:702–23. doi:10.3390/biom5020702
10. Khan MA, Palaniyar N. Transcriptional firing helps to drive NETosis. *Sci Rep* (2017) 7:41749. doi:10.1038/srep41749
11. Hakkim A, Fuchs TA, Martinez NE, Hess S, Prinz H, Zychlinsky A, et al. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol* (2011) 7:75–7. doi:10.1038/nchembio.496
12. Douda DN, Khan MA, Grasemann H, Palaniyar N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc Natl Acad Sci U S A* (2015) 112:2817–22. doi:10.1073/pnas.1414055112
13. Cheng OZ, Palaniyar N. NET balancing: a problem in inflammatory lung diseases. *Front Immunol* (2013) 4:1. doi:10.3389/fimmu.2013.00001
14. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. *Nat Med* (2012) 18:1386–93. doi:10.1038/nm.2847
15. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* (2009) 16:1438–44. doi:10.1038/cdd.2009.96
16. Baker VS, Imade GE, Molta NB, Tawde P, Pam SD, Obadofin MO, et al. Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. *Malar J* (2008) 7:41. doi:10.1186/1475-2875-7-41
17. Guimarães-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceição-Silva F, et al. *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* (2009) 106:6748–53. doi:10.1073/pnas.0900226106
18. Wang Y, Chen Y, Xin L, Beverley SM, Carlsen ED, Popov V, et al. Differential microbicidal effects of human histone proteins H2A and H2B on *Leishmania* promastigotes and amastigotes. *Infect Immun* (2011) 79:1124–33. doi:10.1128/IAI.00658-10
19. Behrendt JH, Ruiz A, Zahner H, Taubert A, Hermosilla C. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. *Vet Immunol Immunopathol* (2010) 133:1–8. doi:10.1016/j.vetimm.2009.06.012
20. Silva LMR, Muñoz-Caro T, Rüdiger G, Vila-Viçosa MJM, Cortes HCE, Hermosilla C, et al. The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. *Parasitol Res* (2014) 113:2797–807. doi:10.1007/s00436-014-3939-0
21. Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, Denkers EY. *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. *Infect Immun* (2012) 80:768–77. doi:10.1128/IAI.05730-11
22. Reichel M, Muñoz-Caro T, Sánchez-Contreras G, Rubio-García A, Magdowski G, Gärtner U, et al. Harbour seal (*Phocavitulina*) PMN and monocytes release extracellular traps to capture the apicomplexan parasite *Toxoplasma gondii*. *Dev Comp Immunol* (2015) 50:106–15. doi:10.1016/j.dci.2015.02.002
23. Sousa-Rocha D, Thomaz-Tobias M, Diniz LFA, Souza PSS, Pinge-Filho P, Toledo KA. *Trypanosoma cruzi* and its soluble antigens induce NET release by stimulating toll-like receptors. *PLoS One* (2015) 10:e0139569. doi:10.1371/journal.pone.0139569
24. Ventura-Juárez J, Campos-Esparza MR, Pacheco-Yepez J, López-Blanco JA, Adabache-Ortíz A, Silva-Briano M, et al. *Entamoeba histolytica* induces human neutrophils to form NETs. *Parasite Immunol* (2016) 38:503–9. doi:10.1111/pim.12332
25. Muñoz-Caro T, Silva LMR, Rentería-Solís Z, Taubert A, Hermosilla C. Neutrophil extracellular traps in the intestinal mucosa of *Eimeria*-infected animals. *Asian Pac J Trop Biomed* (2016) 6:301–7. doi:10.1016/j.apjtb.2016.01.001
26. Canton GJ, Katzer F, Benavides-Silván J, Maley SW, Palarea-Albaladejo J, Pang Y, et al. Phenotypic characterisation of the cellular immune infiltrate in placentas of cattle following experimental inoculation with *Neospora caninum* in late gestation. *Vet Res* (2013) 44:6. doi:10.1186/1297-9716-44-60
27. Abe C, Tanaka S, Ihara F, Nishikawa Y. Macrophage depletion prior to *Neospora caninum* infection results in severe neosporosis in mice. *Clin Vaccine Immunol* (2014) 8:1185–8. doi:10.1128/CVI.00082-14
28. Costa RC, Orlando DR, Abreu CC, Nakagaki KY, Mesquita LP, Nascimento LC, et al. Histological and immunohistochemical characterization of the inflammatory and glial cells in the central nervous system of goat fetuses and adult male goats naturally infected with *Neospora caninum*. *BMC Vet Res* (2014) 10:291. doi:10.1186/s12917-014-0291-7
29. Dubey JP, Hattel AL, Lindsay DS, Topper MJ. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *J Am Vet Med Assoc* (1988) 193:1259–63.
30. Farley K, Stolley JM, Zhao P, Cooley J, Remold-O'Donnell E. A SerpinB1 regulatory mechanism is essential for restricting NETosis. *J Immunol* (2012) 189:4574–81. doi:10.4049/jimmunol.1201167
31. Scapinello S, Brooks AS, MacInnes JI, Hammermueller J, Clark ME, Caswell JL. Bactericidal activity of porcine neutrophil secretions. *Vet Immunol Immunopathol* (2011) 139:113–8. doi:10.1016/j.vetimm.2010.09.004
32. Parker H, Albrett AM, Kettle AJ, Winterbourn CC. Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *J Leukoc Biol* (2012) 91:369–76. doi:10.1189/jlb.0711387
33. Conejeros I, Jara E, Carretta MD, Alarcón P, Hidalgo MA, Burgos RA. 2- Aminoethoxydiphenyl borate (2-APB) reduces respiratory burst, MMP-9 release and CD11b expression, and increases l-selectin shedding in bovine neutrophils. *Res Vet Sci* (2012) 92:103–10. doi:10.1016/j.rvsc.2010.10.005
34. Conejeros I, Patterson R, Burgos RA, Hermosilla C, Werling D. Induction of reactive oxygen species in bovine neutrophils is CD11b, but not dectin-1-dependent. *Vet Immunol Immunopathol* (2011) 139:308–12. doi:10.1016/j.vetimm.2010.10.021
35. Burgos RA, Conejeros I, Hidalgo MA, Werling D, Hermosilla C. Calcium influx, a new potential therapeutic target in the control of neutrophil-dependent inflammatory diseases in bovines. *Vet Immunol Immunopathol* (2011) 143:1–10. doi:10.1016/j.vetimm.2011.05.037
36. Erb L, Liao Z, Seye CI, Weisman GA. P2 receptors: intracellular signaling. *Pflugers Arch* (2006) 452:552–62. doi:10.1007/s00424-006-0069-2
37. Fredholm BB, Ijzerman AP, Jacobson KA, Linden J, Müller CE. International union of basic and clinical pharmacology. LXXXI. Nomenclature and classification of adenosine receptors – an update. *Pharmacol Rev* (2011) 63:1–34. doi:10.1124/pr.110.003285
38. Bao Y, Chen Y, Ledderose C, Li L, Junger WG. Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils. *J Biol Chem* (2013) 288:22650–7. doi:10.1074/jbc.M113.476283
39. McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM. Dogs are definitive hosts of *Neospora caninum*. *Int J Parasitol* (1998) 28:1473–8. doi:10.1016/S0020-7519(98)00138-6
40. Hemphill A, Gottstein B. A European perspective on *Neospora caninum*. *Int J Parasitol* (2000) 30:877–924. doi:10.1016/S0020-7519(00)00072-2

Bovine polymorphonuclear neutrophils cast neutrophil extracellular traps against the abortive parasite *Neospora caninum*

1. Ortega-Mora LM, Fernández-García A, Gómez-Bautista M. Diagnosis of bovine neosporosis: recent advances and perspectives. *Acta Parasitol* (2005) 51:1–14. doi:10.2478/s11686-006-0001-0
2. Dubey JP, Schares G. Neosporosis in animals – the last five years. *Vet Parasitol* (2011) 180:90–108. doi:10.1016/j.vetpar.2011.05.031
3. Arranz-Solís D, Benavides J, Regidor-Cerrillo J, Horcajo P, Castaño P, Del Carmen Ferreras M, et al. Systemic and local immune responses in sheep after *Neospora caninum* experimental infection at early, mid and late gestation. *Vet Res* (2016) 47:2. doi:10.1186/s13567-015-0290-0
4. Porto WJ, Regidor-Cerrillo J, de Cássia Peixoto Kim P, Benavides J, Dos Santos Silva AC, Horcajo P, et al. Experimental caprine neosporosis: the influence of gestational stage on the outcome of infection. *Vet Res* (2016) 47:29. doi:10.1186/s13567-016-0312-6
5. Maksimov P, Hermosilla C, Kleinertz S, Hirzmann J, Taubert A. *Besnoitia besnoiti* infections activate primary bovine endothelial cells and promote PMN adhesion and NET formation under physiological flow condition. *Parasitol Res* (2016) 115:1991–2001. doi:10.1007/s00436-016-4941-5
6. Bottazzi B, Garlanda C, Cotena A, Moalli F, Jaillon S, Deban L, et al. The long pentraxin PTX3 as a prototypic humoral pattern recognition receptor: interplay with cellular innate immunity. *Immunol Rev* (2009) 227:9–18. doi:10.1111/j.1600-065X.2008.00719.x
7. Daigo K, Hamakubo T. Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. *Front Immunol* (2012) 3:378. doi:10.3389/fimmu.2012.00378
8. Silva LMR, Muñoz-Caro T, Burgos RA, Hidalgo MA, Taubert A, Hermosilla C. Far beyond phagocytosis: phagocyte-derived extracellular traps act efficiently against protozoan parasites in vitro and in vivo. *Mediators Inflamm* (2016) 2016:5898074. doi:10.1155/2016/5898074
9. Parseghian MH, Luhrs KA. Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity. *Biochem Cell Biol* (2006) 84:589–604. doi:10.1139/o06-082
10. Chuah C, Jones MK, Burke ML, Owen HC, Anthony BJ, Mcmanus DP, et al. Spatial and temporal transcriptomics of *Schistosoma japonicum*-induced hepatic granuloma formation reveals novel roles for neutrophils. *J Leukoc Biol* (2013) 94:353–65. doi:10.1189/jlb.1212653
11. Bonne-Année S, Kerepesi LA, Hess JA, Wesolowski J, Paumet F, Lok JB, et al. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval *Strongyloides stercoralis*. *Microbes Infect* (2014) 16:502–11. doi:10.1016/j.micinf.2014.02.012
12. Sandoval AJ, Riquelme JP, Carretta MD, Hancke JL, Hidalgo MA, Burgos RA. Store-operated calcium entry mediates intracellular alkalinization, ERK1/2, and Akt/PKB phosphorylation in bovine neutrophils. *J Leukoc Biol* (2007) 82:1266–77. doi:10.1189/jlb.0307196
13. Gupta AK, Giaglis S, Hasler P, Hahn S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One* (2014) 9(5):e97088. doi:10.1371/journal.pone.0097088
14. Leshner M, Wang S, Lewis C, Zheng H, Chen XA, Santy L, et al. PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol* (2012) 3:307. doi:10.3389/fimmu.2012.00307
15. Knight J, Zhao W, Luo W, Subramanian V, O'Dell AA, Yalavarthi S, et al. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest* (2013) 123:2981–93. doi:10.1172/JCI67390
16. Gabl M, Winther M, Welin A, Karlsson A, Oprea T, Bylund J, et al. P2Y2 receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism. *Exp Cell Res* (2015) 336:242–52. doi:10.1016/j.yexcr.2015.07.014
17. Jacobson KA, Ivanov AA, de Castro S, Harden TK, Ko H. Development of selective agonists and antagonists of P2Y receptors. *Purinergic Signal* (2009) 5:75–89. doi:10.1007/s11302-008-9106-2
18. Önnheim K, Christenson K, Gabl M, Burbiel JC, Müller CE, Oprea TI, et al. A novel receptor cross-talk between the ATP receptor P2Y2 and formyl peptide receptors reactivates desensitized neutrophils to produce superoxide. *Exp Cell Res* (2014) 323:209–17. doi:10.1016/j.yexcr.2014.01.023

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4. FIRST REPORT ON THE SEROPREVALENCE OF *NEOSPORA CANINUM* IN GOATS FROM THE FEDERAL STATE OF HESSE, GERMANY

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First report on the seroprevalence of *Neospora caninum* in goats from the Federal State of Hesse, Germany

Erster Bericht über die Seroprävalenz von Neospora caninum in hessischen Ziegenbeständen

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Summary

A total of 41 goat serum samples from 26 flocks in Hesse, Central Germany, were analyzed for the presence of specific antibodies against the abortion-causing apicomplexan parasite *Neospora (N.) caninum* by immuno-enzyme assay (ELISA). In total, three serum samples were seropositive for *N. caninum* with two of them originating from a flock in Middle-Hesse and one sample coming from a flock in Northern Hesse. Western Blotting confirmed two of three ELISA-positive samples proving a low overall prevalence of 2/41 (0.48%) for caprine neosporosis. No clinical signs related to neosporosis were detected in any seropositive animal. Additionally, there was no familiar relationship between them and *N. caninum*-positive goats were purchased from different breeders. The low numbers of *N. caninum*-seropositive animals excluded risk factors assessment. Based on the current seroprevalence data, *N. caninum* infections appear of minor importance in German goat flocks. Nevertheless, taking into account that caprine neosporosis was detected in some European countries bordering Germany, further epidemiological and surveillance studies on caprine *N. caninum* infections are required to complement our findings regarding the current situation in goat populations from the other German Federal States.

Keywords: Survey, goat, Central Germany, epidemiology.

Zusammenfasssung

Insgesamt wurden 41 Ziegenserumproben von 26 Herden in Hessen, Deutschland, auf das Vorhandensein spezifischer Antikörper gegen den abortiven apikomplexen Parasiten *Neospora (N.) caninum* mittels Immunoenzymassay (ELISA) untersucht. Insgesamt konnten nur drei positive Proben ermittelt werden, wobei zwei aus einem Bestand in Mittelhessen und eine aus einem nordhessischen Bestand kamen. Ein anschließend durchgeführter Western Blot bestätigte zwei der drei ELISA-positiven Proben, die auf eine insgesamt niedrige Gesamtprävalenz von 2/41 (0,48 %) für Ziegenneosporose hinweisen. Keines der seropositiven Tiere wies klinische Symptome der Neosporose auf. Darüber hinaus lagen keine verwandtschaftlichen Beziehungen zwischen ihnen vor und die *N. caninum*-positiven Ziegen wurden von unterschiedlichen Züchtern erworben. Die geringe Seroprävalenz der vorliegenden Studie schloss die Möglichkeit, Risikofaktoren zu analysieren, aus. Die tatsächliche Bedeutung der *N. caninum*-Infektionen in deutschen Ziegenbeständen scheint aufgrund der gemessenen geringen Seroprävalenz vernachlässigbar. Da die Neosporose in Ziegen in einigen an Deutschland angrenzenden europäischen Ländern bereits nachgewiesen wurde, sind weitere epidemiologische Studien zu *N. caninum*-Infektionen von Ziegen erforderlich, um

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Introduction

Neospora (N.) caninum is an apicomplexan intracellular parasite responsible for abortions and serious disease in ruminants and dogs worldwide (Dubey, 2003). It shows a wide range of intermediate host species and causes reproductive problems mainly in cattle (Hemphill and Gottstein, 2000), but is also associated with clinical reproductive and neurological infections in dogs, horses, goats, sheep, deer and marine mammals (Dubey and Lindsay, 1996; Anderson et al., 2000; Schares et al., 2001a, b; Dubey, 2003; Omata et al., 2006). Protozoan-induced abortions in sheep and goats have commonly been related to *Toxoplasma (T.) gondii* infections (Buxton et al., 1998; Dubey, 2003), however, *N. caninum* infections can also result in abortion and reproductive disorders in small ruminants with both, economical and clinical consequences (Dubey and Schares, 2011). Clinical manifestations occurring during the course of caprine neosporosis can include abortion and still birth (Barr et al., 1992; Lindsay et al., 1995; Dubey et al., 1996; Koyama et al., 2001; Eleni et al., 2004; Mesquita et al., 2013; Costa et al., 2014). Moreover, enhanced neonatal mortality associated with caprine *N. caninum* infections has been reported from different countries, such as the USA (Barr et al., 1992), Costa Rica (Dubey et al., 1996), Taiwan (Ooi et al., 2000), Brazil (Corbellini et al., 2001) and Spain (Moreno et al., 2012).

For the detection of specific antibodies against *N. caninum*, the following serological assays are commonly used: enzyme-linked immunosorbent assays (ELISAs), indirect fluorescent antibody tests (IFATs) and agglutination test (NAT) (Ortega-Mora et al., 2007; Alvarez-García et al., 2013).

So far, in Europe caprine *N. caninum* seroprevalences have been analyzed in France (8.9%, Chartier et al., 2000), Austria (68.7%, Edelhofer et al., 2005), Poland, (0.47%, Czopowicz et al., 2011), Slovakia (15.5%, Čobádiová et al., 2013), Czech Republic (6%, Bartova and Sedlak, 2012), Romania (2.3%, Iovu et al., 2012), Italy (5.7%, Gazzonis et al., 2016), Turkey (10.2%, Ütük et al., 2011) and Spain (5,6%, García-Bocanegra et al., 2012; 6%, Díaz et al., 2016; 1.08%, Rodríguez-Ponce et al., 2016). In Germany, seroprevalence studies have been performed in other animal species, including cattle (Schares et al., 1998; Weber et al., 2000, Bartels et al., 2006), swine (Damriyasa et al., 2004), south American camelids (Wolf et al., 2005) dogs (Klein and Müller, 2001; Schares et al., 2001a) and foxes (Schares et al., 2001b). Moreover, the clinical and pathological presence of *N. caninum* has been reported in German cattle (Söndgen et al., 2001) and dogs (Peters et al., 2000).

The main objective of this study was to determine the caprine seroprevalence of *N. caninum* in Hesse particularly because of the importance of goat husbandry in this region is strongly associated with landscape preservation, species conservation and milk or meat production through grazing activities, which also represent a possible route of horizontal transmission for this parasite.

Materials and Methods

Analyzed population

The goat flocks in Hesse had similar characteristics regarding biosecurity, production, reproduction and health management. The majority of the analyzed flocks hold a small amount of animals (less than 100 goats, 82.1%), mostly maintained under semi-intensive conditions (78.6%). Most of them (85.7%) were members of the Hessian Goat Breeders (Hessischer Ziegenzuchtverband, HZV), commercially produced milk and meat, and routinely tested their animals for caprine arthritis and encephalitis virus (CAEV) and caseous lymphadenitis (CLA). The animals were kept together with other *N. caninum*-susceptible hosts, especially cattle (10.7%), sheep (46.4%), South American camelids (7.1%) and dogs (67.9%), but also with poultry (46.4%), cats (60.7%) and horses (32.1%). All flocks were routinely controlled by the Veterinary Ambulance of the Clinic for Obstetrics, Gynecology and Andrology of the Justus Liebig University Giessen.

Sample size

The sample size was calculated according to data given by the Hessen Epizootic Fund (Hessische Tierseuchenkasse) which estimated a population of 20 247 animals distributed in 3109 goat farms. Using the formula for freedom of disease (Cameron and Baldock, 1998), with a 2.5% overall expected prevalence, at 95.0% confidence level, and assuming all the sampled animals as a unique population with an identical probability of infection within the flock and among flocks, 402 samples needed to be analyzed to accomplish the objective; however, a total of 415 samples were taken. Due to a small number of goats per flock and in order to determine the presence of *N. caninum*, the Cannon and Roe´s formula (1982) was used (5% expected prevalence inside each flock at 95.0% confidence level). The study was conducted in 26 German goat flocks randomly selected within a list of owners voluntarily willing to participate. According proportional allocation, the farms were distributed as follows: 6 were located in Northern Hesse, 11 in Middle Hesse and 9 in Southern Hesse. The sampled goat breeds were the following: White German Improved Goat (WDE) (23%), German Improved Fawn (BDE) (19.2%), Boer (BRZ) (34.6%), Thuringian Forest (TWZ) (15.4%), Peacock (3.8%) and mixes (3.8%).

Sample collection and survey

The selected animals had to be older than 8 months to participate in this research, in order to avoid false negatives due to the non-detection of the parasite by the immune system (Mesquita et al., 2013). All animals included in this study were examined clinically to detect any signs of infections. The blood sampling was performed by punctuation of the jugular vein. Tubes were transported in coolers for keeping a temperature between 5 to 10°C. In the laboratory, the samples were centrifuged for 5 min at 10000 x g, sera were isolated and frozen at –20°C until further use. Immediately after sampling and in order to assess risk factors associated to *N. caninum* serostatus, a questionnaire was applied to the farmers, to obtain information about housing conditions, goat kids husbandry, management, abortion recurrence and presence of clinical signs related to apicomplexan parasite infections.

Enzyme-linked Immunosorbent Assay (ELISA)

The IDScreen® *Neospora caninum* Indirect Multi-species ELISA from IDVet® (Montpellier, France) was used to detect *N. caninum*-specific antibodies in caprine serum

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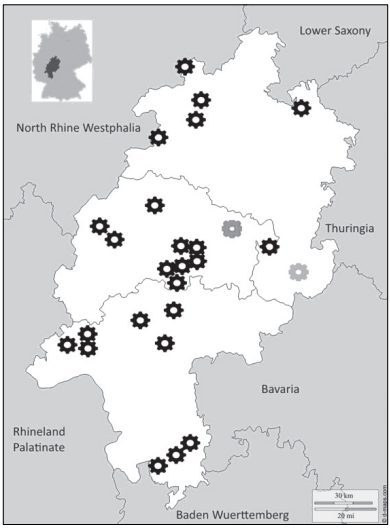

FIGURE 1: *Location of the participating seronegative farms (black gear icons) and flocks with Neospora caninum seropositive and suspect goats (dark grey and light grey gear icons, respectively) within the Northern-, Middle- and Southern-Hesse.*

samples. This assay showed a sensitivity of 99.6% and specificity of 98.9% (Álvarez-García et al., 2013). The samples were processed according to the manufacturer's protocol. For validation, the average of the optical densities (OD) of the positive controls, and the difference between averages of OD of positive and negative control sera were calculated. Applying the optical density data from the different serum samples, Serum Positive Percentages (S/P) were calculated, with respect to the average of the positive control sera, using the following formula: S/P = (OD of sample x 100): (average OD of positive control). As recommended by the manufacturer, serum samples that yielded S/P percentages less than 40% were considered as negative, samples with S/P values between 40–50% were scored as weakly positive and sera with S/P values greater than 50% were determined as positive. The seropositive samples of the current study were additionally analyzed in the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut) using Immunblot techniques.

Statistical analysis

Frequencies of the management conditions inside each goat flock were calculated. The global and specific within-herd seroprevalences with 95% confidence intervals were assessed. At the beginning of the study, based on the hypothesis of at least 2.5% of goats yield positive results, a statistical analysis by logistic regression was planned.

Results

Two goat sera reacted positive with ELISA (S/P 84% and 62%) and the other one with a weak positive result (S/P 44%). Two seropositive animals came from the same flock in Middle Hesse and the weak positive animal was detected in a farm in Northern Hesse; the remaining 24 flocks showed seronegative results (Fig. 1). The Immunblot-based analyses confirmed seropositivity in one positive animal from the flock in Middle Hesse and the weak positive sample from Northern Hesse demonstrating a low global seroprevalence of 0.48% (2/415). As such, these two flocks from Middle and Northern Hesse accounted 5% (1/20) and 7.7% (1/13) intra-flock seroprevalence respectively, whilst all other flocks showed a within-herd *N. caninum* seroprevalence of 0% (Tab. 1).

The descriptive data of all analyzed animals are shown in Table 1. The seropositive goats were two females, between four and six years old, showing neither a history of abortion nor reproductive problems or neurological disorders. At the time of sampling, the clinical examination of these animals showed no signs of infection. According to the flock rearing registers, these goats had no familiar relationship to each other and were introduced to the farm from two different flocks 3 years ago. The weakly positive goat was also a female, more than 4 years old and presented three normal births before. No history of abortions, reproductive or neurological disorders were registered before for this animal. In addition, the animal presented no signs of infection. It was also purchased from another breeder approximately 3 years ago. Owing to the low *N. caninum* seroprevalence found in the present study, no statistical association between the seropositive animals and risk factors could be assessed.

Discussion

The German goat population was estimated 150 000 individuals (Destatis, 2010) and continues growing. This investigation focused mainly on the goat flocks registered in the HZV.

Previously, the presence of *T. gondii*-specific antibodies (a closely related apicomplexan to *N. caninum*) was reported by Lenz (2014) in goats with clinical abortions in one flock in North Hesse. Whilst the presence of *N. caninum*-specific antibodies in bovine hosts is commonly reported in Germany (Söndgen et al., 2001; Schares et al., 2003; Bartels et al., 2006); to our best knowledge this study represents the first report of the presence of caprine specific antibodies against *N. caninum* in goats from Hesse and one of the earliest reports in total Germany. However, it has to be considered that the positive results obtained in the present study could also be due to false positive reactions in the ELISA. Nevertheless, this assay was reported to exhibit a high specificity (Álvarez-García et al., 2013) causing only 1.1% false positive results. However, since it is generally recommended to use at least two different diagnostic methods, such as IFAT, IHA, ELISA, PCR or Western Blot (Czopowicz et

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TABLE 1: *Number and percentage of animals analyzed and distribution of seropositive individuals according to flock, breed and regions*

Flock	Region	Animals	Animals tested (%)	Positive animals	Predominant Breed
1	Middle-Hesse	47	20 (42.50%)	2 (10.0%)	BRZ
2	Middle-Hesse	29	22 (75.90%)	0	BRZ
6	Middle-Hesse	62	15 (24.19%)	0	WDE + BDE
7	Middle-Hesse	98	25 (25.51%)	0	BRZ
12	Middle-Hesse	16	9 (56.52%)	0	BRZ
13	Middle-Hesse	14	10 (71.42%)	0	PZ
15	Middle-Hesse	19	9 (47.40%)	0	BRZ
16	Middle-Hesse	35	23 (65.71%)	0	BRZ
17	Middle-Hesse	31	13 (41.93%)	0	WDE
19	Middle-Hesse	48	22 (45.83%)	0	WDE
24	Middle-Hesse	12	6 (50.0%)	0	WDE
26	Middle-Hesse	321	30 (9.34%)	0	WDE + BDE
3	Northern-Hesse	80	26 (32.50%)	0	BDE
4	Northern-Hesse	6	4 (66.66%)	0	TWZ
9	Northern-Hesse	23	13 (56.52%)	1 (7.7%)	PZ
18	Northern-Hesse	6	6 (100.0%)	0	Omb
5	Northern-Hesse	302	31 (10.26%)	0	WDE
8	South-Hesse	4	4 (100 %)	0	Omb
10	South-Hesse	32	18 (56.25%)	0	BRZ
11	South-Hesse	168	28 (16.67%)	0	TWZ
14	South-Hesse	15	9 (60.0%)	0	BRZ
20	South-Hesse	17	9 (52.94%)	0	TWZ
21	South-Hesse	16	9 (56.52%)	0	BDE
22	South Hesse	17	10 (58.82%)	0	TWZ
23	South Hesse	223	28 (12.55%)	0	BDE
25	South-Hesse	31	16 (51.61%)	0	BRZ
	TOTAL	1672 (100 %)	415 (24.82%)	3 (0.72%)	

White German Improved Goat (WDE), German Improved Fawn (BDE), Boer (BRZ), Thuringian (TWZ) Peacock (PZ), and other mixed breeds (Omb)

al., 2011; Bartova and Sedlak, 2012; Gazzonis et al., 2016) to exclude false positive results, the positive samples obtained in ELISA were additionally analyzed via immunoblotting technique. Here, two out of three positive samples were confirmed in their seropositivity.

Overall, the low global seroprevalence determined in this study (0.48%) was consistent to data published for other European countries (Chartier et al., 2000; Czopowicz et al., 2011; Bartova and Sedlak, 2012; Gazzonis et al., 2016), in which *N. caninum* prevalences in goats remained below 10%. Thus, the prevalence data in this study was lower than those obtained in other European studies such as from Slovakia and Turkey (Čobádiová et al., 2013; Ütük et al., 2011). The intra-flock prevalences (5 and 7.7%) showed that the presence of *N. caninum* in Hesse was lower than expected, which is in agreement with other recently published data in Poland (Czopowicz et al., 2011). However, these authors recommended, that although *N. caninum* prevalences were low, caprine neosporosis should not be underestimated, since higher flock-level seroprevalences with serious repercussions could be emerging in the future. Thus, we also would recommend further serological and surveillance studies to be performed within caprine flocks, particularly in those that did not subscribe to HZV, in order to better understand the actual situation of this abortive disease.

Most of the owners maintained their animals in an intensive and semi-intensive management, grazing in fields close to dense forests or urban areas, where domestic dogs as potential oocyst shedders were commonly observed. As determined in personal interviews with the farmers, they noticed the canids mainly close to water sources and fences, where canid faeces were also frequently found. These conditions may favor the spread of *N. caninum* within the flocks as postulated elsewhere (Czopowicz et al., 2011; Čobádiová et al., 2013; Topazio et al., 2014; Gazzonis et al., 2016). Additionally, the owners described that this situation happened with a higher frequency during summer and autumn than in other seasons, when humidity is higher allowing the oocyst sporulation, which has concordance with the data described by Čobádiová et al. (2013) in Slovakia, Luo et al. (2016) in China and Díaz et al. (2016) in Spain.

Despite the number of positive animals was extremely low and risk factors could not be measured, the survey revealed that most of the caprine flocks in Hesse maintained a semi-intensive system with local breeds, which are environmentally better adapted inside the flock and probably reduced the seroprevalence of *N. caninum*, contributing to the absence of clinical signs associated with neosporosis, as mentioned in other European studies (Díaz et al., 2016; Gazzonis et al., 2016). The differences between the current data and the seroprevalences in goats from other European countries (e.g. Spain or Slovakia) may also depend on different climate conditions, farm management and sensitivity or (and) specificity of the diagnostic assays (Álvarez-García et al.,2013; Čobádiová et al., 2013; Díaz et al., 2016).

To sum up, we conclude that *N. caninum* infections indeed occur in goat flocks from Hesse but at a very low prevalence. Nonetheless, further studies are required to obtain more information on the current situation in small ruminant populations in other German Federal States. To improve the data sets, a higher number of goat flocks not having subscribed to HZV or to the Veterinary Ambulance Service should also be analyzed.

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Conflict of interest statement

The authors ratified that they have no competing interests in the present study.

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References

Álvarez-García G, García-Culebras A, Gutiérrez-Exposito D, Navarro-Lozano V, Pastor-Fernández I, Ortega-Mora LM (2013): Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests. Vet Parasitol 198: 85–95.

Anderson ML, Andrianarivo AG, Conrad PA (2000): Neosporosis in cattle. Anim Reprod Sci 61: 417–431.

Barr BC, Anderson ML, Woods LW, Dubey JP, Conrad PA (1992): Neospora-like protozoal infections associated with abortion in goats. J Vet Diagn Invest 4: 365–367.

Bartels CJ, Arnaiz-Seco JI, Ruiz-Santa-Quitera A, Bjorkman C, Frossling J, von Blumröder D, Conraths FJ, Schares G, van Maanen C, Wouda W, Ortega-Mora LM (2006): Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, The Netherlands, Spain and Sweden. Vet Parasitol 137: 17–27.

Bartova E, Sedlak K (2012): *Toxoplasma gondii* and *Neospora caninum* antibodies in goats in the Czech Republic Vet Med 57: 111–114.

Buxton D, Maley SW, Wright S, Thomson KM, Rae AG, Innes EA (1998): The pathogenesis of experimental neosporosis in pregnant sheep. J Comp Pathol 118: 267–279.

Cameron AR, Baldock FC (1998): A new probability formula for surveys to substantiate freedom from disease. Prev Vet Med 34: 1–17.

Cannon RM, Roe RT (1982): Livestock disease survey: a field manual for veterinarians. Australian Government Publishing Service, Canberra, Australia.

Chartier C, Baudry C, Losson B, De Meerschman F, Romand S, Thuillier P (2000): La néosporose chez la chèvre: résultats de deux enquêtes sérologiques dans l'Ouest de la France. Le Point Vétérinaire 31: 65–70.

Cobádiová A, Reiterová K, Derdáková M, Špilovská S, Turcekova L, Hviščová I, Hisira V (2013): *Toxoplasma gondii*, *Neospora caninum* and tick-transmitted bacterium *Anaplasma phagocytophilum* infections in one selected goat farm in Slovakia. Acta Parasitol 58: 541–546.

Corbellini LG, Colodel EM, Driemeier D (2001): Granulomatous encephalitis in a neurologically impaired goat kid associated with degeneration of *Neospora caninum* tissue cysts. J Vet Diagn Invest 13: 416–419.

Costa RC, Orlando DR, Abreu CC, Nakagaki KY, Mesquita LP, Nascimento LC, Silva AC, Maiorka PC, Peconick AP, Raymundo DL, Varaschin MS (2014): Histological and immunohistochemical characterization of the inflammatory and glial cells in the central nervous system of goat fetuses and adult male goats naturally infected with *Neospora caninum*. BMC Vet Res 10: 291.

Czopowicz M, Kaba J, Szalus-Jordanow O, Nowicki M, Witkowski L, Frymus T (2011): Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections in goats in Poland. Vet Parasitol 178: 339–341.

Damriyasa IM, Bauer C, Edelhofer R, Failing K, Lind P, Petersen E, Schares G, Tenter AM, Volmer R, Zahner H (2004): Cross-sectional survey in pig breeding farms in Hesse, Germany: seroprevalence and risk factors of infections with *Toxoplasma gondii*, *Sarcocystis* spp. and *Neospora caninum* in sows. Vet Parasitol 126:271–286.

Dubey JP, Lindsay DS. (1996): A review of *Neospora caninum* and neosporosis. Vet Parasitol 67:1–59.

Dubey JP, Morales JA, Villalobos P, Lindsay DS, Blagburn BL, Topper MJ (1996): Neosporosis-associated abortion in a dairy goat. J Am Vet Med Assoc 208: 263–265.

Dubey JP (2003): Review of *Neospora caninum* and Neosporosis in animals. Korean J Parasitol 41: 1–16.

Dubey JP, Schares G (2011): Neosporosis in animals – the last five years. Vet Parasitol 180: 90-108.

Diaz P, Cabanelas E, Díaz JM, Viña M, Béjar JP, Pérez-Creo A, Prieto A, López CM, Panadero R, Fernández G, Díez-Baños P, Morrondo P (2016): Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in goats from north-western Spain. Ann Agric Environ Med 23: 643–646.

Destatis (Deutsches Statistisches Bundesamt) (2010): Rund 150 000 Ziegen und 460 000 Einhufer in Deutschland. https://www.destatis.de/DE/ZahlenFakten/Wirtschaftsbereiche/LandForstwirtschaftFischerei/TiereundtierischeErzeugung/AktuellSonstigeNutztiere.html.

Edelhofer R, Peschke R, Perz I (2005): Seroepidemiological studies of *Toxoplasma gondii* and *Neospora caninum* in Austrian sheep and goats. 20th Internat. Conference of the Worls Association for the Advancement of Veterinary Parasitology, Christchurch, New Zealand, 16.–20. Oct. 2005.

Eleni C, Crotti S, Manuali E, Costarelli S, Filippini G, Moscati L, Magnino S. (2004): Detection of *Neospora caninum* in an aborted goat foetus. Vet Parasitol 123: 271–274.

García-Bocanegra I, Cabezon O, Pabon M, Gomez-Guillamon A, Arenas A, Alcaide E, Salas- Vega R, Dubey JP, Almeria S (2012): Prevalence of *Toxoplasma gondii* and *Neospora caninum* antibodies in Spanish ibex (*Capra pyrenaicahispanica*). Vet J 191: 257–260.

Gazzonis AL, Alvarez-Garcia G, Zanzani SA, Ortega Mora LM, Invernizzi A, Manfredi MT (2016): *Neospora caninum* infection in sheep and goats from north-eastern Italy and associated risk factors. Small Ruminant Res 140: 7–12.

Iovu A, Györke A, Mircean V, Gavrea R, Cozma V (2012): Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in dairy goats from Romania. Vet Parasitol 186: 470–474.

Hemphill, A, Gottstein, B (2000): A European perspective on *Neospora caninum*. Int J Parasitol 30: 877–924.

Klein BU, Müller E (2001): Seroprävalenz von Antikörpern gegen *Neospora caninum* bei Hunden mit und ohne klinischem Neosporoseverdacht in Deutschland. Prakt Tierarzt 82: 437–440.

Koyama T, Kobayashi Y, Omata Y, Yamada M, Furuoka H, Maeda R, Matsui T, Saito A, Mikami T (2001): Isolation of *Neospora caninum* from the brain of a pregnant sheep. J Parasitol 87: 1486–1488.

Lindsay DS, Rippey NS, Powe TA, Sartin EA, Dubey JP, Blagburn BL (1995): Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of *Neospora caninum*. Am J Vet Res 56: 1176–1180.

Lenz M (2014): Toxoplasmose in einer Ziegenherde. Landesbetrieb Hessisches Landeslabor. http://www.hessischetierseuchenkasse.de/download/about/8_Lenz_Toxoplasmose.pdf.

Luo HQ, Li K, Zhang H, Wu B, Wang J, Shahzad M, Tu YQ, Song XZ, Sun SW (2016): Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* infections in goats in Hubei province, China. Trop Biomed 33: 285–289.

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Mesquita LP, Nogueira CI, Costa RC, Orlando DR, Bruhn FRP, Lopes PFR, Nakagaki KYR, Peconick AP, Seixas JN, Bezerra OS Jr, Raymundo DL, Varaschin MS (2013): Antibody kinetics in goats and conceptuses naturally infected with *Neospora caninum*. Vet Parasitol 196: 327–333.

Moreno B, Collantes-Fernandez E, Villa A, Navarro A, Regidor-Cerrillo J, Ortega-Mora LM (2012): Occurrence of *Neospora caninum* and *Toxoplasma gondii* infections in ovine and caprine abortions. Vet Parasitol 187: 312–318.

Omata Y, Umeshita Y, Watarai M, Tachibana M, Sasaki M, Murata K, Yamada TK (2006): Investigation for presence of *Neospora caninum*, *Toxoplasma gondii* and Brucella-species infection in killer whales (*Orcinus orca*) mass-stranded on the coast of Shiretoko, Hokkaido. Jpn J Vet Med Sci 68: 523–526.

Ooi HK, Huang CC, Yang CH, Lee SH (2000): Serological survey and first finding of *Neospora caninum* in Taiwan, and the detection of its antibodies in various body fluids of cattle. Vet Parasitol 90: 47–55.

Ortega-Mora LM, Gottstein B, Conraths FJ, Buxton D (Eds) (2007): Protozoal abortions in farm ruminants: Guidelines for diagnosis and control. CAB International, doi: 10.1079/9781845932114.0000.

Peters M, Wagner F, Schares G (2000): Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. Parasitol Res 86: 1–7.

Rodríguez-Ponce E, Conde M, Corbera JA, Jaber JR, Ventura MR, Gutiérrez C (2016): Serological survey of antibodies to *Toxoplasma gondii* and *Neospora caninum* in goat population in Canary Islands (Macaronesia Archipelago, Spain). Small Ruminant Res147: 73–76.

Schares G, Peters M, Wurm R, Bärwald A, Conraths FJ (1998): The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet Parasitol 80: 87–98.

Schares G, Heydorn AO, Cuppers A, Conraths FJ, Mehlhorn H (2001a): *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. Parasitol Res 87: 808–816.

Schares G, Wenzel U, Müller T, Conraths FJ (2001b): Serological evidence for naturally occurring transmission of *Neospora caninum* among foxes (*Vulpes vulpes*). Int J Parasitol 31:418–423.

Schares G, Bärwald A, Staubach C, Ziller M, Klöss D, Wurm R, Rauser M, Labohm R, Dräger K, Fasen W, Hess RG, Conraths FJ (2003): Regional distribution of bovine *Neospora caninum* infection in the German state of Rhineland-Palatinate modelled by Logistic regression. Int J Parasitol 33 (14): 1631–1640.

Söndgen P, Peters M, Bärwald A, Wurm R, Holling F, Conraths FJ, Schares G (2001): Bovine neosporosis: immunoblot improves foetal serology. Vet Parasitol 102: 279–290.

Topazio JP, Weber A, Camillo G, Vogel FF, Machado G, Ribeiro A, Moura AB, Lopes LS, Tonin AA, Solda NM, Braunig P, Da Silva AS (2014): Seroprevalence and risk factors for *Neospora caninum* in goats in Santa Catarina state, Brazil. Braz J Vet Parasitol 23: 360–366.

Ütük AE, Şimşek S, Pişkin Ç, Balkaya İ (2011): Detection of *Neospora caninum* IgG antibodies in goats in Elazig, Erzurum and Kırsehir provinces of Turkey. Isr J Vet Med. 66: 157-160.

Weber A, Zetzmann K, Ewringmann T (2000): Vorkommen von Antikörpern gegen *Neospora caninum* bei Kühen in nordbayerischen Beständen mit Abortproblemen. Tierärztl Umsch 55: 28–29.

Wolf D, Schares G, Cardenas O, Huanca W, Cordero A, Bärwald A, Conraths FJ, Gauly M, Zahner H, Bauer C (2005): Detection of specific antibodies to *Neospora caninum* and *Toxoplasma gondii* in naturally infected alpacas (*Lama pacos*), llamas (*Lama glama*) and vicuñas (*Lama vicugna*) from Peru and Germany. Vet Parasitol 130: 81–87.

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5. SEROPREVALENCE AND RISK FACTORS ASSOCIATED WITH *NEOSPORA CANINUM*-, *TOXOPLASMA GONDII*- AND *COXIELLA BURNETTI*-INFECTIONS IN DAIRY GOAT FLOCKS FROM COSTA RICA

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Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

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Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

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Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

Abstract

A total of 391 goats from 13 dairy flocks from all Costa Rican regions were analyzed for *Toxoplasma gondii*-, *Neospora caninum*- and *Coxiella burnetii*-related seroprevalence by enzyme-linked immunosorbent assays (ELISA). Additionally, a risk factor analysis for these parasitic infections was performed based on a questionnaire considering several environmental and housing/management factors. A total of 62.1% (243/391) of individual serum samples revealed seropositive for *T. gondii,* 7.9% (31/391) for *N. caninum,* and 1.8% (7/391) for *C. burnetii*. At herd level, the overall seroprevalence for *T. gondii* was 100%, for *N. caninum* 69.2% and for *C. burnetii* 7.7%. However, no clinical signs related to toxoplasmosis, neosporosis or Q fever were apparent in these flocks. *T. gondii*-related risk factors were the contact with cats (OR= 3.44; CI 95%; 2.0 – 5.91), dogs (OR= 5.75; CI 95%; 2.84 – 11.66), and white-tailed deer (*Odocoileus virginianus*) (OR= 0.15; CI 95%; 0.08 – 0.26) within or around the farms. The presence of reproductive males in each flock (OR = 0.32; CI 95%; 0.14 – 0.74) and the coexistence of sheep (OR = 0.46; CI 95%; 0.2 – 1.08) and cattle (OR = 5.94; CI 95%; 1.70 – 20.78) revealed as protective and risk factors respectively for *N. caninum* infections. This study determined for the first time the seroprevalences of *N. caninum, T. gondii* and *C. burnetii* in Costa Rican goat flocks. Particularly, the high within-herd seroprevalences determined for *T. gondii* requires further surveillance to complement these findings.

Keywords: Goats, ELISA, zoonosis, public-health, Costa Rica.

Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

Introduction

Toxoplasma gondii and *Neospora caninum* are two closely related apicomplexan parasites associated with reproductive disorders in ruminants, such as foetal reabsorption, mummification, abortion, stillbirth and neonatal losses, leading to substantial economic losses in livestock production (Reichel et al., 2013). *Toxoplasma gondii* also plays a considerable zoonotic role since the consumption of infected raw or undercooked meat or milk from ruminants has been demonstrated to cause human toxoplasmosis (Tenter et al., 2000). *Coxiella burnetii* is an intracellular gamma proteobacterium of the family *Coxiellaceae*, which causes reproductive disorders in small ruminants and Q fever in humans (Van den Brom et al., 2015). This pathogen can be transmitted by ticks and other arthropods, but the main source of infection for domestic animals and humans is exposure to parturient secretions by inhalation of contaminated aerosols (Woldehiwet, 2004).

In Costa Rica, seroprevalences of *T. gondii* were reported, so far, only in rodents (5% in mice and 30.4% in rats; Chinchilla, 1978), cattle (34.4%; Arias et al., 1994) and chicken (40.6%; Abrahams-Sandí and Vargas-Brenes, 2005). The presence of *Neospora*-associated abortion was firstly described in Costa Rica in a dairy goat by Dubey et al. (1996). Further studies determined a dairy herd seroprevalence of 94.7% (89/94), showing an overall individual seroprevalence

of 43.3% (1185/2743) (Romero et al., 2005). Infections with *N. caninum* occur rather by vertical (64% of cases) than by horizontal transmission (22% of cases) in Costa Rica, however the latter value is much higher than reported in other countries, probably due to the ecology and biodiversity of this country (Romero and Frankena, 2003). With respect to *C. burnetii,* respective DNA was not detected in milk powder samples from Costa Rica using real-time PCR analysis (Tilburg et al., 2012). So far and to the best of our knowledge, there are no data available on *T. gondii* and *C. burnetii* seroprevalences in small ruminants from Costa Rica. To fill this gap, the current study aimed to determine the seroprevalence of *T. gondii, N. caninum and C. burnetii* in goat flocks from Costa Rica, and to identify risk or protective factors being associated to seropositivity for these three pathogens.

Materials and Methods

Ethic statement

The present study was conducted under the protocols established by the Animal Welfare Board (Comisión de Bienestar Animal) of the Universidad Nacional (Heredia, Costa Rica) and adhered to the legal requirements of the Animal Welfare Law (Ley 7451 de Bienestar Animal) of Costa Rica.

Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

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Study population

In the current study, exclusively flocks with dairy goat keeping of typical breeds, such as Saanen, Toggenburg, Anglo-Nubian and Alpine were included. The different flocks were registered in the database of the Small Ruminant Program of the National Animal Health Service (SENASA) of Costa Rica or affiliated to independent local caprine associations. The majority of analyzed flocks (69.2%) kept a small number of animals (< 100 goats), mostly maintained under semi-intensive conditions (61.5%). These animals were kept together with other domestic species, such as dogs (84.6%), cattle (61.5%), horses (53.8%), pigs (53.8%), poultry (46.2%), cats (46.2%), and sheep (38.5%).

Required sample sizes were calculated according to data published by the National Institute of Statistics and Census (INEC) of Costa Rica in 2014, who reported a population of 12.852 goats, kept in 2.348 farms. The expected prevalence of anti-*T. gondii (*40%*)*, anti-*N. caninum* (7%) and anti-*C. burnetii* antibodies (25%) was estimated with 95.0% confidence level and using Win Episcope 2.0 (Thrusfield et al., 2001) to determine the representative number of animals to be tested. The serological survey was conducted in 391 goats for the three abortive agents using farms sampled nationwide as part of the surveillance

program against brucellosis during 2013-2017 (Hernández-Mora et al., 2017). The Cannon and Roe´s formula (1982) was used to determine the sample size to be analyzed in each flock (5% expected prevalence at 95.0% confidence level). The study was conducted in 13 Costa Rican goat flocks selected by their broodstock activities, where the abortions were more frequent to happen and with owners who were willing to participate (Hernández-Mora et al., 2017). For proportional allocation, the sample flocks were present along the six regions of Costa Rica: Central (five farms), North Huetar (three farms), Atlantic Huetar (two flocks), Central Pacific (one flock), Chorotega (one flock) and Brunca (one flock).

Sample collection and survey

The selection of the animals inside each flock was randomly performed. Blood sampling was performed by bleeding from the jugular vein using BD Vacutainer® 22Gx1" needles with their respective plastic cap, adjusted to 6 ml vacuum tubes for serum (without anticoagulant). Tubes were transported in coolers keeping a temperature between 5 to 10°C. For serum isolation, blood samples were centrifuged for 8 min at 3500 x *g.* Serum was frozen at -20°C until further use. A questionnaire was applied to the farmers to assess possible risk factors being associated with *T. gondii, N. caninum* and *C. burnetii* serostatus. Therefore, information on housing conditions, management, animal feeding habits, goat kid

husbandry, abortions and contact with other domestic/wild animals on the farm or/and the surroundings was prompted.

Enzyme-linked immunosorbent assay (ELISA)

The IDScreen® *Toxoplasma gondii, Neospora caninum* and *Coxiella burnetii* Indirect Multispecies ELISAs (IDVet®, Montpellier, France) were used to detect parasite-specific antibodies in the caprine serum samples. These assays were reported to have a high sensitivity (*T. gondii*: 100%; *N. caninum*: 99.6%; *C. burnetii*: 100%) and high specificity (*T. gondii*: 100%; *N. caninum*: 98.9%; *C. burnetii*: 100%) (Proctor et al., 2008; Álvarez-García et al., 2013; Sidibe et al., 2013; IDVET, 2016). Serum samples were processed according to the manufacturer's protocol. The sera were diluted 1:10 for the analysis of each agent. For an adequate interpretation, the average of the optical densities (OD) of the positive controls, and the difference between averages of ODs of positive and negative control sera were calculated. Serum positive percentages (S/P) were calculated according to OD data from the different serum samples and the average of OD of the positive control sera, using the following formula: S/P = (OD of sample x 100): (average OD of positive control). As recommended by the manufacturer, the serum samples with S/P percentages <40% were considered as negative; samples with S/P values between 40-50% were scored as

inconclusive (considered negative in this study) and sera with S/P values >50% were determined as positive.

Statistical analysis

The overall and specific within-herd seroprevalences were assessed; besides, frequencies of the general characteristics and management conditions inside each goat flock were calculated. Factors associated with the agents were assessed by odds ratio (OR) estimation with the goat flock serving as the random variable. The causal variables with inferior and superior confidence intervals (CI 95%) ≤ 1 were considered as risk variable/factors, meanwhile protective variables/factors contained CI 95% ≥ 1. A non conditional logistic regression in two steps was used and first, an univariate analysis was performed for each independent variable and those ones with p ≤0.25 were retained and selected for the multivariate logistic regression model performed by a step-wise backward elimination (Hosmer and Lemeshow, 2005), which was evaluated by likelihood ratio tests. The data were analyzed using EGRET for Windows version 9.2 (Cytel Software Corporation).

Results

From a total of 391 caprine serum samples analyzed by ELISA, 243 reacted positive to *T. gondii* (62.14%), 31 to *N. caninum* (7.92%) and 7 to *C. burnetii* (1.79%). Moreover, 20 animals were found positive for *N. caninum* and *T. gondii* (5.12%) and just two goats (0.5%) were positive for all three pathogens. *T. gondii*-specific antibodies were detected in all analyzed flocks (herd prevalence: 100%), *N. caninum*-specific antibodies in nine flocks (69.2%) from five regions, and *C. burnetii*-specific antibodies only in one flock (7.7%) in the North Huetar Region. In general, the regional seropositivity varied considerably: 39.1% - 88.3% for *T. gondii*, 0% - 17.6% for *N. caninum* and 0% - 5.5% for *C. burnetii*. Within each herd, the prevalences range from 13.3% to 95.3% for *T. gondii* and 0% to 23.5% for *N. caninum.* In the single seropositive flock, 15.2% of the goat samples contained *C. burnetii*-specific antibodies (Table 1). No clinical signs related to toxoplasmosis, neosporosis or Q fever were apparent in any flock under investigation.

The univariate analysis revealed the extensive farm management and the contact with domestic animals (pigs, sheep, dogs, cats) in the farm, as well as wild animals (coyotes, opossums, coatis, peccaries, raccoons, white-tailed deer) around the farms as risk factors for *T. gondii* seropositivity. Respective factors for *N. caninum* seropositivity were the presence of reproductive males (protective

variable), a farm size with 10 - 50 goat kids (protective variable), adequate disposal of abortive materials (protective variable), and the co-existence of goats with bovines (risk factor) and sheep (protective variable) (Table 2). Since only seven goats from one single flock were positive for *C. burnetii*, no risk factor analysis could be performed.

Following the backward process, the final multivariate logistic regression model for *T. gondii* confirmed that contact with cats, dogs and white-tailed deer as risk factors to seropositivity. Meanwhile, three variables were confirmed for *N. caninum* seropositivity: the presence of more than two reproductive males (protective variable) and the co-existence with bovine (risk variable) and with sheep (protective factor) (Table 3).

Discussion

The current seroprevalence study provides data on *T. gondii, N. caninum*, and *C. burnetii* infections in domestic goats in Costa Rica. Thus, a high proportion of goats were detected seropositive for *T. gondii* (62.14%). In line, similar *T. gondii* seroprevalences were recently reported in four Caribbean islands (58% in Dominica, 57% in Grenada, 80% in Montserrat and 42% in Saint Kitts and Nevis) and Colombia (58%) whilst in Mexico fewer animals revealed infected (31%) (Alvarado-Esquivel et al. 2011; Cañon-Franco et al. 2014; Hamilton et al. 2014).

All these authors agreed with a broad environmental contamination of *T. gondii* oocysts in soil, water, vegetables or fruits affecting small ruminant flocks.

In case of *T. gondii* infections, poor management practices (e. g. poor hygiene, lack of proper feeding, irregular vaccination, and deworming) and goat farming in close contact with other productive, companion or wild animals (univariate analysis), especially cats, dogs and white-tailed deer (multivariate analysis) were here identified as risk factors for *T. gondii* seropositivity, which is in agreement to other reports (Dubey et al. 1995; Liu et al., 2015; Bawm et al. 2016.). The most obvious risk factor is related to the co-existence of goats with cats since these may shed high numbers of oocysts which contaminate the environment. In agreement, Frenkel and Ruiz (1981) assumed that the endemicity of *T. gondii* in Costa Rica is due to the high cat population. The unlimited contact between goats and free-roaming cats has been described as important risk factor in extensive management systems related to high herd-level seroprevalences (Czopowicz et al., 2011). In the other hand, dogs may also play an important role on the transmission of *T. gondii* since they could be affected by coprophagia when they have ingested feces of infected cats (Schares et al., 2005). Here is important to clarify, despite dogs themselves do not produce oocysts, high contaminated environments with *T. gondii* and *H. hammondi* oocysts represent a source for other animals, particularly in areas with dense populations of stray and

guard dogs, who shed coprophaged/excreted oocysts after intestinal passage, which possibly explains the roll of dogs as a risk factor of *T. gondii* in the surrounding goat populations (Schares et al., 2005; Hosseininejad et al., 2009). However, further deep analysis on the mechanisms involving coprophaged/excreted oocysts by dogs and their posterior infection in small ruminants should be performed. Additionally, wildlife mammals were also identified as risk factor of toxoplasmosis, since these animals are also susceptible wild intermediate hosts of this polyxenous apicomplexan parasite, which prolong the parasite's life cycle when wild felines feed on their infected carcases, as documented by several studies (Solorio et al., 2010; Dubey et al., 2014).

In case of *N. caninum*, the lower overall seroprevalence observed in the present study (7.92%) agreed with data published in other Latin American reports, such as Moore et al. 2007 in Argentina (6.6%), Topazio et al. (2014) in Brazil (4.58%) and Sharma et al (2015) in Grenada (5.8%). Moreover, Dubey et al (2017) reported that worldwide caprine *N. caninum* seroprevalences range between 0 to 26%, which agree with our results. In contrast to goats, *N. caninum* infections are well established in cattle, thus a considerably higher overall *N. caninum* seroprevalence of 39.7% was found in Costa Rican dairy cattle (Romero and Frankena, 2003).

The lower caprine *N. caninum* seroprevalence might be influence by the duration of the infection, the seroconversion and the applied serological tests, modifying the dynamics of the antibodies (Dubey et al., 2017).

As expected, instant removal of placentas and fetuses performed by most Costa Rican goat farmers (OR= 0.37; CI 95%; 0.15 - 0.95) revealed as an important protective factor for caprine neosporosis, as already described by Dubey et al. (2007). However, *N. caninum* infections in Costa Rica seemed mainly to be influenced by ruminant sharing grazing (Liu et al., 2015), either through the permanence of caprine with bovine (risk variable) or/and ovine (protective variable) species. Recent literature associated pastures highly contaminated with *N. caninum* oocysts with flocks where the animal density was extremely elevated and ruminant sharing grazing practices were common, thus promoting horizontal transmission of this parasite (Haddad et al., 2005; Armengol et al., 2007).

The presence of reproductive males was found as a protective factor against caprine neosporosis; which may be based on the assumption that bucks may be less susceptible to this infection than females, due to the absence of immunosuppression periods during pregnancy (Huerta-Peña et al., 2011). Enhanced innate immune responses against *T. gondii* and *N. caninum* infections have been mentioned in caprine males than in females in numerous studies, therefore female goats demonstrated higher seropositivity (van der Puije et al.,

2000; Uzêda et al., 2004; Sharma et al., 2015). Moreover, as the risk of sexual transmission of N. caninum is relative low (Ortega-Mora et al., 2003), we suggest that transportation of goats for mating would break the biosecurity cycle of *N. caninum*-free farms, particularly when goats are brought into flocks with reproductive bucks under different management conditions. This situation might justify the presence of males as a protective variable in the present study. However, the biological role of sex in apicomplexan infection and transmission in the caprine systems must be investigated further.

In eight flocks, *N. caninum* seropositive goats (20/31; 64.5%) were also *T. gondii* positive. These mixed infections were most probably due to management practices, abortions and significant contact with cats (*T. gondii,* OR= 3.44; CI 95%; 2.0 – 5.91) and dogs (*N. caninum*, OR= 5.75; CI 95%; 2.84 – 11.66) (Topazio et al., 2014; Gos et al., 2017).

The low seroprevalence of *C. burnetii* was similar to that determined by Gardon et al. (2001) in livestock from French Guiana (1.7%). Interestingly, the only flock with positive animals was located near to the border with Nicaragua. In this country, *C. burnetii* was recently reported to cause acute febrile illness in humans (Reller et al., 2016). In future work, *C. burnetii*-related data should be verified additionally via molecular techniques.

Finally, no clinical signs related to toxoplasmosis, neosporosis or Q fever were detected in any analyzed flocks, which can be related to several variables, such as stage of infection, pathogen strains, seroconversion and the animal immunity, as previously reported in the literature (Bezerra et al., 2013; Porto et al., 2016; Muleme et al.,2017). The absence of clinical reproductive signs (e. g. abortions and infertility) and their relationship with these mentioned variables (particularly with seroconversion) suggests also the presence of persistent or latent infected goats in these flocks, but their confirmation using immunoblot techniques are necessary to assess this hypothesis. Furthermore, the elevated cost in the importation of further diagnostic tools in Costa Rica was the principal limitation of our study that impeded the use of other detection methods as confirmation of our results. Therefore, we highly recommend the use of alternative diagnostic tools, such as PCR or immunoblot in further studies to confirm and complement the obtained data through the identification of particular pathogen-antigens.

Conclusions

This study determined for the first time seroprevalences of *N. caninum, T. gondii* and *C. burnetii* in goat flocks from all Costa Rican regions: all flocks were infected with *T. gondii*; *N. caninum* was found in nine flocks (69.2%), and *C. burnetii* only in one flock. Furthermore, risk and protective factors associated to seropositivity were identified: *T. gondii* infection was related with the contact of

goats with cats, dogs and white-tailed deer in the farms, whereas *N. caninum* was linked to joint grazing of goats with cattle and sheep. It is important to inform the caprine producers on these results; to keep definitive hosts (dogs and cats) separated from the grazing fields, prevent them (and wild animals) to wander in pastures and feed on abortive material. It is also recommended to avoid the sharing of paddocks between cows, goats and wild ruminants, especially the maternity paddocks, and adequate management practices inside the flock to prevent transmission of apicomplexan parasites. Complementary molecular detection is necessary to confirm the presence of *C. burnetii* in Costa Rica. Finally, further epidemiological surveillance for caprine toxoplasmosis and coxiellosis is recommended.

Declaration

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study were included in this published article.

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Author´s contributions

RVB, GD and AES collaborated with the sample collection and survey. RVB and GD performed the ELISA analysis. RVB, HW, JJR, AW, AT, CH and GD cooperated in research design, data analysis and manuscript’s review. All the authors corrected and accepted the final manuscript.

Conflict of interest statement

The authors ratified that they have no competing interests in the present study.

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References

Abrahams-Sandi, E., Vargas-Brenes, O. 2005. Serological prevalence of *Toxoplasma gondii* in free-range chickens from Costa Rica. Trop Anim Health Prod 37(5): 369-372.

Alvarado-Esquivel, C., García-Machado, C., Vitela-Corrales, J., Villena, I., Dubey, J.P. 2011. Seroprevalence of *Toxoplasma gondii* infection in domestic goats in Durango State, Mexico. Vet Parasitol. 183:43–46.

Álvarez-García, G., García-Culebras, A., Gutiérrez-Exposito, D., Navarro-Lozano, V., Pastor-Fernández, I., Ortega-Mora, L.M. 2013. Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests. Vet Parasitol 198: 85–95.

Arias, M.L., Chinchilla, M., Reyes, L., Sabah, J., Guerrero, O.M. 1994. Determination of *Toxoplasma gondii* in several organs of cattle by carbon immunoassay (CIA) testing. Vet Parasitol 55:133-136.

Armengol, R., Pabon, M., Santolaria, P., Cabezon, O., Adelantado, C., Yaniz, J., Lopez- Gatius, F., Almeria, S., 2007. Low seroprevalence of *Neospora caninum* infection associated with the limousin breed in cow-calf herds in Andorra, Europe. J. Parasitol. 93, 1029–1032.

Bawm, S., Maung, W.Y., Win, M.Y., Thu, M.J., Chel, H.M., Khaing, T.A., Wai, S.S., Htun, L.L., Myaing, T.T., Tiwananthagorn, S., Igarashi, M., Katakura, K. 2016. Serological survey and factors associated with *Toxoplasma gondii* infection in domestic goats in Myanmar. Scientifica (Cairo), 4794318.

Bezerra, M. J. G., P. C. P. Kim, E. P. B. X. Moraes, S. G. Sa, P. P. F. Albuquerque, J. G. Silva, B. H. L. S. Alves, and R. A. Mota. 2013. Detection of *Toxoplasma gondii* in the milk of naturally infected goats in the Northeast of Brazil. Transbound Emerg. Dis. 62(4):421-4. doi: 10.111/tbed.12160.

Cannon, R.M., Roe, R.T. 1982. Livestock disease survey: a field manual for veterinarians. Australian Government Publishing Service, Canberra, Australia.

Cañón-Franco, W. A., López-Orozco, N., Gómez-Marín, J. E., Dubey, J.P. 2014. An overview of seventy years of research (1944 – 2014) on toxoplasmosis in Colombia, South America. Parasit Vectors 7: 427.

Chinchilla, M. 1978. Epidemiología de la toxoplasmosis en Costa Rica y la importancia de los roedores domésticos. Rev Biol Tropl 26: 113-24.

Czopowicz, M., Kaba, J., Szalus-Jordanow, O., Nowicki, M., Witkowski, L., Frymus, T. 2011. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections in goats in Poland. Vet Parasitol. 178: 339–341.

Dubey, J.P., Weigel, R.M., Siegel, A.M., Thulliez, P., Kitron, U.D., Mitchell, M.A., Mannelli, A., Mateus-Pinilla, N.E., Shen, S.K., Kwok, O.C., Todd, K.S. 1995. Sources of reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. J Parasitol. 81: 723-729.

Dubey, J.P., Morales, J.A., Villalobos, P., Lindsay, D.S., Blagburn, B.L., Topper, M.J. 1996. Neosporosis-associated abortion in a dairy goat. J. Am. Vet. Med. Assoc. 208: 263–265.

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20, 323–367.

Dubey, J.P., Dennis, P.M., Verma, S.K., Choudhary, S., Ferreira, L.R., Oliveira, S., Kwok, O.C., Butler E., Carstensen, M., Su, C. 2014. Epidemiology of toxoplasmosis in white tailed deer (*Odocoileus virginianus*): occurrence, congenital transmission, correlates of infection, isolation, and genetic characterization of *Toxoplasma gondii*. Vet Parasitol 202(3-4): 270-275.

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G. 2017. Neosporosis in animals. Boca Raton Florida, USA: CRC Press.

Frenkel, J.K., Ruiz, A. 1981. Endemicity of toxoplasmosis in Costa Rica. Transmission between cats, soil, intermediate hosts and humans. Am. J. Epidemiol. 113: 254-269.

Gardon, J., Héraud, J.M., Laventure, S., Ladam, A., Capot, P., Fouquet, E., Favre, J., Weber, S., Hommel, D., Hulin, A., Couratte, Y., Talarmin, A. 2001. Suburban transmission of Q fever in French Guiana: evidence of a wild reservoir. J. Infect. Dis. 184:278–284.

Gos, M. L., Manazza, J. A., Späth, E. J. A., Pardini, L., Fiorentino, M. A., Unzaga, J. M., More, G. Venturini, M.C. 2017. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections in goats from two Argentinean provinces. Open Vet. J. 7(4): 319–322.

Haddad, J. P. A., Dohoo, I. R., VanLeewen, J. A. 2005. A review of *Neospora caninum* in dairy and beef cattle — a Canadian perspective. Can Vet J. 46(3), 230–243.

Hamilton, C.M., Katzer, F., Innes, E.A., Kelly, PJ. 2014. Seroprevalence of *Toxoplasma gondii* in small ruminants from four Caribbean islands. Parasit Vectors 7:449.

Hernández-Mora, G., Bonilla-Montoya, R., Barrantes-Granados, O., Esquivel-Suárez, A., Montero-Caballero, D., González-Barrientos, R., Fallas-Monge, Z., Palacios-Alfaro, J.D., Baldi, M., Campos, E., Chanto, G., Barquero-Calvo, E., Chacón-Díaz, C., Chaves-Olarte, E., Guzmán-Verri, C., Romero-Zúñiga, J.J. Moreno, E. 2017. Brucellosis in mammals of Costa Rica: An epidemiological survey. PLoS ONE 12(8): e0182644.

Hosseininejad, M., Malmasi, A., Hosseini, F., Selk-Ghaffari, M., Khorrami, N., Mohebali, M., Shojaee, S., Mirani, A., Azizzadeh, M., Mirshokraei, P., Aliari, A. 2011. Seroprevalence of *Toxoplasma gondii* infection in dogs in Tehran, Iran. Iranian J Parasitol. 6: 81–85.

Hosmer, D.W., Lemeshow, S. 2005. Wiley Series in Probability and Statistics, in Applied Logistic Regression, Second Edition, John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: https://doi.org/10.1002/0471722146.scard.

Huerta-Peña, J.C., Martínez-Herrera, D.I., Peniche-Cardeña, A.E.J., Villanueva-Valencia, M., Hernández-Ruiz, S.G., Villagómez-Cortes, J.A., Barradas-Piña, F.T., Morales-Álvarez, J.F., Flores-Castro, R. 2011. Seroprevalence and risk factors associated with *Neospora caninum* in goats from municipalities of central region of Veracruz. Trop. Subtrop. Agroecosystem 13: 445-454.

IDVET. 2016. ID Screen® Q Fever Indirect Multi-species sensitivity Quality Control Data Sheet [Pamphlet]. Montpellier, France. https://www.id-vet.com/produit/id-screen-q-fever-indirect-multi-species/

Liu, Z.K., Li, J.Y., Pan, H. 2015. Seroprevalence and risk factors of *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants in China. Prev. Vet. Med. 118: 488–492.

Moore, D.P., de Yañiz, M.G., Odeón, A.C., Cano, D., Leunda, M.R., Späth, E.A.J., Campero, C.M. 2007. Serological evidence of *Neospora caninum* infections in goats from La Rioja Province, Argentina. Small Rumin Res. 73, 256-258.

Muleme, M., Campbell, A., Stenos, J., Devlin, J. M., Vincent, G., Cameron, A., Graves, S., Wilks, C.R., Firestone, S. 2017. A longitudinal study of serological responses to *Coxiella burnetii* and shedding at kidding among intensively-managed goats supports early use of vaccines. Vet Res. 48, 50.

Ortega-Mora, L. M., I. Ferre, I. del Pozo, A. Caetano da Silva, E. Collantes-Fernández, J. Regidor-Cerrillo, C. Ugarte-Garagalza, Aduriz, G. 2003. Detection of *Neospora caninum* in semen of bulls. Vet. Parasitol. 117:301– 308.

Porto, W.J., Regidor-Cerrillo, J., de Cassia Peixoto Kim, P., Benavides, J., Dos Santos Silva, A.C., Horcajo, P., da Fonseca-Oliveira, A.A., Ferre, I., Mota, R.A., Ortega- Mora, L.M., 2016. Experimental caprine neosporosis: the influence of gestational stage on the outcome of infection. Vet. Res. 47, 29.

Proctor, A.F., O'Donovan, J., Marques, P.X., Gutierrez, J., Sammin, D., Brady, C., Worrall, S., Nally, J.E., Bassett, H., Markey, B.K. 2008. Detection of antibodies to *Toxoplasma gondii* in serum from experimentally infected pregnant ewes. University College Dublin, UCD, School of Agriculture, Food Science and Veterinary Medicine Research Day, 4th December.

Reichel M.P., Alejandra Ayanegui-Alcerreca M., Gondim L.F., Ellis J.T. 2013. What is the global economic impact of *Neospora caninum* in cattle – the billion dollar question. Int. J. Parasitol 43:133–142.

Reller, M.E., Chikeka, I., Miles, J.J., Dumler, J.S, Woods, C.W., Mayorga, O., Matute, A.J. 2016. First identification and description of Rickettsioses and Q fever as causes of acute febrile illness in Nicaragua. PLoS Negl Trop Dis 10(12): e0005185.

Romero, J. J., Frankena, K. 2003. The effect of the dam-calf relationship on serostatus to *Neospora caninum* on 20 Costa Rican dairy farms. Vet. Parasitol. 114**:**159-171.

Romero, J.J., van Breda, S., Vargas, B., Dolz, G., Frankena, K. 2005. Effect of neosporosis on productive and reproductive performance of dairy cattle in Costa Rica. Theriogenol 64(9):1928-1939.

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C., Conraths, F.J. 2005. Oocysts of *Neospora caninum, Hammondia heydorni, Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int J Parasitol. 35(14):1525-37.

Sharma, R.N., Bush, J., Tiwari, K., Chikweto, A., Bhaiyat, M.I. 2015. Seroprevalence of *Neospora caninum* in sheep and goats from Grenada, West Indies. Open J. Vet. Med. 5: 219-223.

Sidibe, S.S., Coulibaly, K.W., Dakouo, M., Tarnagda, Z., Sery, A., Niang, M., Traore, K., Nantoume, H., Diarra, S., Seyni, H. 2013. Q fever in small ruminants in Mali. Results of a serological survey. Revue d'élevage et de médicine vétérinaire des pays tropicaux, 66(1): 11-18.

Solorio, M.R., Gennari, S.M., Soares, H.S., Dubey, J.P., Hartley, A.C.Z, Ferreira, F. 2010. *Toxoplasma gondii* antibodies in wild white-lipped peccary (*Tayassu pecari*) from Peru. J Parasitol. 96(6):1232.

Tenter, A. M., Heckeroth, A. R., Weiss, L. M. 2000. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 30(12-13): 1217–1258.

Tilburg, J.J., Roest, H.J., Nabuurs-Franssen, M.H., Horrevorts, A.M., Klaassen, C.H. 2012. Genotyping reveals the presence of a predominant genotype of *Coxiella burnetii* in consumer milk products. J Clin Microbiol 50: 2156–2158.

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J.P., Frankena, K. 2001. WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Veterinary Record 148: 567-572.

Topazio, J.P., Weber, A., Camillo, G., Vogel, F.F., Machado, G., Ribeiro, A., Moura, A.B., Lopes, L.S., Tonin, A.A., Soldá, N.M., Bräunig, P., Silva, A.S. 2014. Seroprevalence and risk factors for *Neospora caninum* in goats in Santa Catarina state, Brazil. Rev Bras Parasitol Vet. *23*: 360-366. https://dx.doi.org/10.1590/S1984-29612014062.

Uzêda, R.S., Fernández, S.Y., Jesus, E.E.V., Pinheiro, A.M., Ayres, M.C.C., Spinola, S., Barbosa Junior, H.V., Almeida, M.A.O., 2004. Fatores relacionados á presença de anticorpos IgG anti- *Toxoplasma gondii* em caprinos leiteiros do Estado da Bahia. Rev. Bras. Saude Prod. Anim. 5, 1–8.

Van den Brom, R., van Engelen, E., Roest, H.I.J., van der Hoek, W., Vellema, P. 2015. *Coxiella burnetii* infections in sheep or goats: an opinionated review. Vet. Microbiol. 181(1-2):119-29.

van der Puije, W.N.A., Bosompem, K.M., Canacoo, E.A., Wastling, J.M., Akanmori, B.D., 2000. The prevalence of anti-*Toxoplasma gondii* antibodies in Ghanaian sheep and goats. Acta Trop. 76, 21– 26.

Woldehiwet, Z. 2004. Q fever (coxiellosis): epidemiology and pathogenesis. Res. Vet. Sci. 77: 93–100.

Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica

TABLE 1: Distribution of seronegative and seropositive sera to *N. caninum*, *T. gondii* and *C. burnetii* from goats of Costa Rica according to flock and region.

Flock	Region	Animals tested / Animals in the flock (%)	Positive animals (%)			Regional seropositivity n (%)		
			N. caninum	*T. gondii*	*C. burnetii*	*N. caninum*	*T. gondii*	*C. burnetii*
1	Central	21/23 (91.3)	3 (14.3)	5 (23.8)	0 (0.0)	7.7	54.2	0.0
2		46/145 (31.7)	4 (8.7)	9 (19.6)	0 (0.0)			
3		17/18 (94.4)	4 (23.5)	4 (23.5)	0 (0.0)			
4		38/101 (37.6)	2 (5.3)	32 (84.2)	0 (0.0)			
5		46/64 (71.9)	0 (0.0)	41 (89.1)	0 (0.0)			
6	Brunca	9/13 (69.2)	1 (11.1)	4 (44.4)	0 (0.0)	11.0	44.4	0.0
7	Atlantic Huetar	15/15 (100)	2 (13.3)	2 (13.3)	0 (0.0)	8.7	39.1	0.0
8		8/9 (88.9)	0 (0.0)	7 (87.5)	0 (0.0)			
9	Central Pacific	17/18 (94.4)	3 (17.6)	9 (52.9)	0 (0.0)	17.6	52.9	0.0
10	Chorotega	46/66 (69.7)	0 (0.0)	17 (37.0)	0 (0.0)	0.0	37.0	0.0
11	North Huetar	39/91 (42.9)	6 (15.4)	37 (94.8)	0 (0.0)	9.4	88.3	5.47
12		46/213 (21.6)	6 (13.0)	35 (76.1)	7 (15.2)			
13		43/102 (42.2)	0 (0.0)	41 (95.3)	0 (0.0)			
	TOTAL	**391 (44.7)**	**31 (7.92)**	**243 (62.2)**	**7 (1.79)**			

TABLE 2: Univariate analyses of risk factors associated with *N. caninum* and *T. gondii* seropositivity in 13 goat flocks from Costa Rica.

Variable	Category	*Neospora caninum*			
			CI 95%		
		***p*-value**	**OR**	**IL**	**UL**
Number of goat kids	11-50	0.031	0.23	0.05	1.23
Number of males	> 2	0.038	0.44	0.18	1.01
Disposal of abortive materials	Burial	0.019	0.37	0.15	0.95
Co-existence with bovines	Yes	0.007	4.55	1.28	19.19
Co-existence with sheep	Yes	0.062	0.46	0.18	1.11
Variable code	**Category**	***Toxoplasma gondii***			
			CI 95%		
		***p*-value**	**OR**	**IL**	**UL**
Husbandry system	Extensive	< 0.001	0.09	0.01	0.44
Contact with swine	Yes	< 0.001	2.05	1.32	3.20
Co-existence of sheep	Yes	< 0.001	0.35	0.22	0.55
Contact with cats	Yes	< 0.001	5.19	3.22	8.38
Contact with dogs	Yes	< 0.001	4.39	2.42	8
Contact with coyotes	Yes	< 0.001	0.24	0.14	0.39
Contact with opossums	Yes	< 0.001	0.17	0.10	0.27
Contact with coatis	Yes	< 0.001	0.15	0.09	0.25
Contact with peccaries	Yes	< 0.001	0.15	0.09	0.24
Contact with raccoons	Yes	< 0.001	0.09	0.05	0.16
Contact with white-tailed deer	Yes	< 0.001	0.30	0.19	0.48

Codes: OR= Odds ratio; IL= Inferior limit; UL= Upper limit; CI= Confidential interval.

TABLE 3: Risk factors associated with *N. caninum* and *T. gondii* seropositivity in 13 goat flocks from Costa Rica according to the multivariate logistic regression model.

Variable	*N. caninum*				*T. gondii*			
		CI 95%				CI 95%		
	***p*-value**	**OR**	**IL**	**UL**	***p*-value**	**OR**	**IL**	**UL**
More than two reproductive bucks	0.008	0.32	0.14	0.74	-	-	-	-
Co-existence of bovine	0.005	5.94	1.70	20.78	-	-	-	-
Co-existence of sheep	0.076	0.46	0.20	1.08	-	-	-	-
Contact with cats	-	-	-	-	< 0.001	3.44	2.00	5.91
Contact with dogs	-	-	-	-	< 0.001	5.75	2.84	11.66
Contact with white-tailed deer	-	-	-	-	< 0.001	0.15	0.08	0.26

Codes: OR= Odds ratio; IL= Inferior limit; UL= Upper limit; CI= Confidential interval.

6. SEROPREVALENCE OF *NEOSPORA CANINUM*-SPECIFIC ANTIBODIES IN GERMAN BREEDING BITCHES

This chapter is based on the following published paper:

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Parasites & Vectors

SHORT REPORT Open Access

Seroprevalence of *Neospora caninum*-specific antibodies in German breeding bitches

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Abstract

Background: *Neospora caninum* is an intracellular obligate apicomplexan parasite responsible for multisystemic lesions in dogs. Being definitive hosts and reservoirs, dogs excrete environmentally resistant oocysts. Breeding bitches represent a susceptible dog group and infected bitches may spread this parasite through transplacental transmission.
Results: A total of 218 serum samples of German breeding bitches were collected to determine the presence of *N. caninum*. Antibodies were detected in 16 (7.33%) bitches using a commercial indirect enzyme-linked immunosorbent assay (ELISA). Immunoblotting analysis confirmed all seropositive samples detected by ELISA, proving that the animals were infected with *N. caninum*. The owners were interviewed regarding breed, age, environment, type, vaccine status, feeding habits and the presence of reproductive disorders. Seropositive animals were between the ages of two to seven years; three of them were kept in kennels while the others were household dogs, one of which was additionally a hunting dog. Owners of four seropositive bitches reported one gestation, while multiple pregnancies had been recorded for the other twelve bitches. Fourteen bitches were regularly vaccinated and six were fed with fresh raw meat.
Conclusions: Although the results confirmed a low incidence of *N. caninum* seropositive German breeding bitches, further epidemiological and surveillance studies are required to complement our findings regarding the current situation of neosporosis in this specific canine population of Germany.
Keywords: *Neospora caninum*, Reproduction, Breeding bitches, Germany

Background

Neospora caninum is an apicomplexan obligate intracellular parasite that causes multisystemic lesions in dogs [1–5]. Dogs can act as definitive as well as intermediate hosts during *N. caninum* infections [6, 7]. Canine neosporosis is characterized by neuromuscular symptoms, such as ataxia, ascending paralysis, and other general nervous clinical signs [8]. Other manifestations include myocardial, pulmonary, dermatological, as well as reproductive disorders [3, 9–12]. *Neospora caninum* infections can occur through horizontal and vertical transmission of the parasite, i.e. a foetus may become infected transplacentally. In addition, dogs can be postnatally infected through oral uptake of cysts from infected tissue material or sporulated *N. caninum* oocysts in contaminated food or water sources [11, 13, 14]. Oocysts are greatly significant in the spread and maintenance of this abortive agent, which is known to be highly tenacious [6, 7, 15].

Female dogs that have given birth to pups congenitally infected with *N. caninum* do not present any clinical signs [13]. Nevertheless, transmission of the protozoan to offspring in succeeding generations can occur [3, 16].

There are many diagnostic methods used to detect this parasite, such as histology, immunochemistry, serology, and conventional and real-time PCR [5, 17, 18]. Despite the fact that clinical canine neosporosis is rare, there are many re- ports on the seroprevalence of N. caninum in domestic and wild canines [10–13, 15]. Even among different canine populations with diverse roles and environments, distinct

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Seroprevalences have been reported in stray [19], farm-rural[20–24], kennel [20] and urban dogs [21, 23–25].

European studies revealed differences in *N. caninum* seroprevalence; three of them were kept in kennels while the others were household dogs, one of which was additionally a hunting dog of various canine populations, presenting with15.3% seroprevalence in Denmark [9], 3.6% in Austria [26], 2.6–19.2% in Czech Republic [27], 17.2% in Serbia [28], 32.7% in Romania [29], 16.36% or 21.7% in Poland [25, 30], 10.9% in Italy [31], 12.2% in Spain [32],0.5% in Sweden [33] and 4% or 13% in Germany [34].

The aim of the present study was to determine the presence of *N. caninum* antibodies in German breeding female dogs and describe the characteristics of seropositive animals that may be correlated with this parasite and their potential involvement in reproductive disease.

Methods

Analysed population and sample size

Female dogs that showed optimal health parameters were presented for routine progesterone concentration measurements for ovulation determination at the Clinic for Obstetrics, Gynaecology and Andrology of the Justus Liebig University (JLU) Giessen, Germany. All bitches participating in this study were previously subjected to a clinical examination. A total of 218 samples were collected from March 2016 to June 2017 to determine the presence of N. caninum and the correlation between a current infection and reproductive disorders. Owners of seropositive animals were contacted and requested to complete a questionnaire that asked about breed, age, environment (indoors or outdoors, urban or rural), type of dog (farm, hunting, kennel, police, rescue, household/pet dogs), vaccination status (e.g. vaccinated against distemper virus, canine hepatitis virus, canine parvovirus, parainfluenza virus, Leptospira spp. and rabies), feeding habits, and reproductive disorders.

Sample collection and additional information

Blood was collected by puncture of the cephalic vein. Then, the samples were transported at 5–10 °C. In the laboratory, samples were centrifuged for 5 min at 10000×g, and then sera were separated and frozen at -20°C until further analysis.

Enzyme-linked immunosorbent assay (ELISA)

The IDScreen® Neospora caninum Indirect Multi-species ELISA from IDVet® (Montpellier, France) was used for the detection of *N. caninum*-specific antibodies in canine serum samples. The same assay was employed in the studies by Sharma et al. [19], and Enăchescu et al. [35]. Sera were analysed, according to the ELISA-manufacturer's instructions. For validation, positive control optical density (OD) averages and the difference between positive and negative control OD averages were evaluated. According to OD data of different serum samples, serum positive percentages (S/P) were calculated with respect to the average of the positive control sera using the following formula: S/P = (sample OD× 100) / (average OD of positive control). As recommended by the ELISA-manufacturer, samples that yielded S/P percentages of less than 40% were classified negative, samples with S/P values between 40–50% were weakly positive, and those with S/P values higher than 50% were assumed positive for *N. caninum* infection. The seropositive samples detected by ELISA and 10% of the remaining negative samples were further validated by immunoblotting assays.

Immunoblot assays

Two immunoblot assays were performed: one immunoblot was based on total tachyzoite antigen (NC-1 strain of N. caninum; Dubey et al. [36] cultivated in MARC145 cells), while the second immunoblot relied on p38 tachyzoite antigen (NcSRS2) application after affinity purification, as previously described [37].

Total tachyzoite antigen immunoblot was performed as described previously [38] using 8×10^7 tachyzoite pellets of *N. caninum* or purified NcSRS2 (p38, 0.05 µg per SDS-PAGE protocol) [37, 39]. Antigens were incubated in non-reducing sample buffer [2% (w/v) SDS, 10% (v/v) glycerol, 62 mM Tris-HCl, pH 6.8] for 1 min at 94°C, separated on12% SDS polyacrylamide minigels (60 × 70 × 1 mm), and transferred to PVDF membranes (Immobilon-P, Merck Chemicals GmbH, Darmstadt, Germany). After the transfer, membranes were blocked in PBS-TG consisting of PBS with 0.05% (v/v) Tween 20 (Sigma-Aldrich, Taufkirchen, Germany) and 2% (v/v) liquid fish gelatin (Serva, Heidelberg, Germany), cut into 50 strips, and examined as described below. To detect antibodies against *N. caninum* tachyzoite antigens, western blot membrane strips were incubated as previously described [38]. Dog sera were diluted 1:100 in PBS-TG, and then immunoreactions were detected using a peroxidase anti-dog IgG conjugate (Dianova, Hamburg, Germany) diluted 1:1000 in PBS-TG. Sera of naturally *N. caninum*-infected and non-infected dogs [40] were used as positive and negative control, respectively. In the case of total antigen detection, reactivity of the sera with non-reduced immunodominant *N. caninum* tachyzoite antigens (NC-IDA) of 17–19, 29, 30, 33, and 37 kDa Mr was examined. For purified NcSRS2, reactivity at 37–39 kDa was analysed [37].

Results

Out of 218 analysed samples, 16 (7.33%) were positive for *N. caninum*-specific antibodies as determined by ELISA and reported S/P values higher than 50% (Table 1).

Table 1 Distribution of seronegative and seropositive sera of *N. caninum* according to the serum positive percentage (S/P) values determined with ELISA

Positivity percentage (SP)	*Neospora caninum* (%)
≤30 (negative serorreactors)	202 (92.6)
31–50 (low serorreactors)	0 (0)
51–70 (high positive serorreactors)	1 (0.6)
≥ 71 (very high positive serorreactors)	15 (6.8)
Total	218 (100.0)

Immunoblot-based analyses confirmed seropositivity of all samples detected positive by ELISA (Fig. 1) and the 20 representative samples found negative by ELISA (10%).

Characteristics of the seropositive bitches are summarised in Table 2. The positive bitches were between two and seven years old, three (18.8%) were kept in kennels and the remaining were household animals (75%), including one (6.2%) that was also used for hunting. During sampling, four (25%) positive female dogs had been pregnant once, while the other 12 (75%) had had more than one birth as reported by the owners. Six (37.5%) seropositive individuals were fed with fresh raw meat not treated by cooking. Finally, 14 (87.5%) of the 16 seropositive bitches had a full vaccination program recorded.

Discussion

The present study confirmed the presence of *N. caninum* antibodies in German breeding female dogs, which represent a susceptible *N. caninum*-infection dog group. Infected bitches may spread this parasite through transplacental transmission during successive pregnancies [41–43]. Immunoblot assays were used as a validation method for ELISA-positive and some ELISA-negative animals with the main purpose of avoiding false positive serological results and verifying the presence or absence of specific antibodies against *N. caninum* [25, 44].

The clinical and pathological isolation of *N. caninum* in an 11-week-old German puppy was previously reported [40]. Moreover, *N. caninum* faecal oocysts were found and cysts of this parasite were identified in German dogs [45, 46]. Previously, serological analyses of three German Doberman puppies from an infected bitch demonstrated the vertical transmission of *N. caninum* [42]. The low number of serologically positive dogs in this study (7.33%) is in agreement with previous seroprevalence obtained for German dogs with (13%) and without (4%) clinical signs of neosporosis [34] and in dogs from the German Federal State of Rhineland-Palatinate (4.45%) [47]. However, it should be noted that the novelty of this study relies on the low seroprevalence determined in canine breeding populations in Germany, specifically in the reproductive bitches population for which an *N. caninum* seroprevalence has not yet been described in the literature.

Transplacental transmission in dogs has been reported for experimental infections [48]; however, natural-neonatal canine neosporosis is rare and findings are variable, as not all litter puppies become seropositive [3]. Thus, frequent

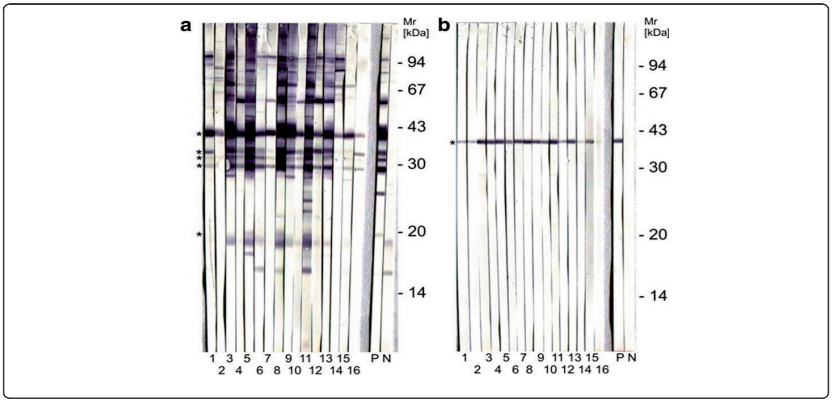

Fig. 1 Immunoblot analysis of 16 *N. caninum*-seropositive German bitches. a Immunoblot with total tachyzoite antigen. Immunodominant antigens of 17–19, 29, 30, 33 and 37 kDa Mr are marked by asterisks. b Immunoblot using NcSRS2;the antigen is indicated with an asterisk. Abbreviations: P, positive control; N, negative control

Table 2 Characteristics of *N. caninum* seropositive breeding bitches

Animal	Breed	Age	Environment	Vaccination status	Feeding	Previous births
1	Rhodesian ridgeback	2	Household	DHPPi+LR	Raw meat	1
2	Shepherd dog	6	Household and kennel	DHPPi + LR	Dry food and raw meat	> 1
3	Giant schnauzer	6	Household	DHPPi + LR	Raw meat	> 1
4	Norwich terrier	7	Household	DHPPi + LR	Dry and wet food	> 1
5	Rhodesian ridgeback	5	Household	DHPPi + LR	Raw meat	> 1
6	German shepherd	4	Household	DHPPi + LR	na	> 1
7	Boston terrier	4	Household	DHPPi + LR	Dry food	> 1
8	English bulldog	-	Household	DHPPi + LR	Dry food	> 1
9	Giant schnauzer	6	Household	DHPPi + LR	Raw meat	> 1
10	Bernese mountain	6	Kennel	na	Dry food and raw meat	>1
11	English bulldog	-	Household	DHPPi + LR	Dry food	> 1
12	Bulldog	5	Household	DHPPi + LR	Dry food	> 1
13	Greater Swiss Mountain dog	4	Household	DHPPi + LR	Dry food	> 1
14	Rottweiler	7	Kennel	na	na	1
15	Unknown	4	Household	DHPPi + LR	Dry food	1
16	Austrian black and tan hound	2	Household and hunting	DHPPi + LR	Dry food	1

Abbreviations: na no answer, DHPPi + LR vaccination against distemper virus, canine hepatitis virus, canine parvovirus, parainfluenza virus, *Leptospira spp*. and rabies

canine transplacental transmission is unlikely to occur in the absence of horizontal infection [3, 49], highlighting the importance of investigating additional canine horizontal infection routes of *N. caninum* in seropositive breeding bitches [3, 50]. All infection routes should be considered during the reproductive cycle of subclinical Neospora-infected bitches, especially considering that no drugs are known to prevent transplacental transmission [49]. Therefore, we also consulted with the owners of seropositive animals regarding risk factors of canine neosporosis identified in previous studies, such as breed [31], age [50], environment [51], type [20], vaccine status [52], and feeding habits [53].

Most of the individuals analysed were household, breeding female dogs. Several studies have demonstrated that European farm dogs have higher *N. caninum* seroprevalences than kennel, rescue, household, or urban dogs [20, 21, 54]. Seroprevalence was especially high in farm dogs that were kept with highly specialised dairy herds [22] or even with small ruminant flocks [23]; however, most of the studies mainly focused on this type of canine population, with only a few investigating household breeding dogs [3].

In the present study, four of the female dogs studied were found to have received raw meat as part of their diet. Horizontal transmission of *N. caninum* occurs through the intake of tissue cysts [55, 56]. Infection, as evidenced by shedding oocysts, was demonstrated in dogs after experimentally feeding them infected meat from goat and sheep [57].

Moreover, one positive household bitch was used for hunting proposes. Hunting dogs have an increased risk of being *N. caninum*-seropositive [24]. Possible contact with eviscerated infected wild animal carcasses (e.g. deer) might represent a potential source of infection [15, 29, 47]. In contrast with this observation, however, a serological study [9] found no statistical significance be- tween seroprevalence of hunting and non-hunting dogs.

The vaccination status of the animals was also recorded to assure proper health status. In the present study, 14 out of 16 seropositive bitches were vaccinated. These data are in contrast with previous observations, in which vaccinated dogs had significantly lower seroprevalence compared with non-vaccinated canines [52]. The level of care provided by the dog owners regarding vaccination and diet were not correlated to *N. caninum* seropositivity.

Little is known about the clinical and economic consequences of canine neosporosis on the reproductive performance of breeding bitches and their progeny; therefore, further long-term studies are necessary to better understand the impact of neosporosis on breeding dog populations.

Conclusions

We concluded that *N. caninum* infections exist in German breeding bitches at a very low prevalence. Nonetheless, further epidemiological studies are required to obtain more information regarding the seroprevalence of other German canine populations.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors' contributions
TC contributed in sample collection and surveys. RVB and LA performed ELISA analysis. GS and AB conducted immunoblot analysis. RVB, LA, TC, GS, AW, AT and CH cooperated in research design, data analysis, and manuscript review. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Competing interests
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References

1. Dubey JP, Metzger FL, Hattel AL, Lindsay DS, Fritz DL. Canine cutaneous neosporosis: clinical improvement with clindamycin. Vet Dermatol. 1995;6: 37–43.
2. Barber JS, Trees AJ. Clinical aspects of 27 cases of neosporosis in dogs. Vet Rec. 1996;139:439–43.
3. Barber JS, Trees AJ. Naturally occurring vertical transmission of *Neospora caninum* in dogs. Int J Parasitol. 1998;28(1):57–64.
4. Garosi L, Dawson A, Couturier J, Matiasek L, de Stefani A, Davies E, et al. Necrotizing cerebellitis and cerebellar atrophy caused by *Neospora caninum* infection: magnetic resonance imaging and clinico-pathologic findings in seven dogs. J Vet Intern Med. 2010;24:571–8.
5. Legnani S, Pantchev N, Forlani A, Zini E, Schares G, Balzer J, et al. Emergence of cutaneous neosporosis in a dog receiving immunosuppressive therapy: molecular identification and management. Vet Dermatol. 2016;27(1):49–e14.
6. Dubey JP. Neosporosis in cattle. J Parasitol. 2003a;89:42–56.
7. Dubey JP. Review of *Neospora caninum* and neosporosis in animals.Korean
J Parasitol. 2003b; 41:1–16.
8. Lindsay DS, Dubey JP. Canine neosporosis. J Vet Parasitol. 2000;14:1–11.
9. Rasmussen K, Jensen AL. Some epidemiologic features of canine neosporosis in Denmark. Vet Parasitol. 1996;62:345–9.
10. Lindsay DS, Dubey JP, Duncan RB. Confirmation that the dog is a definitive host for *Neospora caninum*. Vet Parasitol. 1999;82(4):327–33.
11. Dubey JP, Schares G, Ortega-Mora LM. Epidemiology and control of neosporosis and *Neospora caninum*. Clin Microbiol Rev. 2007a;20:323–67.
12. Dubey JP, Jenkins MC, Rajendran C, Miska K, Ferreira LR, Martins J, et al. Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*. Vet Parasitol. 2011;181:382–7.
13. Dubey JP, Vianna MCB, Kwok OCH, Hill DE, Miska KB, Tuo W, et al. Neosporosis in Beagle dogs: clinical signs, diagnosis, treatment, isolation and genetic characterization of *Neospora caninum*. Vet Parasitol. 2007b;149: 158–66.
14. Cavalcante GT, Monteiro RM, Soares RM, Nishi SM, Alves Neto AF, Esmerini Pde O, et al. Shedding of *Neospora caninum* oocysts by dogs fed different tissues from naturally infected cattle. Vet Parasitol. 2011;179:220–3.
15. Gondim LF, McAllister MM, Mateus-Pinilla NE, Pitt WC, Mech LD, Nelson ME. Transmission of *Neospora caninum* between wild and domestic animals. J Parasitol. 2004;90(6):1361–5.
16. Crookshanks JL, Taylor SM, Haines DM, Shelton GD. Treatment of canine pediatric *Neospora caninum* myositis following immunohistochemical identification of tachyzoites in muscle biopsies. Can Vet J. 2007; 48:506–8.
17. Dubey JP, Schares G. Diagnosis of bovine neosporosis. Vet Parasitol. 2006;
140:1–34.
18. Cavalcante GT, Soares RM, Nishi SM, Hagen SCF, Vannucchi CI, Maiorka PC, et al. Experimental infection with *Neospora caninum* in pregnant bitches. Rev Bras Parasitol Vet. 2012; 21(3):232–6.
19. Sharma R, Kimmitt T, Tiwari K, Chikweto A, Thomas D, Lanza Perea M, et al. Serological evidence of antibodies to *Neospora caninum* in stray and owned
Grenadian dogs. Trop Biomed. 2015; 32:286–90.
20. Paradies P, Capelli G, Testini G, Cantacessi C, Trees AJ, Otranto D. Risk factors for canine neosporosis in farm and kennel dogs in southern Italy. Vet Parasitol. 2007; 145:240–4.
21. Ferroglio E, Pasino M, Ronco F, Bena A, Trisciuoglio A. Seroprevalence of antibodies to *Neospora caninum* in urban and rural dogs in north-west Italy. Zoonoses Public Health. 2007; 54:135–9.
22. Palavicini P, Romero JJ, Dolz G, Jiménez AE, Hill DE, Dubey JP. Fecal and serological survey of *Neospora caninum* in farm dogs in Costa Rica. Vet Parasitol. 2007;149:265–70.
23. Antony A, Williamson NB. Prevalence of antibodies *to Neospora caninum* in dogs of rural or urban origin in central New Zealand. NZ Vet J. 2003;51(5):232–7.
24. Nogueira CI, Mesquita LP, Abreu CC, Nakagaki KY, Seixas JN, Bezerra PS, etal. Risk factors associated with seroprevalence of *Neospora caninum* in dogs from urban and rural areas of milk and coffee production in Minas Gerais state, Brazil. Epidemiol Infect. 2013;141(11):2286–93.
25. Goździk K, Wrzesien R, Wielgosz-Ostolska A, Bien J, Kozak-Ljunggren M, Cabaj W. Prevalence of antibodies against *Neospora caninum* in dogs from urban areas in Central Poland. Parasitol Res. 2011;108:991–6.
26. Wanha K, Edelhofer R, Gabler-Eduardo C, Prosl H. Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. Vet Parasitol. 2005;128:189–93.
27. Vaclavek P, Sedlak K, Hurkova L, Vodrazka P, Sebesta R, Koudela B. Serological survey of *Neospora caninum* in dogs in the Czech Republic and a long-term study of dynamics of antibodies. Vet Parasitol. 2007;143:35–41.
28. Kuruca L, Spasojević-Kosić L, Simin S, Savović M, Lauš S, Lalošević V. *Neospora caninum* antibodies in dairy cows and domestic dogs from Vojvodina. Serbia. Parasite. 2013;20:40.
29. Gavrea R, Mircean V, Pastiu A, Cozma V. Epidemiological survey of *Neospora caninum* infection in dogs from Romania. Vet Parasitol. 2012;188(3–4):382–5.
30. Ploneczka K, Mazurkiewicz M. Seroprevalence of *Neospora caninum* in dogs in southwestern Poland. Vet Parasitol. 2008;153:168–71.
31. Capelli G, Nardelli S, di Regalbono AF, Scala A, Pietrobelli M. Sero- epidemiological survey of *Neospora caninum* infection in dogs in north- eastern Italy. Vet Parasitol. 2004;123(3–4):143–8.
32. Ortuño A, Castella J, Almeria S. Seroprevalence of antibodies to *Neospora caninum* in dogs from Spain. J Parasitol. 2002;88:1263–6.
33. Björkman C, Lunden A, Uggla A. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in Swedish dogs. Acta Vet Scand. 1994;35:445–7.
34. Klein BU, Müller E. Seroprevalence of antibodies to *Neospora caninum* in dogs with and without clinical suspicion for neosporosis in Germany. Der praktische Tierarzt. 2001;82:437–40. (in German)
35. Enăchescu V, Ioniţă M, Mitrea IL. Preliminary data in comparative serodiagnostic of *Neospora caninum* in dogs. Scientific Works. C Series. Vet Med. 2012;58(4):128–35.

36. Dubey JP, Hattel AL, Lindsay DS, Topper MJ. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med Assoc. 1988;193:1259–63.
37. Schares G, Rauser M, Söndgen P, Rehberg P, Bärwald A, Dubey JP, et al. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. Int J Parasitol. 2000;30:1123–30.
38. Schares G, Peters M, Wurm R, Bärwald A, Conraths FJ. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet Parasitol. 1998;80:87–98.
39. Azevedo SS, Pena HF, Alves CJ, Guimaraes Filho AA, Oliveira RM, Maksimov P, et al. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in swine from Northeastern Brazil. Rev Bras Parasitol Vet. 2010;19:80–4.
40. Peters M, Wagner F, Schares G. Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. Parasitol Res. 2000;86(1):1–7.
41. Reichel MP, Thornton RN, Morgan PL, Mills RJ, Schares G. Neosporosis in a pup. N Z Vet J. 1998;46(3):106–10.
42. Heckeroth AR, Tenter AM. Immunoanalysis of three litters born to a Doberman bitch infected with *Neospora caninum*. Parasitol Res. 2007;100: 837–46.
43. Dubey JP, Koestner A, Piper RC. Repeated transplacental transmission of *N. caninum* in dogs. J Am Vet Med Assoc. 1990;197:857–60.
44. Björkman C, Sager H, Schares G. Serology. In: Ortega-Mora LM, et al. editors. Protozoal abortion in farm ruminants. Guidelines for diagnosis and control. Wallingford: CAB International;2007. p. 63–75.
45. Peters M, Lütkefels E, Heckeroth AR, Schares G. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. Int J Parasitol. 2001;31(10): 1144–8.
46. Schares G, Pantchev N, Barutzki D, Heydorn AO, Bauer C, Conraths FJ. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int J Parasitol. 2005; 35(14):1525–37.
47. Staub M. Investigation on the prevalence and epidemiology of Neospora infections in dogs in Rhineland-Palatinate, district Mayen-Koblenz (Germany). University, Hannover, Germany: Vet Med Diss; 2004. (in German)
48. Dubey JP, Lindsay DS. Transplacental *Neospora caninum* infection in dogs. Am J Vet Res. 1989; 50(9):1578–9.
49. Dubey JP, Knickman E, Greene CE. Neonatal *Neospora caninum* infections in dogs. Acta Parasitol. 2005; 50:176–9.
50. Dubey JP, Hemphill A, Calero-Bernal R, Schares G. Neosporosis in animals. 1st ed. Boca Raton: Taylor & Francis Inc; 2017.
51. Robbe D, Passarelli A, Gloria A, Di Cesare A, Capelli G, Iorio R, et al. *Neospora caninum* seropositivity and reproductive risk factors in dogs. Exp Parasitol.2016;164:31–5.
52. Ghalmi F, China B, Kaidi R, Losson B. First epidemiological study on exposure to *Neospora caninum* in different canine populations in the Algiers District (Algeria). Parasitol Int. 2009;58:444–50.
53. Kramer L, de Risio L, Tranquilo VM, Magnino S, Genchi C. Analysis of risk factors associated with seropositivity to *Neospora caninum* in dogs. Vet Rec.2004;154:692–3.
54. Collantes-Fernández E, Gómez-Bautista M, Miró G, Álvarez-García G, Perciro- Bueno J, Frisuelos C, et al. Seroprevalence and risk factors associated with *Neospora caninum* infection in different dog populations in Spain. Vet Parasitol. 2008; 152:148–51.
55. Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis.Vet Parasitol. 1996 ;67(1–2):1–59.
56. Wouda W, Dijkstra T, Kramer AM, van Maanen C, Brinkhof JM. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. Int J Parasitol. 1999;29(10):1677–82.
57. Schares G, Heydorn AO, Cüppers A, Conraths FJ, Mehlhorn H. *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. Parasitol Res. 2001; 87(10):808–16.

7. GENERAL DISCUSSION AND OUTLOOK

Findings contained in this doctoral thesis pretended to enrich further our knowledge in different innate effector mechanisms involved in *N. caninum* host-parasite interactions. Therefore, we presented here for first time early host innate immune reactions through NETosis in response against this abortive apicomplexan parasite in three different mammalian hosts, e. g. caprine (chapter 2; Villagra-Blanco et al., 2017a), bovine (chapter 3; Villagra-Blanco et al., 2017b) and cetaceans (please see Supplementary data). Moreover, we assessed current seroepidemiolgical situation on *N. caninum* infections in goat flocks from German Federal State Hesse (chapter 4; Villagra-Blanco et al., 2017c), Costa Rica (chapter 5; Villagra-Blanco et al., 2018a) as well as first investigation on German breeding bitches (chapter 6; (Villagra-Blanco et al., 2018b).

So far, little is known on NETosis-mediated immune reactions against *N. caninum* and to our best knowledge there is only one report on *N. caninum*-triggered canine NETosis *in vitro* published by Wei et al. (2016).

Caprine NETs were described for first time during early innate leucocyte-mediated reactions against *Eimeria ninakohlyakimovae*-, *E. arloingi*- and *E.bovis*-infection performed by Ruiz et al. (2013); Silva et al. (2014) and Muñoz-Caro et al (2016), respectively. Later on, Perez et al. (2016) demonstrated NOX-dependency of caprine monocyte-mediated ETs stimulated with sporozoites of *E. ninakohlyakimovae,* an observation that contrasted with findings obtained with caprine PMN described in Chapter 2, because *N. caninum*-triggered NETosis appears to be influenced by MPO and reacted in a NOX-independent manner since no reduction on NETs formation were

detected during functional inhibition experiments using DPI. Similarly, Wei et al (2016) reported that DPI and SOCE inhibitor, 2-APB, significantly reduced *N. caninum*-triggered NETosis in canine system, affirmation that differs with our results, too. These observations strongly suggest existence of remarkable individual differences within various kind of leukocytes during ETs formation, for example, animal species, nature of cellular organelles from where DNA backbone originates, i. e. nucleus or mitochondria, the amount of vital and suicidal leukocytes inducing ETs within cellular pool (Yousefi et al., 2008) and molecular pathways involved in ETosis (Goldmann and Medina, 2013; Muniz et al., 2013; Hermosilla et al., 2014; Douda et al., 2015, Stoiber et al., 2015).

Furthermore, the participation of cathepsin B and pentraxin were identified for first time in caprine *N. caninum*-induced NETosis (please see Chapter 2). Both enzymes together with NE are implicated in protective immune responses against some bacterial infections (Hahn et al., 2011; Walter et al., 2015); but these enzymes also promote active recruitment of PMN, vascular permeability and are involved during cell embryogenesis, angiogenesis, hematopoiesis, tumor growth and metastasis (Styrt et al., 1989; Vandercappellen et al., 2008; Fortelny et al., 2014; Repnik et al., 2015). In human medicine literature, cathepsins and pentraxins families have been recognized as biomarkers with proteolytic activity associated with lung injuries and inflammation in cases of chronic pulmonary diseases, where NET formation were also seen and discussed as pivotal in pneumonia pathogenesis (Schraufstatter et al., 1984; Borregaard et al., 2007; Cheng and Palaniyar, 2013; Kurt et al., 2015). If these markers could be used as biomarkers during acute ruminant/canine neosporosis needs further clarification.

On the other hand, bovine leukocytes forming ETs (please see Chapter 3), have recently evidenced a crucial role against bacterial (Aulik et al., 2012; Hellenbrand et al., 2013) and viral pathogens (Cortjens et al., 2016). As such, together with phagocytosis and oxidative burst, NETs found in milk represented a novel defense mechanism of bovine PMN in mammary gland during clinical mastitis (Kehrli et al., 1989; Lippolis et al., 2006; Grinberg et al., 2008). Nevertheless, while most of NETosis-related studies in cattle have been concentrated on pathways of NET formation against bacterial pathogens; little attention has been paid to the role of NETs in early host innate immune response against protozoan and metazoan parasites. By now, bovine NETosis has been described to be induced by different protozoan parasites *in vitro* and *in vivo*, such as *Besnoitia besnoiti* (Muñoz-Caro et al., 2014), *E. bovis* (Behrendt et al., 2010; Muñoz-Caro et al., 2015a) and *Cryptosporidium parvum* (Muñoz-Caro et al., 2015b). Like in the mammary gland (Lippolis et al., 2006), ruminant NETosis can occur in other organs such as intestinal mucosa. Consistently, Muñoz-Caro et al. (2016) proved that apicomplexan parasite-triggered NETosis occurred in intestinal tissue samples of *Eimeria*-infected goats and cattle *in vivo*.

Although PMN and other leukocytes, such as monocytes, macrophages and NK cells, seem to play a pivotal functions during bovine neosporosis *in vivo* (Boysen et al., 2006; Canton et al., 2013; Abe et al., 2014; Costa et al., 2014), nothing was so far known on NET-based host innate immune reactions against *N. caninum*. Current data demonstrated vital *N. caninum* tachyzoites as potent inducers of NET formation in cattle (Chapter 3). These structures illustrated a fine network of grosser and slimmer strands of DNA fibers produced by bovine PMN being solidly attached to *N. caninum*

tachyzoites. These SEM findings agreed with other apicomplexan-triggered NETosis observations (Baker et al., 2008; Behrendt et al., 2010; Abi Abdallah et al., 2012; Muñoz-Caro et al., 2014, 2015a, b, 2016; Silva et al., 2014; Reichel et al., 2015; Maksimov et al., 2016). Additionally, resolution of these extracellular chromatin-structures networks by DNase I treatments confirmed DNA basis of *N. caninum*-triggered NETosis (Villagra-Blanco et al., 2017b). Co-localization assays corroborated the concomitant existence of H3, NE and MPO in *N. caninum*-caused NETs confirming molecular characteristics of NETosis in bovine system, which had concordance with other reports on apicomplexan-induced NETosis (Abi Abdallah et al., 2012; Muñoz-Caro et al., 2014, 2015a, b, 2016; Silva et al., 2014; Reichel et al., 2015; Maksimov et al., 2016; Muñoz-Caro et al., 2016). Just as seen in caprine system, pentraxin (PTX), another relevant component of mammalian host innate immune system and responsible of microbial recognition, was here also described for first time as a novel component of bovine *N. caninum*-triggered NETosis (Villagra-Blanco et al., 2017b). This protein is particularly important on inflammatory regulation and female fertility too (Bottazzi et al., 2006; Garlanda et al., 2009; Inforzato et al., 2011; Cieslik and Hrycek, 2012; Daigo and Hamakubo, 2012) and might be related to local defence mechanisms of genital tract as reported elsewhere (Cieslik and Hrycek, 2012; Daigo and Hamakubo, 2012). It is stored in PMN granules and binds with another antimicrobial proteins such as MPO, azurocidin 1, neutrophil defensin 1 and proteinase 3 during formation of NETs and expressed on apoptotic neutrophil surface while fighting against pathogens (Bottazzi et al., 2009; Daigo and Hamakubo, 2012).

Recent analyses on *Eimeria* spp.- and *C. parvum*-induced NETosis also validated a time- (Muñoz-Caro et al., 2015a), dose-dependent (Muñoz-Caro et al., 2015b) manner on production of bovine NETosis and their dependency on NOX, NE, MPO, CD11b, ERK1/2, p38 MAPK and SOCE (Behrendt et al., 2010; Silva et al., 2014; Muñoz-Caro et al., 2015a;b), characteristics that agreed with results obtained in chapter 3, since *N. caninum* tachyzoites-triggered NETosis behaved in a time- and dose-dependent ways and showed a slightly inhibition of bovine *N. caninum*-induced NETosis in presence of specific inhibitors of these enzymes (i. e. DPI, CMK, ABAH, anti-CD11b, UO126, SB202190 and 2-APB, respectively).

Furthermore, the enzyme PAD4 is known to be a vital enzyme participating as catalyzer on hypercitrullination of histones correlated with chromatin decondensation during the extrusion of NETs against pathogens (Li et al., 2010; Leshner et al., 2012; Knight et al., 2013). As such, patients with a lack of adequate PAD4 activities are more susceptible to bacterial infection because of their deficiency in NETs production (Li et al., 2010). Consistently, functional inhibition of this enzyme using Cl-amidine confirmed critical role of PAD4-mediated histone hypercitrunillation for *N. caninum*-triggered NETosis in the bovine system as it resulted in a barely no-significant diminishment of tachyzoite-mediated NETosis.

Additionally, we assayed for role of P2Y2 in bovine *N. caninum*-induced NETosis through functional blockage of P2Y2 via NF-449, which resulted in a decrease of NET production, demonstrating for the first time importance of this energy and metabolic-related receptor in parasite-triggered NETosis (Villagra-Blanco et al., 2017b). Stimulation of purinergic receptors (e. g. P2X, P1, P2Y) generally promotes or inhibits

cell responses through different signaling events in all kinds of mammalian cells and tissue inflammation (Erb et al., 2006; Fredholm et al., 2011). Therefore, it requires the local release of extracellular ATP via pannexin 1 (PANX1) channels and/or autocrine feedback regulation of this mechanism involving G-protein coupled receptors (GPCR), such as P2Y2 (Bao et al., 2013; Gabl et al., 2015). These processes may result in amplification of chemotactic signals through binding to ATP and triggering PMN polarization/activation (Gabl et al., 2015). Given that P2Y2 also regulates PMN adhesion onto endothelial cells through binding of ATP and UTP (Jacobson et al., 2009; Önnheim et al., 2014) and since NETs were recently found adhering to *B. besnoiti*-infected endothelium (Maksimov et al., 2016) further investigation on interrelationship of P2Y2 with other PMN-related energetic pathways and *N. caninum*-induced NETosis will be of interest.

We here demonstrate that NOX participated in *N. caninum* tachyzoite-exposed bovine PMN activation and NETs extrusion since DPI treatments resulted in a decrease of parasite-driven NETosis. Similar findings have been reported from *E. bovis*- (Behrendt et al., 2010; Muñoz-Caro et al., 2015a), *T. gondii*- (Reichel et al., 2015), *B. besnoiti*- (Muñoz-Caro et al., 2014) and *C. parvum*-triggered NETosis (Muñoz-Caro et al., 2015b), emphasizing importance of NOX-influence in apicomplexan-mediated NETosis (Hermosilla et al., 2014; Silva et al., 2016).

In contrast to *E. bovis*- (Behrendt et al., 2010) and *C. parvum*-related NETosis data (Muñoz-Caro et al., 2015b), but according to *B. besnoiti*-induced NETosis (Muñoz-Caro et al., 2014), neither a time- nor a dose-dependency of *N. caninum*-triggered NETosis was demonstrated as significant values were obtained only when each period of

incubation (one and two hours) and last two highest infection ratios (1:3 and 1:4) were compared with negative controls.

Moreover, NET structures were demonstrated being firmly attached to tachyzoites of *N. caninum* thereby supporting quantitative data on efficient tachyzoite entrapment and being immobilized by extruded NETs. Consequently, *in vitro* host cell invasion experiments involving PMN-pre-exposed *N. caninum* tachyzoites unveiled a significant diminishment of their infectivity (40% reduction) for endothelial host cells. The crucial role of NETosis in this process was proven by the fact that reduced infectivity could be restored by DNase I treatments. Moreover, this result proved that tachyzoites were indeed not killed by extruded NETs as also demonstrated for several bacteria (Parseghian and Luhrs, 2006), protozoan parasites (Behrendt et al., 2010; Silva et al., 2014; Muñoz-Caro et al., 2014, 2015a, b; Reichel et al., 2015) as well as metazoan parasites (Chuah et al., 2013; Bonne-Année et al., 2014; Muñoz-Caro et al., 2015b).

Taken together, these data confirm capacity of NETs to hamper *N. caninum* tachyzoites from active host cell invasion *in vitro* by immobilizing them. Taking into account, that tachyzoites of *N. caninum* obligatory must infect endothelial host cells *in vivo* it seems reasonable to speculate that NETosis might represent an efficient defense mechanism during acute cattle neosporosis.

Considering that PMN-derived NOX-activation and subsequent ROS production is known to be Ca^{++}/SOCE-dependent (Sandoval et al., 2007), we here employed the SOCE inhibitor 2-APB in NET-related functional studies, as described elsewhere (Conejeros et al., 2011; Burgos et al., 2011; Muñoz-Caro et al., 2015b). *N. caninum*-triggered NET formation proved to be influenced by SOCE since 2-APB applications

limited tachyzoite-induced NET formation. A Ca^{++}-dependency on NET extrusion was also recently published for *E. bovis*- (Muñoz-Caro et al., 2015a) and *C. parvum*-mediated NETosis (Muñoz-Caro et al., 2015b) and for NETs release by human PMN in response to other non-parasitic stimulators (Gupta et al., 2014).

The pivotal role of Raf-MEK-ERK signaling pathways in the process of NETosis was firstly proven by Hakkim et al. (2011). Here, functional inhibition experiments confirmed importance of ERK1/2- and p38 MAPK-signaling pathways also for *N. caninum*-triggered NET formation. Thus, functional interference of these routes produced a reduction of tachyzoite-mediated NETosis (Muñoz-Caro et al., 2015b). Corresponding findings on ERK1/2 and p38 MAPK have recently been reported on *T. gondii*- (Abi Abdallah et al., 2012), *E. bovis*- (Muñoz-Caro et al., 2015a) and *C. parvum*-induced NETosis (Muñoz-Caro et al., 2015b), evidencing a general role of these signaling pathways in apicomplexan-derived NETosis.

Considering life cycle of *N. caninum,* which includes endogenous parasite stages, such as tachyzoites and bradyzoites, exhibiting an obligatory intracellular replication, extracellular immobilization via NETosis might have implications in host cell invasion and therefore affecting replication and outcome of acute cattle neosporosis as previously postulated for closely related apicomplexan protozoa (Muñoz-Caro et al., 2014; 2015a; Silva et al., 2014, 2016; Hermosilla et al., 2014).

Comparative differences in NETs formation between caprine (Chapter 2) and bovine system (Chapter 3) were principally dose- and time-dependency observed only in bovine PMN and NOX- and SOCE-independent mechanisms observed in goat PMN, probably influenced by alternative activation routes associated with MPO (Stoiber et al.,

2015) and mitochondrial ROS production via calcium-activated potassium channels (e. g. SK3 channels) (Fay et al., 2006; Douda et al., 2015; Khan and Palaniyar, 2017). In addition, variations were seen in shape and size of NET structures: caprine *N. caninum*-triggered NETs were short but robustly thick and solidly attached to tachyzoites with presence of either parasite-stimulated or non-activated PMN, which have concordance to data obtained in caprine ETosis described by Silva et al. (2014) and Perez et al. (2016). Instead, SEM analyses revealed that bovine *N. caninum*-mediated NETs were delicate-PMN derived filaroid networks (long drawn-out fibers) grouping together and forming later a large-, chunky-meshwork of PMN filaments similarly to descriptions reported in other parasite-stimulated ETs studies performed with bovine PMN (Behrendt et al., 2010; Muñoz-Caro et al., 2014; 2015a, b).

In the same way, caprine and bovine *N. caninum*-triggered NETosis showed some similarities too: for example, all activated and non-activated PMN were able to entrap, to phagocyte and to attach tachyzoites in both systems, as it was also described in canine leukocytes stimulated with viable *N. caninum* tachyzoites (Wei et al., 2016). Likewise, MPO activity have been described in ruminant MPO (sheep, goat and cattle) as important component of ETs principally against bacteria (e. g. intramammary infections during clinical and subclinical mastitis) (Lippolis et al 2006; Pisanu et al., 2015) and even parasites (Yildiz et al., 2017). NET-derived mechanisms and their proteolytic activity seem to occur in a MPO-dependent manner, since its specific inhibitor, ABAH demonstrated to reduce *N. caninum*-triggered NETs formation in cattle and goats. This affirmation has concordance with findings reported by other authors (Metzler et al., 2011, 2014), indicating that MPO and ROS are obligatory required for translocation and

release of active NE and other proteases from PMN azurophilic granules into cytosol during NETosis (Metzler et al., 2014).

Interestingly, to best of our knowledge, in Germany there were no serological studies performed neither on presence/absence nor on seroprevalences of *N. caninum*-specific antibodies produced by goats. Thus, in Chapter 4, we focused our interest in the Federal State Hesse (middle Germany), specifically on goat flocks subscribed to Hessian Goat Breeders (Hessischer Ziegenzuchtverband or HZV) or/and to the Veterinary Ambulance Service of Clinic for Obstetrics, Gynecology and Andrology at the JLU-Giessen (Villagra-Blanco et al., 2017c). Most of evidence on *N. caninum* infections in European goats during last years have demonstrated low seroprevalences which did not exceed 10%, e. g. in France (Chartier et al., 2000); Poland (Czopowicz et al., 2011); Czech Republic (Bartova and Sedlak, 2012); Spain (García-Bocanegra et al., 2012; Díaz et al., 2016; Rodríguez-Ponce et al., 2016); Rumania (Iovu et al., 2012) and Italy (Gazzonis et al., 2016). This observation has also concordance with our results in which presence of specific antibodies raised against *N. caninum* in two ELISA-positive animals and one suspicious goat indicated an overall seroprevalence of 0.48%. Later on, immunoblot analysis confirmed two out of these three *N. caninum* positive samples. In most of analyzed flocks, semi-extensive farming conditions together with use of local-resistant goat breeds, such as White German Improved Goat (WDE), German Improved Fawn (BDE) or Thuringian (TWZ), probably diminished obtained seroprevalence in Hesse, as it was previously reported in some studies in other countries (Nasir et al., 2012; Gazzonis et al., 2016; Díaz et al., 2016). Obviously, no statistical association

between seropositive animals and risk factors could be assessed because of low *N. caninum* seroprevalence detected in this study.

Then, *N. caninum* seroprevalence obtained in goats from Costa Rica (7.92%, please see Chapter 5) contrasted with that one obtained in Hessian goats. Despite clinical and pathological evidence of *N. caninum* in one goat farm of Central Region of Costa Rica were previously reported (Dubey et al., 1996), this survey represents first published epidemiological study in this Central American country which evidently includes caprine flocks from all Costa Rican regions (Villagra-Blanco et al., 2018a). In general, Costa Rican goat flocks were focused on dairy production, mostly maintained under semi-intensive conditions and using breeds such as Saanen, Toggenburg, Anglo-Nubian and Alpine (Navarro, 1983; Vallejo, 1989) were found in local caprine production systems. Obtained seroprevalence agrees with those reported in goats along other Latin American countries, e. g. Argentina (6.6%, Moore et al., 2007), Brazil (6.4%, 10.7% and 4.58%, Figliuolo et al., 2004; Andrade et al., 2013; Topazio et al., 2014), Mexico (3.8%, Huerta-Peña et al., 2011) and Grenada (5.8%, Sharma et al, 2015). Furthermore, risk factors associated with goat neosporosis in Costa Rica were evaluated; the univariate analysis considered as *N. caninum*-risks factors: the number of males (multiple bucks), goat kids (groups between 11 to 50 individuals), co-existence of bovine and ovine species in caprine flocks and burial as disposal method of placentas and aborted tissues. These variables agreed with risk factor findings obtained in South- and North-American serological goat studies published by Topazio et al. (2014), Barr et al. (1992) and Lindsay et al. (1995). For example, superficial burial of infected placentas and other aborted tissues represented an important risk issue that agreed with data mentioned by

Dubey et al. (2007a) and Palavicini et al. (2007), since unsafe disposal of infected fetal membranes from *N. caninum*-intermediate hosts enables infection of dogs and coyotes which might have access to them. Particularly in Costa Rica, dairy farm adult dogs have an uncontrollable access to paddocks with highly oocyst-infected pastures and having close contact to *N. caninum*-infected placentas as previously reported (Romero-Zúñiga et al., 2006; Palavicini et al., 2007). These authors suggested that farm dogs (usually older than nine months) frequented these areas searching for fresh food, social and sexual activities.

Flocks with multiple reproductive males and co-existence of cattle and sheep were also related with *N. caninum* infections in final logistic regression models (multivariate analysis) (Villagra-Blanco et al., 2018a). In case of reproductive males, results contrasted with the study performed by Huerta-Peña et al. (2011) in Mexican goat flocks, because bucks were identified as protective factor of *N. caninum* infections. Otherwise, Sharma et al. (2015) mentioned that this risk factor related with higher *N. caninum*-seropositivity in goat flocks from Grenada, although in this former study no significant differences were detected. The role of males on caprine neosporosis is still unclear and controversial, however, venereal transmission of apicomplexan parasites (e. g. *T. gondii*), acquired via semen during natural matinghas been previously described (Wanderley et al., 2015). Meanwhile, co-existence of cattle and sheep in caprine flocks could be associated with large significant high-performance dairy cattle population present in Costa Rica, especially in Central Region, where infections with *N. caninum* have been serologically reported (Romero-Zúñiga et al., 2002, 2003, 2005). Topazio et al. (2015) warned that regions with dairy cattle often faced difficulties to

control bovine neosporosis, which impact directly on geographical and epidemiological distribution of *N. caninum* into small ruminant industry of these regions. Moreover, communal grazing habits of cattle and goats have been described as an additional risk factor which can influence contact of these animals with patent *N. caninum*-infected dogs shedding oocysts, however this variable is still controversial as most authors consider it as a relevant risk factor in caprine neosporosis (Abo-Shehada and Abu-Halaweh, 2010; Liu et al., 2015) whereas others disagree (Machado et al., 2011; Gazzonis et al., 2016).

Concerning detection of *N. caninum*-specific antibodies in German breeding bitch populations (Chapter 6), European studies revealed a great discrepancy on *N. caninum* seroprevalences obtained in various canine populations, e. g. 3.6% in Austria (Wanha et al., 2005), 2.6-19.2% in Czech Republic (Vaclavek et al., 2007), 15.3% in Denmark (Rasmussen and Jensen), 17.2% in Serbia (Kuruca et al., 2013), 32.7% in Romania (Gavrea et al., 2012), 16.36% or 21.7% in Poland (Ploneczka and Mazurkrewicz, 2008 andGoździk et al., 2011, respectively), 10.9% in Italy (Capelli et al., 2004), 12.2% in Spain (Ortuño et al., 2002), 0.5% in Sweden (Björkman et al., 1994) and 4% or 13% in Germany (Klein and Müller, 2001). Collected data in Chapter 6 was focused on a vulnerable dog population since persistent infected bitches may spread this parasite in successive pregnancies by transplacental transmission (Reichel et al., 1998; Heckeroth and Tenter, 2007). Little is known on clinical and economical impact of canine neosporosis in performance of reproductive dogs and their progeny when compared with *N. caninum* infections in breeding cattle (Santolaria et al., 2010; Nazir et al., 2013; Reichel et al., 2013). Furthermore, most widely *N.caninum*-studied infections in dogs

have been performed in farm dogs, since these animals are more likely to hunt, feed on *N. caninum*-infected tissues of domestic and wild animals (Dubey et al., 2017). In similar way, stray dogs represented another highly-exposed dog population described in literature, because these dog population have full access to infected waste and/or prey animals as postulated elsewhere (Benetti et al., 2008; Gennari et al., 2002; Fridlund-Plugge et al., 2008; Yakhchali et al., 2010). Therefore, this study provided for first time seroepidemiological information on breeding bitches, since *N. caninum* seroprevalences in this canid group have been poorly described in literature and therefore demanding further attention (Villagra-Blanco et al., 2018b) to better understand complex epidemiology of canine neosporosis.

Detection of canine neosporosis have been successful using IFAT-, ELISA-, PCR- and immunoblot-techniques due to development of specific IgG *N. caninum* antibodies in infected dogs even in cases of prenatally seroconversion when non-specific IgM antibodies were difficult to distinguish for specific ones (Dubey et al., 1998). Additional immunblot results confirmed all ELISA-positive animals and 10% of remaining negative samples giving support to serological reactions demonstrating presence or absence of *N. caninum* infections in each sample avoiding chance of false positive/negative results (Björkman et al. 2007; Goździk et al., 2011).

In this PhD work, we presented novel results that contribute to build up an innovative knowledgement in differente fields of neosporosis, since the determination of different seroepidemiological prevalences in intermediate (caprine) and definitive hosts (canine) from two different global regions (Germany and Costa Rica), and further better understanding of early host innate immune reactions against this apicomplexan parasite. Nevertheless,

these new insights represented only the beginning of current understanding of complex of host-parasite mechanisms and thus need to be investigated in depth in near future. Further scientific research on abortive nesoporosis is still necessary and next challenges start now by the chance/opportunity of using abundant new analytic tools to contribute in this scientific field.

8. ZUSAMMENFASSUNG

Neosporose ist eine bedeutsame Protozoenerkrankung, die für viele Fortpflanzungsstörungen in der Wiederkäuerhaltung verantwortlich ist. Die Reproduktionsleistung von Schafen und Ziegen kann durch die Infektion mit *N. caninum* durch Aborte, Totgeburten und Geburten lebensschwacher Lämmern stark beeinträchtigt werden. Trotz dieser Bedeutung der Neosporose sind die verfügbaren Untersuchungen zu *N. caninum*-Infektionen in Ziegenpopulationen weltweit rar. Ziel der vorliegenden Studie war es daher Daten zur Seroprävalenz und Informationen zur Pathogenese von *N. caninum* bei Ziegen aus Costa Rica und dem Bundesland Hessen zu erhalten. Da der Hund im Vermehrungszyklus von *N. caninum* eine entscheidene Rolle spielt, wurde zusätzlich die Seroperavalenz in einer ausgewählten Hundepopulation in Deutschland untersucht.

Folgende relevanten Ergebnisse wurden erzielt:

N. caninum-Infektionen wurden bei Ziegen mit niedriger Prävalenz sowohl in Deutschland (Hessen) (0,48%) als auch in Costa Rica (7,9%) gefunden. In Costa Rica konnten seropositive Herden mit gemeinsamen Weidegang mit Rindern und Schafen in Verbindung gebracht werden. Die Daten deuten auf eine niedrige vertikale Übertragungsrate hin. Vorsorgemaßnahmen, wie z. B. die Verwendung von separaten Koppeln und die Kontrolle von heimischen und Wildfleischfressern und deren Fäkalien auf den Weiden, werden für die zukünftige Überwachung der Ziegenneosporose

essentiell sein. In einer ausgewählten Hundepopulation in Deutschland wurden 7 % seropositive Tiere gefunden, die über Western Blot bestätigt werden konnten.

Ein umfassendes Wissen über die Rolle des frühen angeborenen Immunsystems gegen die Protozoenparasitosen ist von entscheidender Bedeutung, um die Mechanismen der Wirt-Parasit-Interaktion und weiterer zellvermittelter adaptiver Immunantworten besser zu verstehen, die in gleicher Weise mit dem klinischen Schweregrad und den klinischen Ergebnissen assoziiert sind. Polymorphonukleare Neutrophilen (PMN) und andere Leukozyten (wie z. B. Eosinophilen, Monozyten und Makrophagen) sind in der Lage, ihre DNA in einem kontrollierten Prozess in die extrazelluläre Umgebung auszustossen und pathogene Mikroorganismen einzufangen, um sogenannte „neutrophil extracellular traps“(NETs) zu bilden. Diese Strukturen zeigten, dass viele *N. caninum*-Tachyzoiten fest angeheftet, immobilisiert und dann eingeschlossen waren, was zum ersten Mal die Bedeutsamkeit von NETosis als Effektormechanismus für die frühe Wirtsimmunantwort während einer *Neospora*-Infektion bei Rind und Ziege belegt. Darüber hinaus wies *N. caninum*-getriggerte NETosis in beiden Spezies eine Co-Lokalisierung von DNA mit antimikrobiellen Molekülen auf, die in den Neutrophilengranula vorhanden sind, wie Histone (H1, H2A / H2B, H3, H4), Neutrophile Elastase (NE), Myeloperoxidase (MPO), Pentraxin (PTX) und Cathepsine bezüglich der klassischen Natur dieses extrazellulären Abwehrmechanismus. Die Hauptunterschiede in der NET-Bildung zwischen diesen Tierarten waren die kurze, aber breite Form der caprinen *N. caninum*-ausgelösten NET-Strukturen; die Dosis- und Zeitabhängigkeit, die nur in den bovinen PMN beobachtet wurde, und die Unabhängigkeit von Nikotinamidadenindinucleotidphosphatoxydase

(NOX) und speicherbetriebenen Calciumeintrittsmechanismen (SOCE), die ausschließlich in der Ziegen-PMN nachgewiesen wurden.

Zusammenfassend tragen die vorliegenden Ergebnisse zum Verständnis der komplexen Wechselwirkungen zwischen Parasit und Wirt *in vitro* / *in vivo* im Verlauf der Wiederkäuer-Neosporose bei und geben erstmals epidemiologische Informationen über die Infektion bei Ziegen in Deutschland und Costa Rica.

Schlüsselwörter: *Neospora caninum*, neutrophil extracellular traps, innate Immunität des Wiederkäuers, Nicotinamidadenindinucleotidphosphatoxidase.

9. ABSTRACT

Neosporosis is serious protozoan disease responsible of many reproductive disorders in ruminant industry. Productivity of sheep and goat is also seriously affected by infectious abortions, stillbirth and birth of weak kids with congenital neosporosis, impacting also small ruminant productive systems. Despite of this background, available investigations on importance of *N. caninum* infections on caprine populations worldwide are still scarce. Here we demonstrated for first time, novel data on seroprevalence and pathogenesis of *N. caninum* in goats from Costa Rica and Federal State Hesse (Germany) collecting current epidemiological information on this abortive apicomplexan parasite and analyzing their impact on caprine production in these two participating countries for their respective geographical areas [Central America (tropical climate) and Central Europe (template climate)]. Since the dog plays a crucial role in the reproductive cycle of *N. caninum*, additionally seroprevalence in a selected dog population in Germany was examined.

The following relevant results were achieved:

N. caninum infections were found in low-prevalence goats in both Germany (Hesse) (0.48%) and Costa Rica (7.9%). In Costa Rica, seropositive flocks with common pastures were associated with cattle and sheep. The data indicates a low vertical transmission rate. Preventive measures, such as the use of separate paddocks and the control of domestic and wild carnivores and their faeces on the pastures, will be essential for the future monitoring of caprine neosporosis. In a selected dog population

in Germany, 7% seropositive animals were found, which could be confirmed by Western Blot.

A wide knowledge on the role of early innate immune system against protozoan parasitoses is crucial to better understand molecular mechanisms involved in resulting host-parasite interactions and further cell-mediated adaptive immune responses, associated in the same way with clinical severity and outcome of this parasitic disease. Polymorphonuclear neutrophils (PMN) and other leukocytes (such as eosinophils, monocytes, and macrophages) are able to expel their DNA in a controlled process into extracellular environment and trapping pathogenic microorganisms by extruding neutrophil extracellular traps (NETs). These structures demonstrated to firmly attach, immobilize and entrap *N. caninum* tachyzoites, evidencing for the first time the importance of NETosis, as an additional effector mechanism in early host immune response during acute bovine and caprine neosporosis. Moreover, in both species, *N. caninum*-triggered NETosis illustrated a co-localization of DNA with antimicrobial molecules present in PMN nucleus and granules, such as histones (H1, H2A/H2B, H3, H4), neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin (PTX) and cathepsins demonstrating classical nature of these extracellular defense structures. The main differences on NETs formation between these species were the short but thick size of caprine *N. caninum*-triggered NET structures; dose- and time-dependency was exclusively observed in bovine PMN whereas nicotinamide adenine dinucleotide phosphate-oxidase (NOX)- and store-operated calcium entry (SOCE)–independency were detected exclusively for caprine PMN.

In general, obtained results have an important implication in the better understanding of complex pasarite-host interactions *in vitro*/*in vivo* during ruminant neosporosis, providing for the first time epidemiological information on the infection in goats from Germany and Costa Rica. Further more investigations on this topic are required, which might reduce initial infection rates during acute phase of neosporosis.

Keywords: *Neospora caninum*, neutrophil extracellular traps, ruminant innate immunity, nicotinamide adenine dinucleotide phosphate-oxidase

10. REFERENCES

ABE, C., TANAKA, S., IHARA, F. AND NISHIKAWA, Y. 2014. Macrophage depletion prior to *Neospora caninum* infection results in severe neosporosis in mice. Clin Vaccine Immunol, 21(8), 1185-8.

ABI ABDALLAH, D.S., LIN, C., BALL, C.J., KING, M.R., DUHAMEL, G.E. AND DENKERS, E.Y. 2012. *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. Infect Immun, 80(2), 768-77.

ABI ABDALLAH, D.S. AND DENKERS, E.Y. 2012. Neutrophils cast extracellular traps in response to protozoan parasites. Front Immunol, 14, 3:382.

ABO-SHEHADA, M.N. AND ABU-HALAWEH, M.M. 2010. Flock-level seroprevalence of, and risk factors for *Neospora caninum* among sheep and goats in northern Jordan. Prev Vet Med, 93(1), 25-32.

ADL, S.M., LEANDER, B.S., SIMPSON, A.G., ARCHIBALD, J.M., ANDERSON, O.R., BASS, D., BOWSER, S.S., BRUGEROLLE, G., FARMER, M.A., KARPOV, S., KOLISKO, M., LANE, C.E., LODGE, D.J., MANN, D.G., MEISTERFELD, R., MENDOZA, L., MOESTRUP, Ø., MOZLEY-STANDRIDGE, S.E., SMIMOV, A.V. AND SPIEGEL, F. 2007. Diversity, nomenclature, and taxonomy of protists. Syst Biol, 56(4), 684-9.

ALGHAMDI, A.S. AND FOSTER, D.N. 2005. Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. Biol Reprod, 73(6), 1174-81.

ALMERÍA, S., FERRER, D., PABÓN, M., CASTELLÁ, J. AND MAŃAS, S. 2002. Red foxes (*Vulpes vulpes*) are a natural intermediate host of *Neospora caninum*. Vet Parasitol,107(4), 287-94.

ALTINCICEK, B., STÖTZEL, S., WYGRECKA, M., PREISSNER, K.T.AND VILCINSKAS, A. 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J Immunol, 181(4), 2705-12.

ANDERSON, M.L., REYNOLDS, J.P., ROWE, J.D., SVERLOW, K.W., PACKHAM, A.E., BARR, B.C.AND CONRAD, P.A. 1997. Evidence of vertical transmission of *Neospora sp* infection in dairy cattle. J Am Vet Med Assoc, 210(8), 1169-72.

ANDRADE, G.S., BRUHN, F.R., ROCHA, C.M., DE SÁ GUIMARÂES, A., GOUVEIA, A.M.AND GUIMARÂES, A.M. 2013. Seroprevalence for *Neospora caninum* in goats of Minas Gerais state, Brazil. Res Vet Sci, 94(3), 584-6.

ANDRIANARIVO, A.G., BARR, B.C., ANDERSON, M.L., ROWE, J.D., PACKHAM, A.E., SVERLOW, K.W. AND CONRAD, P.A. 2001. Immune responses in pregnant cattle and bovine fetuses following experimental infection with *Neospora caninum*. Parasitol Res, 87(10), 817-25.

AULIK, N.A., HELLENBRAND, K.M., KLOS, H. AND CZUPRYNSKI, C.J. 2010. *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun, 78(11), 4454-66.

AULIK, N.A., HELLENBRAND, K.M. AND CZUPRYNSKI, C.J. 2012. *Mannheimia haemolytica* and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. Infect Immun, 80(5), 1923-33.

BAHARI, P., SALEHI, M., SEYEDABADI, M.AND MOHAMMADI, A. 2014. Molecular identification of macroscopic and microscopic cysts of sarcocystis in sheep in north khorasan province, Iran. Int J Mol Cell Med, 3(1), 51-6.

BAILLARGEON, P., FECTEAU, G., PARÉ, J., LAMOTHE, P. AND SAUVÉS, R. 2001. Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of controlling vertical transmission of *Neospora caninum* in cattle. J Am Vet Med Assoc, 218(11), 1803-6.

BAINTON, D.F., ULLYOT, J.L. AND FARQUHAR, M.G. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med,134(4), 907-34.

BAKER, V.S., IMADE, G.E., MOLTA, N.B., TAWDE, P., PAM, S.D., OBADOFIN, M.O., SAGAY, S.A., EGAH, D.Z., IYA, D., AFOLABI, B.B., BAKER, M., FORD, K., FORD, R., ROUX, K.H.AND KELLER, T.C. 3rd. 2008. Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. Malar J, 29, 7:41.

BAO, Y., CHEN, Y., LEDDEROSE, C., LI, L. AND JUNGER, W.G. 2013. Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils. J Biol Chem,288(31), 22650-7.

BARR, B.C., ANDERSON, M.L., BLANCHARD, P.C., DAFT, B.M., KINDE, H. AND CONRAD, P.A. 1990. Bovine fetal encephalitis and myocarditis associated with protozoal infections. Vet Pathol, 27(5), 354-61.

BARR, B.C., ANDERSON, M.L., DUBEY, J.P. AND CONRAD, P.A. 1991. *Neospora*-like protozoal infections associated with bovine abortions. Vet Pathol, 28(2), 110-6.

BARR, B.C., ANDERSON, M.L., WOODS, L.W., DUBEY, J.P. AND CONRAD, P.A. 1992. *Neospora*-like protozoal infections associated with abortion in goats. J Vet Diagn Invest, 4(3), 365-7.

BARRATT, J., AL QASSAB, S., REICHEL, M.P. AND ELLIS, J.T. 2008. The development and evaluation of a nested PCR assay for detection of *Neospora caninum* and *Hammondia heydorni* in feral mouse tissues. Mol Cell Probes, 22(4), 228-33.

BARTELS, C.J., VAN SCHAIK, G., VELDHUISEN, J.P., VAN DEN BORNE, B.H., WOUDA, W. AND DIJKSTRA, T. 2006. Effect of *Neospora caninum*-serostatus on culling, reproductive performance and milk production in Dutch dairy herds with and without a history of Neospora caninum-associated abortion epidemics. Prev Vet Med, 77(3-4), 186-98.

BARTELS, C.J., HUININK, I., BEIBOER, M.L., VAN SCHAIK,G., WOUDA, W., DIJKSTRA, T. AND STEGEMAN, A. 2007. Quantification of vertical and horizontal transmission of *Neospora caninum* infection in Dutch dairy herds. Vet Parasitol, 148(2), 83-92.

BÁRTOVÁ, E. AND SEDLÁK, K. 2012. *Toxoplasma gondii* and *Neospora caninum* antibodies in goats in the Czech Republic. Vet Med Czech, 57, 111-114.

BASSO, W., SCHARES, S., BÄRWALD, A., HERRMANN, D.C., CONRATHS, F.J., PANTCHEV, N., VRHOVEC, M.G. AND SCHARES, G. 2009. Molecular comparison of *Neospora caninum* oocyst isolates from naturally infected dogs with cell culture-derived tachyzoites of the same isolates using nested polymerase chain reaction to amplify microsatellite markers. Vet Parasitol, 160(1-2), 43-50.

BEHRENDT, J.H., HERMOSILLA, C., HARDT, M., FAILING, K., ZAHNER, H. AND TAUBERT, A. 2008. PMN-mediated immune reactions against *Eimeria bovis*. Vet Parasitol, 151(2-4), 97-109.

BEHRENDT, J.H., RUIZ, A., ZAHNER, H., TAUBERT, A. AND HERMOSILLA, C. 2010. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. Vet Immunol Immunopathol, 133(1):1-8.

BEITER, K., WARTHA, F., ALBIGER, B., NORMARK, S., ZYCHLINSKY, A. AND HENRIQUES-NORMARK, B. 2006. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. Curr Biol, 16(4), 401-7.

BENETTI, A.H., TONIOLLO, G.H., SANTOS, T.R., GENNARI, S.M., COSTAC, A.J. AND DIAS, R.A. 2008. Ocorrência de anticorpos anti-*Neospora caninum* em cães no município de Cuiabá, Mato Grosso. Cienc Anim Bras, 9(1), 177-180.

BIANCHI, M., HAKKIM, A., BRINKMANN, V., SILER, U., SEGER, R.A., ZYCHLINSKY, A. AND REICHENBACH, J. 2009. Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood, 114(13), 2619-22.

BJÖRKMAN, C., HOLMDAHL, O.J. AND UGGLA, A. 1997. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. Vet Parasitol, 68(3), 251-60.

BJÖRKMAN, C., SAGER, H. AND SCHARES, G., 2007. Serology. In: Ortega-Mora, L.M., et al. (Eds.), Protozoal Abortion in Farm Ruminants. Guidelines for Diagnosis and Control. CAB International, Wallingford, 63-75.

BONNE-ANNÉE, S., KEREPESI, L.A., HESS, J.A., WESOLOWSKI, J., PAUMET, F., LOK, J.B., NOLAN, T.J. AND ABRAHAM, D. 2014. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval *Strongyloides stercoralis*. Microbes Infect, 16(6), 502-11.

BORREGAARD, N. AND COWLAND, J.B. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood, 89(10), 3503-21.

BORREGAARD, N., SØRENSEN, O.E., THEILGAARD-MÖNCH, K. 2007. Neutrophil granules: a library of innate immunity proteins. Trends Immunol, 28(8), 340-5.

BOTTAZZI, B., BASTONE, A., DONI, A., GARLANDA, C., VALENTINO, S., DEBAN, L., MAINA, V., COTENA, A., MOALLI, F., VAGO, L., SALUSTRI, A., ROMANI, L. AND MANTOVANI, A. 2006. The long pentraxin PTX3 as a link among innate immunity, inflammation, and female fertility. J Leukoc Biol, 79(5), 909-12.

BOTTAZZI, B., GARLANDA, C., COTENA, A., MOALLI, F., JAILLON, S., DEBAN, L. AND MANTOVANI, A. 2009. The long pentraxin PTX3 as a prototypic humoral pattern recognition receptor: interplay with cellular innate immunity. Immunol Rev, 227(1), 9-18.

BOYSEN, P., KLEVAR, S., OLSEN, I. AND STORSET, A.K. 2006. The protozoan *Neospora caninum* directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. Infect Immun, 74(2), 953-60.

BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D.S., WEINRAUCH, Y. AND ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. Science, 303(5663), 1532-5.

BRINKMANN, V. AND ZYCHLINSKY, A. 2007. Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol, 5(8), 577-82.

BRINKMANN, V. AND ZYCHLINSKY, A. 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol, 198(5), 773-83.

BRUNS, S., KNIEMEYER, O., HASENBERG, M., AIMANIANDA, V, NIETZSCHE, S., THYWISSEN, A., JERON, A., LATGÉ, J.P., BRAKHAGE, A.A. AND GUNZER, M. 2010. Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. PLoS Pathog, 6(4), e1000873.

BUCHANAN, J.T., SIMPSON, A.J., AZIZ, R.K., LIU, G.Y., KRISTIAN, S.A., KOTB, M., FERAMISCO, J. AND NIZET, V. 2006. DNase expression allows the pathogen

group A *Streptococcus* to escape killing in neutrophil extracellular traps. Curr Biol, 16(4), 396-400.

BUCHTA, R. 1990. Functional and biochemical properties of ovine neutrophils. Vet Immunol Immunopathol, 24(2), 97-112.

BULUA, A.C., SIMON, A., MADDIPATI, R., PELLETIER, M., PARK, H., KIM, K.-Y., SACK, M.N., KASTNER, D.L. AND SIEGEL, R.M. 2011. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med, 208, 519-533.

BURGOS, R.A., CONEJEROS, I., HIDALGO, M.A., WERLING, D. AND HERMOSILLA, C. 2011. Calcium influx, a new potential therapeutic target in the control of neutrophil-dependent inflammatory diseases in bovines. Vet Immunol Immunopathol, 143(1-2),1-10.

BURVENICH, C., BANNERMAN, D.D., LIPPOLIS, J.D., PEELMAN, L., NONNECKE B.J., KEHRLI, M.E. Jr. AND PAAPE, M.J. 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. J Dairy Sci, 90 Suppl 1, E39-54.

BUXTON, D., MALEY, S.W., PASTORET, P.P., BROCHIER, B. AND INNES, E.A. 1997. Examination of red foxes (*Vulpes vulpes*) from Belgium for antibody to *Neospora caninum* and *Toxoplasma gondii*. Vet Rec, 141(12), 308-9.

CANTÓN, G.J., KATZER, F., BENAVIDES-SILVÁN, J., MALEY, S.W., PALAREA-ALBALADEJO, J., PANG, Y., SMITH, S., BARTLEY, P.M., ROCCHI, M., INNES, E.A.

AND CHIANINI, F. 2013. Phenotypic characterisation of the cellular immune infiltrate in placentas of cattle following experimental inoculation with *Neospora caninum* in late gestation. Vet Res, 44:60.

CAPELLI, G., NARDELLI, S., DI REGALBONO, A.F., SCALA, A. AND PIETROBELLI, M. 2004. Sero-epidemiological survey of *Neospora caninum* infection in dogs in north-eastern Italy. Vet Parasitol, 123(3-4), 143-8.

CHAPMAN, H.D. 2014. Milestones in avian coccidiosis research: a review. Poult Sci, 93(3), 501-11.

CHARTIER, C., BAUDRY, C., LOSSON, B., DE MEERSCHMAN, F., ROMAND, S. AND THUILLIER, P. 2000. La néosporose chez la chèvre: résultats de deux enquêtes sérologiques dans l'Ouest de la France. Le Point Vétérinaire, 31, 65-70.

CHARTIER, C. AND PARAUD, C. 2012. Coccidiosis due to *Eimeria* in sheep and goats: a review. Small Rumin Res, 103, 84–92.

CHENG, O.Z. AND PALANIYAR, N. 2013. NET balancing: a problem in inflammatory lung diseases. Front Immunol, 4:1.

CHUAH, C., JONES, M.K., BURKE, M.L., OWEN, H.C., ANTHONY, B.J., McMANUS, D.P., RAMM, G.A. AND GOBERT, G.N. 2013. Spatial and temporal transcriptomics of *Schistosoma japonicum*-induced hepatic granuloma formation reveals novel roles for neutrophils. J Leukoc Biol, 94(2), 353-65.

CHUAMMTRI, P., OSTOJIĆ, J., ANDREASEN, C.B., REDMOND, S.B., LAMONT, S.J. AND PALIĆ, D. 2009. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. Vet Immunol Immunopathol, 129(1-2), 126-31.

CIEŚLIK, P. AND HRYCEK, A. 2012. Long pentraxin 3 (PTX3) in the light of its structure, mechanism of action and clinical implications. Autoimmunity, 45(2), 119-28.

COGEN, A.L., YAMASAKI, K., MUTO, J., SANCHEZ, K.M., CROTTY ALEXANDER, L., TANIOS, J., LAI Y., KIM, J.E., NIZET, V. AND GALLO, R.L. 2010. *Staphylococcus* epidermidis antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A *Streptococcus*. PLoS One, 5(1), e8557.

CONEJEROS, I., VELÁSQUEZ, Z.D., CARRETTA, M.D., ALARCÓN, P., HIDALGO, M.A. AND BURGOS, R.A. 2012. 2-Aminoethoxydiphenyl borate (2-APB) reduces alkaline phosphatase release, CD63 expression, F-actin polymerization and chemotaxis without affecting the phagocytosis activity in bovine neutrophils. Vet Immunol Immunopathol, 145(1-2), 540-5.

CONRATHS, F.J., BAUER, C. AND BECKER, W. 1996. Detection of antibodies against *Neospora caninum* in cows on Hessian farms with abortion and fertility problems. Dtsch Tierarztl Wochenschr, 103(6), 221-4.

CONRATHS, F.J., SCHARES, G., ORTEGA-MORA, L. M. AND GOTTSTEIN, B. 2007. Control Measures: Neosporosis. CAB International, Oxfordshire, UK. 276–284.

CONSTANTIN, E.M., SCHARES, G., GROSSMANN, E., SAUTER, K., ROMIG, T. AND HARTMANN, S. 2011. Studies on the role of the red fox (*Vulpes vulpes*) as a

potential definitive host of *Neospora caninum*. Berl Munch Tierarztl Wochenschr, 124(3-4), 148-53.

COPPENS, I. 2013. Targeting lipid biosynthesis and salvage in apicomplexan parasites for improved chemotherapies. Nat Rev Microbiol, 11(12), 823-35.

CORTES, H., LEITÂO, A., GOTTSTEIN, B. AND HEMPHILL, A. 2014. A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by *Besnoitia besnoitii*. Parasitology, 141(11), 1406-17.

CORTJENS, B., DE BOER, O.J., DE JONG, R., ANTONIS, A.F., SABOGAL PIŃEROS, Y.S., LUTTER, R., VAN WOENSEL, J.B. AND BEM, R.A. 2016. Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. J Pathol, 238(3), 401-11.

COSTA, R.C., ORLANDO, D.R., ABREU, C.C., NAKAGAKI, K.Y., MESQUITA, L.P., NASCIMENTO, L.C., SILVA, A.C., MAIORKA, P.C., PECONICK, A.P., RAYMUNDO, D.L. AND VARASCHIN, M.S. 2014. Histological and immunohistochemical characterization of the inflammatory and glial cells in the central nervous system of goat fetuses and adult male goats naturally infected with *Neospora caninum*. BMC Vet Res, 10, 291.

CZOPOWICZ, M., KABA, J., SZALUŚ-JORDANOW, O., NOWICKI, M., WITKOWSKI L. AND FRYMUS, T. 2011. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections in goats in Poland. Vet Parasitol, 178(3-4), 339-41.

DAIGO, K. AND HAMAKUBO, T. 2012. Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. Front Immunol, 3, 378.

DAVIES, M.J. 2011. Myeloperoxidase-derived oxidation: mechanisms of biological damage and its prevention. J Clin Biochem Nutr, 48(1), 8-19.

DAVISON, H.C., OTTER, A. AND TREES, A.J. 1999. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. Int J Parasitol, 29(10), 1683-9.

DE MOURA, L., BAHIA-OLIVEIRA, L.M., WADA, M.Y., JONES, J.L., TUBOI, S.H., CARMO, E.H., RAMALHO, W.M., CAMARGO, N.J., TREVISAN, R., GRAÇA, R.M., DA SILVA, A.J., MOURA, I., DUBEY, J.P. AND GARRETT, D.O. 2006. Waterborne toxoplasmosis, Brazil, from field to gene. Emerg Infect Dis,12(2), 326-9.

DÍAZ, P., CABANELAS, E., DÍAZ-CAO, J.M., VIÑA, M., BÉJAR, J.P., PÉREZ-CREO, A., PRIETO, A., LÓPEZ, C.M., PANADERO, R., FERNÁNDEZ, G., DÍEZ-BAÑOS, P. AND MORRONDO, P. 2016. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in goats from north-western Spain. Ann Agric Environ Med, 23(4), 587-590.

DIJKSTRA, T., LAM, T.J., BARTELS, C.J., EYSKER, M. AND WOUDA W. 2008. Natural postnatal *Neospora caninum* infection in cattle can persist and lead to endogenous transplacental infection. Vet Parasitol, 152(3-4), 220-5.

DOUDA, D.N., KHAN, M.A., GRASEMANN, H. AND PALANIYAR, N. 2015. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. Proc Natl Acad Sci USA, 112(9), 2817-22.

DUBEY, J.P., LEATHERS, C.W. AND LINDSAY, D.S. 1989. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. J Parasitol, 75(1), 146-8.

DUBEY, J.P. AND LINDSAY, D.S. 1990. *Neospora caninum* induced abortion in sheep. J Vet Diagn Invest, 2(3), 230-3.

DUBEY, J.P., MILLER, S., LINDSAY, D.S. AND TOPPER, M.J. 1990a. *Neospora caninum*-associated myocarditis and encephalitis in an aborted calf. J Vet Diagn Invest, 2(1); 66-9.

DUBEY, J.P., HARTLEY, W.J, LINDSAY, D.S. AND TOPPER, M.J. 1990b. Fatal congenital Neospora caninum infection in a lamb. J Parasitol, 76(1), 127-30.

DUBEY, J.P. AND PORTERFIELD, M.L. 1990. *Neospora caninum* (Apicomplexa) in an aborted equine fetus. J Parasitol, 76(5), 732-4.

DUBEY, J.P., ACLAND, H.M. AND HAMIR, A.N. 1992. *Neospora caninum* (Apicomplexa) in a stillborn goat. J Parasitol, 78(3), 532-4.

DUBEY, J.P., RIGOULET, J., LAGOURETTE, P., GEORGE, C., LONGEART, L. AND LENET, J.L. 1996. Fatal transplacental neosporosis in a deer (*Cervus eldi siamensis*). J Parasitol, 82(2), 338-9.

DUBEY, J.P., BARR, B.C., BARTA, J.R., BJERKÁS, I., BJÖRKMAN, C., BLAGBURN, B.L., BOWMAN, D.D., BUXTON, D., ELLIS, J.T., GOTTSTEIN, B.,

HEMPHILL, A., HILL, D.E., HOWE, D.K., JENKINS, M.C., KOBAYASHI, Y., KOUDELA, B., MARSH, A.E., MATTSSON, J.G., McALLISTER, M.M., MODRÝ, D., OMATA, Y., SIBLEY, L.D., SPEER, C.A., TREES, A.J., UGGLA, A., UPTON, S.J., WILLIAMS, D.J.AND LINDSAY, D.S. 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int J Parasitol, 32(8), 929-46.

DUBEY, J. P., ZARNKE, R., THOMAS, N. J., WONG, S. K., VAN BONN, W., BRIGGS, M.J., DAVIS, W., EWING, R., MENSEA, M.,. KWOK, O. C. H, ROMAND, S. AND THUELLIEZ, P. 2003. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. Vet. Parasitol. 116**,** 275-296.

DUBEY, J.P. 2004. Toxoplasmosis - a waterborne zoonosis. Vet Parasitol, 126(1-2), 57-72.

DUBEY, J.P., BUXTON, D. AND WOUDA, W. 2006. Pathogenesis of bovine neosporosis. J Comp Pathol, 134(4), 267-89.

DUBEY, J.P. AND SCHARES, G. 2006. Diagnosis of bovine neosporosis. Vet Parasitol, 140(1-2), 1-34.

DUBEY, J.P., SCHARES, G. AND ORTEGA-MORA, L.M. 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin Microbiol Rev, 20(2), 323-67.

DUBEY, J.P. AND SCHARES, G. 2011. Neosporosis in animals-the last five years. Vet Parasitol, 180(1-2), 90-108.

DUBEY, J.P., HEMPHILL, A., CALERO-BERNAL, R. AND SCHARES, G. 2017. Neosporosis in animals. Boca Raton Florida, USA: CRC Press.

ELENI, C., CROTTI, S., MANUALI, E., COSTARELLI, S., FILIPPINI, G., MOSCATI, L. AND MAGNINO, S. 2004. Detection of *Neospora caninum* in an aborted goat foetus. Vet Parasitol, 123(3-4), 271-4.

ERB, L., LIAO, Z., SEYE, C.I. AND WEISMAN, G.A. 2006. P2 receptors: intracellular signaling. Pflugers Arch, 452, 552-62.

ERMERT, D., ZYCHLINSKY, A. AND URBAN, C. 2009. Fungal and bacterial killing by neutrophils. Methods Mol Biol, 470, 293-312.

FAY, A.J., QUAN, X., JAN, Y.N. AND JAN, L.Y. 2006. SK channels mediate NADPH oxidase-independent reactive oxygen species production and apoptosis in granulocytes. Proc Natl Acad Sci USA, 103(46), 17548-53.

FERRE, I., SERRANO-MARTÍNEZ, E., MARTÍNEZ, A., OSORO, K., MATEOS-SANZ, A., DEL-POZO, I., ADURIZ, G., TAMARGO, C., HIDALGO, C.O.AND ORTEGA-MORA, L.M. 2008. Effects of re-infection with *Neospora caninum* in bulls on parasite detection in semen and blood and immunological responses. Theriogenology, 69(7), 905-11.

FIGLIUOLO, L.P., KASAI, N., RAGOZO, A.M., DE PAULA, VS, DIAS, R.A., SOUZA, S.L.AND GENNARI, S.M. 2004. Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in ovine from São Paulo State, Brazil. Vet Parasitol, 123(3-4), 161-6.

FORTELNY, N., COX, J.H., KAPPELHOFF, R., STARR, A.E., LANGE, P.F., PAVLIDIS, P.AND OVERALL, C.M. 2014. Network analyses reveal pervasive functional regulation between proteases in the human protease web. PLoS Biol, 12(5), e1001869.

FRANCIA, M.E. AND STRIEPEN, B. 2014. Cell division in apicomplexan parasites. Nat Rev Microbiol, 12(2), 125-36.

FREDHOLM, B.B., IJZERMAN, A.P., JACOBSON, K.A., LINDEN, J. AND MÜLLER, C.E. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. Pharmacol Rev, 63(1), 1-34.

FRIDLUND-PLUGGE, N., MONTIANI-FERREIRA, F., RICHARTZ, R.R., DAL PIZZOL, J., MACHADO, P.C. Jr., PATRÍCIO, L.F., ROSINELLI, A.S. AND LOCATELLILI-DITTRICH, R. 2008. Frequency of antibodies against *Neospora caninum* in stray and domiciled dogs from urban, periurban and rural areas from Paraná State, Southern Brazil. Rev Bras Parasitol Vet, 17(4), 222-6.

FRIEND, S.C. AND STOCKDALE, P.H. 1980. Experimental *Eimeria bovis* infection in calves: a histopathological study. Can J Comp Med,44(2), 129-40.

FUCHS, T.A., ABED, U., GOOSMANN, C., HURWITZ, R., SCHULZE, I., WAHN, V., WEINRAUCH, Y., BRINKMANN, V. AND ZYCHLINSKY, A. 2007. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol, 176(2), 231-41.

GABL, M., WINTHER, M., WELIN, A., KARLSSON, A., OPREA, T., BYLUND, J., DAHLGREN, C. AND FORSMAN, H. 2015. P2Y2 receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism. Exp Cell Res, 336(2), 242-52.

GABRIEL, C., MCMASTER, W.R., GIRARD, D. AND DESCOTEAUX, A. 2010. *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol, 185(7), 4319-27

GARCÍA-BOCANERA, I., CABEZÓN, O., PABÓN, M., GÓMEZ-GUILLAMÓN, F., ARENAS, A., ALCAIDE, E., SALAS-VEGA, R., DUBEY, J.P.AND ALMERÍA, S. 2012. Prevalence of *Toxoplasma gondii* and *Neospora caninum* antibodies in Spanish ibex (*Capra pyrenaica hispanica*). Vet J, 191(2), 257-60.

GARLANDA, C., MAINA, V., COTENA, A. AND MOALLI, F. 2009. The soluble pattern recognition receptor pentraxin-3 in innate immunity, inflammation and fertility. J Reprod Immunol, 83(1-2), 128-33.

GAVREA, R., MIRCEAN, V., PASTIU, A. AND COZMA, V. 2012. Epidemiological survey of *Neospora caninum* infection in dogs from Romania. Vet Parasitol, 188(3-4), 382-5.

GAZZONIS, A.L., ALVAREZ-GARCÍA, G., ZANZANI, S.A., ORTEGA-MORA, L.M., INVERNIZZI, A. AND MANFREDI, M.T. 2016. *Neospora caninum* infection in sheep and goats from north-eastern Italy and associated risk factors. Small Rumin Res, 140, 7-12.

GENNARI, S.M., YAI, L.E., D'AURIA, S.N., CARDOSO, S.M., KWOK, O.C., JENKINS, M.C. AND DUBEY, J.P. 2002. Occurrence of *Neospora caninum* antibodies in sera from dogs of the city of São Paulo, Brazil. Vet Parasitol,106(2), 177-9.

GIBNEY, E.H., KIPAR, A., ROSBOTTOM, A., GUY, C.S., SMITH, R.F., HETZEL, U., TREES, A.J. AND WILLIAMS, D.J. 2008. The extent of parasite-associated necrosis

in the placenta and foetal tissues of cattle following *Neospora caninum* infection in early and late gestation correlates with foetal death. Int J Parasitol, 38(5), 579-88.

GOHIL, S., HERRMANN, S., GÜNTHER, S. AND COOKE, B.M. 2013. Bovine babesiosis in the 21st century: advances in biology and functional genomics. Int J Parasitol, 43(2), 125-32.

GOLDMANN, O. AND MEDINA, E. 2013. The expanding world of extracellular traps: not only neutrophils but much more. Front Immunol, 3, 420.

GONDIM, L.F., McALLISTER, M.M., PITT, W.C.AND ZEMLICKA, D.E. 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int J Parasitol, 34(2), 159-61.

GOŹDZIK, K., WRZESIEŃ, R., WIELGOSZ-OSTOLSKA, A., BIEŃ, J., KOZAK-LJUNGGREN, M.AND CABAJ, W. 2011. Prevalence of antibodies against *Neospora caninum* in dogs from urban areas in Central Poland. Parasitol Res, 108(4), 991-6.

GREENLEE-WACKER, M.C., RIGBY, K.M., KOBAYASHI, S.D., PORTER, A.R., De LEO, F.R.AND NAUSEEF, W.M. 2014. Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. J Immunol, 192(10), 4709-17.

GRINBERG, N., ELAZAR, S., ROSENSHINE, I. AND SHPIGEL, N.Y. 2008. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*. Infect Immun, 76(6), 2802-7.

GUIMARÃES-COSTA, A.B., NASCIMENTO, M.T., FROMENT, G.S., SOARES, R.P., MORGADO, F.N., CONCEIÇÃO-SILVA, F. AND SARAIVA, E.M. 2009. *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci USA, 106(16), 6748-53.

GUIMARÃES-COSTA, A.B., NASCIMENTO, M.T., WARDINI, A.B., PINTO-DA-SILVA, L.H.AND SARAIVA, E.M. 2012. ETosis: A Microbicidal Mechanism beyond Cell Death. J Parasitol Res, 929743.

GUPTA, A.K., HASLER, P., HOLZGREVE, W., GEBHARDT, S.AND HAHN, S. 2005. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol, 66(11), 1146-54.

GUPTA, A.K., GIAGLIS, S., HASLER, P., AND HAHN, S. 2014. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. PLoS One, 9(5), e97088.

HAHN, S., GIAGLIS, S., CHOWDHURY, C.S., HÖSLI, I. AND HASLER, P. 2013. Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin Immunopathol, 35(4), 439-53.

HAKKIM, A., FUCHS, T.A., MARTINEZ, N.E., HESS, S., PRINZ, H., ZYCHLINSKY, A. AND WALDMANN, H. 2011. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol, 7(2), 75-7.

HALL, C.A., REICHEL, M.P. AND ELLIS, J.T. 2005. *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. Vet Parasitol, 128(3-4), 231-41.

HAMILTON, W.D. AND ZUK, M. 1982. Heritable true fitness and bright birds: a role for parasites? Science, 218(4570), 384-7.

HÄSLER, B., REGULA, G., STÄRK, K.D., SAGER, H., GOTTSTEIN, B.AND REIST, M. 2006a. Financial analysis of various strategies for the control of *Neospora caninum* in dairy cattle in Switzerland. Prev Vet Med, 77(3-4), 230-53.

HÄSLER, B., STÄRK, K.D., SAGER, H., GOTTSTEIN, B.AND REIST, M. 2006b. Simulating the impact of four control strategies on the population dynamics of *Neospora caninum* infection in Swiss dairy cattle. Prev Vet Med, 77(3-4), 254-83.

HECKEROTH, A.R. AND TENTER, A.M. 2007. Immunoanalysis of three litters born to a Doberman bitch infected with *Neospora caninum*. Parasitol Res, 100(4), 837-46.

HELLENBRAND, K.M., FORSYTHE, K.M., RIVERA-RIVAS, J.J., CZUPRYNSKI, C.J.AND AULIK, N.A. 2013. *Histophilus somni* causes extracellular trap formation by bovine neutrophils and macrophages. Microb Pathog, 54, 67-75.

HEMPHILL, A., DEBACHE, K., MONNEY, T., SCHORER, M., GUIONAUD, C., ALAEDDINE, F., MUELLER, N. AND MUELLER, J. 2013. Proteins mediating the *Neospora caninum*-host cell interaction as targets for vaccination. Front Biosci (Elite Ed), 5, 23-36.

HERMOSILLA, C., ZAHNER, H. AND TAUBERT, A. 2006. *Eimeria bovis* modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. Int J Parasitol, 36(4), 423-31.

HERMOSILLA, C., CARO, T.M., SILVA, L.M., RUIZ, A.AND TAUBERT, A. 2014. The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology, 141(11), 1489-98.

HILALI, M., ROMAND, S., THULLIEZ, P., KWOK, O.C.H AND DUBEY, JP. 1998. Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in sera from camels from Egypt. Vet Parasitol, 75, 269-71.

HORAK, I.G., BEAUCOURNU, J.C.AND BRAACK, L.E. 2004. Parasites of domestic and wild animals in South Africa. XLIV. Fleas (Insecta: Siphonaptera*:* Pulicidae) collected from 15 carnivore species. Onderstepoort J Vet Res, 71(1), 9-14.

HUERTA-PEÑA, J.C., MARTÍNEZ-HERRERA, D.I., PENICHE-CARDEÑA, A.E., VILLANUEVA-VALENCIA, M., HERNÁNDEZ-RUIZ, S.G., VILLAGÓMEZ-CORTÉS, J.A., BARRADAS-PIÑA, F.T., MORALES-ÁLVAREZ, J.F. AND FLORES CASTRO, R. 2011. Seroprevalence and risk factors associated with *Neospora caninum* in goats from municipalities of the central region of Veracruz. Tropical and Subtropical Agroecosystems, 13, 445-454.

HUONG, L.T., LJUNGSTRÖM, B.L., UGGLA, A., BJÖRKMAN, C. 1998. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes in southern Vietnam. Vet Parasitol, 75(1), 53-7.

INFORZATO, A., JAILLON, S., MOALLI, F., BARBATI, E., BONAVITA, E., BOTTAZZI, B., MANTOVANI, A.AND GARLANDA, C. 2011. The long pentraxin PTX3 at the crossroads between innate immunity and tissue remodelling. Tissue Antigens, 77(4), 271-82.

INNES, E.A., WRIGHT, S., BARTLEY, P., MALEY, S., MACALDOWIE, C., ESTEBAN-REDONDO, I. AND BUXTON, D. 2005. The host-parasite relationship in bovine neosporosis. Vet Immunol Immunopathol, 108(1-2), 29-36.

IOVU, A., GYÖRKE, A., MIRCEAN, V., GAVREA, R. AND COZMA, V. 2012. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in dairy goats from Romania. Vet Parasitol, 186, 470-474.

JACOBSON, K.A., IVANOV, A.A., DE CASTRO, S., HARDEN, T.K. AND KO, H. 2009. Development of selective agonists and antagonists of P2Y receptors. Purinergic Signal, 5(1), 75-89.

JANEWAY, C.K., TRAVERS, P., WALPORT, M. AND SCHLOMCHIK, M. 2001. Failure of the host defense mechanism. *In* P. Austin and E. Lawrence (eds) Immunobiology the Immune System in Health and Disease. Garland, New York, 425-469.

KEHRLI, M.E. Jr, NONNECKE, B.J., WOOD, R.L. AND ROTH, J.A. 1989. In vivo effects of a thymosin alpha 1-containing colostral whey product on neutrophils and lymphocytes from lactating cows without and with experimentally induced *Staphylococcus aureus* mastitis. Vet Immunol Immunopathol, 20(2), 149-63.

KENNEDY, A.D. AND DeLEO, F.R. 2009. Neutrophil apoptosis and the resolution of infection. Immunol Res, 43(1-3), 25-61.

KHAN, M.A. AND PALANIYAR, N. 2017. Transcriptional firing helps to drive NETosis. Sci Rep, 7, 41749.

KING, J.S., SLAPETA, J., JENKINS, D.J., AL-QASSAB, S.E., ELLIS, J.T.AND WINDSOR, P.A. 2010. Australian dingoes are definitive hosts of *Neospora caninum*. Int J Parasitol, 40(8), 945-50.

KLEIN, B.U. AND MÜLLER, E. 2001. Seroprävalenz von Antikörpern gegen *Neospora caninum* bei Hunden mit und ohne klinischem Neosporoseverdacht in Deutschland. Prakt Tierarzt, 82, 437-440.

KNIGHT, J.S., ZHAO, W., LUO, W., SUBRAMANIAN, V., O'DELL, A.A., YALAVARTHI, S., HODGIN, J.B., EITZMAN, D.T., THOMPSON, P.R. AND KAPLAN, M.J. 2013. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. J Clin Invest, 123(7), 2981-93.

KOBAYASHI, S.D., VOYICH, J.M., BURLAK, C.AND DeLEO, F.R. 2005. Neutrophils in the innate immune response. Arch Immunol Ther Exp (Warsz), 53(6), 505-17.

KOBAYASHI, S.D., MALACHOWA, N. AND DeLEO, F.R. 2017. Influence of Microbes on Neutrophil Life and Death. Front Cell Infect Microbiol, 7, 159.

KURT, O.K., TOSUN, M., KURT, E.B.AND TALAY, F. 2015. Pentraxin 3 as a novel biomarker of inflammation in chronic obstructive pulmonary disease. Inflammation, 38(1), 89-93.

KURUCA, L., SPASOJEVIĆ-KOSIĆ, L, SIMIN, S., SAVOVIĆ, M., LAUŠ, S. AND LALOŠEVIĆ, V. 2013. *Neospora caninum* antibodies in dairy cows and domestic dogs from Vojvodina, Serbia. Parasite, 20:40.

LALLY, N.C., JENKINS, M.C. AND DUBEY, J.P., 1996a. Development of a polymerase chain reaction assay for the diagnosis of neosporosis using the *Neospora caninum* 14-3-3 gene. Mol Biol Parasites, 75, 169-178.

LANDMANN, J.K., JILLELLA, D., O'DONOGHUE, P.J.AND McGOWAN, M.R. 2002. Confirmation of the prevention of vertical transmission of *Neospora caninum* in cattle by the use of embryo transfer. Aust Vet J, 80(8), 502-3.

LANDMANN, J.K., GUNN, A.A., O'DONOGHUE, P.J., TRANTER, W.P. AND McGOWAN, M.R. 2011. Epidemiology and impact of *Neospora caninum* infection in three Queensland tropical dairy herds. Reprod Domest Anim, 46(4), 734-7.

LARSON, R.L., HARDIN, D.K. AND PIERCE, V.L. 2004. Economic considerations for diagnostic and control options for *Neospora caninum*-induced abortions in endemically infected herds of beef cattle. J Am Vet Med Assoc, 224(10), 1597-604.

LEE, A., WHYTE, M.K. AND HASLETT, C. 1993. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol, 54(4), 283-8.

LEMBERGER, K.Y., GONDIM, L.F., PESSIER, A.P., McALLISTER, M.M. AND KINSEL, M.J. 2005. *Neospora caninum* infection in a free-ranging raccoon (*Procyon lotor*) with concurrent canine distemper virus infection. J Parasitol, 91(4), 960-1.

LESHNER, M., WANG, S., LEWIS, C., ZHENG, H., CHEN, X.A., SANTY, L. AND WANG, Y. 2012. PAD4 mediated histone hypercitrullination induces

heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. Front Immunol, 3, 307.

LI, P., LI, M., LINDBERG, M.R., KENNETT, M.J., XIONG, N. AND WANG, Y. 2010. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med, 207(9), 1853-62.

LI, Y., CHEN, Z., LIU, Z., LIU, J., YANG, J., LI, Q., LI, Y., CEN, S., GUAN, G., REN, Q., LUO, J. AND YIN. H. 2014. Molecular identification of *Theileria* parasites of northwestern Chinese *Cervidae*. Parasit Vectors, 7, 225.

LINDSAY, D.S., RIPPEY, N.S., POWE, T.A., SARTIN, E.A., DUBEY, J.P. AND BLAGBURN, B.L. 1995. Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of *Neospora caninum*. Am J Vet Res, 56(9), 1176-80.

LINDSAY, D.S., UPTON, S.J. AND DUBEY, J.P. 1999. A structural study of the *Neospora caninum* oocyst. Int J Parasitol, 29(10), 1521-34.

LIPPOLIS, J.D., REINHARDT, T.A., GOFF, J.P. AND HORST, R.L. 2006. Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. Vet Immunol Immunopathol, 113(1-2), 248-55.

LIU, Z.K., LI, J.Y. AND PAN, H. 2015. Seroprevalence and risk factors of *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants in China. Prev Vet Med,118(4), 488-92.

LÓPEZ-GATIUS, F., SANTOLARIA, P., YÁNIZ, J.L., GARBAYO, J.M. AND ALMERÍA, S. 2005. The use of beef bull semen reduced the risk of abortion in *Neospora*-seropositive dairy cows. J Vet Med B Infect Dis Vet Public Health, 52(2), 88-92.

LÖGTERS, T., MARGRAF, S., ALTRICHTER, J., CINATL, J., MITZNER, S., WINDOLF, J.AND SCHOLZ, M. 2009. The clinical value of neutrophil extracellular traps. Med Microbiol Immunol, 198(4), 211-9.

MACALDOWIE, C., MALEY, S.W., WRIGHT, S., BARTLEY, P., ESTEBAN-REDONDO, I., BUXTON, D. AND INNES, E.A. 2004. Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. J Comp Pathol, 131(2-3), 142-56.

MACHADO, G.P., KIKUTI, M., LANGONI, H. AND PAES, A.C. 2011. Seroprevalence and risk factors associated with neosporosis in sheep and dogs from farms. Vet Parasitol, 182(2-4), 356-8.

MAKSIMOV, P., HERMOSILLA, C., KLEINERTZ, S., HIRZMANN, J.AND TAUBERT, A. 2016. *Besnoitia besnoiti* infections activate primary bovine endothelial cells and promote PMN adhesion and NET formation under physiological flow condition. Parasitol Res, 115(5), 1991-2001.

MALEY, S.W., BUXTON, D., RAE, A.G., WRIGHT, S.E., SCHOCK, A., BARTLEY, P.M., ESTEBAN-REDONDO, I., SWALES, C., HAMILTON, C.M., SALES, J.AND INNES, E.A. 2003. The pathogenesis of neosporosis in pregnant cattle: inoculation at mid-gestation. J Comp Pathol, 129(2-3), 186-95.

MARSH, A.E., BARR, B.C., MADIGAN, J., LAKRITZ, J., NORDHAUSEN, R. AND CONRAD, P.A. 1996. Neosporosis as a cause of equine protozoal myeloencephalitis. J Am Vet Med Assoc. 209(11), 1907-13.

McALLISTER, M.M., DUBEY, J.P., LINDSAY, D.S., JOLLEY, W.R., WILLS, R.A. AND McGUIRE, A.M. 1998. Dogs are definitive hosts of *Neospora caninum*. Int J Parasitol, 28(9), 1473-8.

McALLISTER, M.M., BJÖRKMAN, C., ANDERSON-SPRECHER, R., ROGERS, D.G. 2000. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. J Am Vet Med Assoc, 217(6), 881-7.

McCANN, C.M., McALLISTER, M.M., GONDIM, L.F., SMITH, R.F., CRIPPS, P.J., KIPAR, A., WILLIAMS, D.J. AND TREES, A.J. 2007. *Neospora caninum* in cattle: experimental infection with oocysts can result in exogenous transplacental infection, but not endogenous transplacental infection in the subsequent pregnancy. Int J Parasitol,37(14), 1631-9.

METZLER, K.D., FUCHS, T.A., NAUSEEF, W.M., REUMAUX, D., ROESLER, J., SCHULZE, I., WAHN, V., PAPAYANNOPOULOS, V. AND ZYCHLINSKY, A. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood, 117(3), 953-9.

MOORE, D.P., De YANIZ, M.G., ODEÓN, A.C., CANO, D., LEUNDA, M.R., SPÄTH, E.A.J. AND CAMPERO, C.M. 2007. Serological evidence of *Neospora caninum* infections in goats from La Rioja Province, Argentina. Small Rumin Res, 73, 256-258.

MOORE, D.P., PÉREZ, A., AGLIANO, S., BRACE, M., CANTÓN, G., CANO, D., LEUNDA, M.R., ODEÓN, A.C., ODRIOZOLA, E.AND CAMPERO, C.M. 2009. Risk factors associated with *Neospora caninum* infections in cattle in Argentina. Vet Parasitol, 161(1-2), 122-5.

MORENO, B., COLLANTES-FERNÁNDEZ, E., VILLA, A., NAVARRO, A., REDIGOR-CERRILLO, J.AND ORTEGA-MORA, L.M. 2012. Occurrence of *Neospora caninum* and *Toxoplasma gondii* infections in ovine and caprine abortions. Vet Parasitol,187(1-2), 312-8.

MORIN-ADELINE, V., VOGELNEST, L., DHAND, N.K., SHIELS, M., ANGUS, W. AND ŠLAPETA, J. 2011. Afternoon shedding of a new species of *Isospora* (Apicomplexa) in the endangered Regent Honeyeater (*Xanthomyza phrygia*). Parasitology, 138(6), 713-24.

MUNIZ, V.S., BAPTISTA-DOS-REIS, R. AND NEVES, J.S. 2013. Functional extracellular eosinophil granules: a bomb caught in a trap. Int Arch Allergy Immunol, 162(4), 276-82.

MUÑOZ-CARO, T., SILVA, L.M., RITTER, C., TAUBERT, A., HERMOSILLA, C. 2014. *Besnoitia besnoiti* tachyzoites induce monocyte extracellular trap formation. Parasitol Res, 113(11), 4189-97.

MUÑOZ-CARO, T., MENA HUERTAS, S.J., CONEJEROS, I., ALARCÓN, P., HIDALGO, M.A., BURGOS, R.A., HERMOSILLA, C.AND TAUBERT, A. 2015a. *Eimeria bovis*-triggered neutrophil extracellular trap formation is CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent. Vet Res, 46, 23.

MUÑOZ-CARO, T., RUBIO, R.M.C., SILVA, L.M., MAGDOWSKI, G., GÄRTNER, U., McNEILLY, T.N., TAUBERT, A.AND HERMOSILLA, C. 2015b Leucocyte-derived extracellular trap formation significantly contributes to *Haemonchus contortus* larval entrapment. Parasit Vectors, 8, 607.

MUÑOZ-CARO, T., SILVA, L.M., RENTERÍA-SOLIS, Z., HERMOSILLA, C. AND TAUBERT, A. 2016. Neutrophil extracellular traps in the intestinal mucosa of *Eimeria*-infected animals. Asian Pac J Trop Med, 6, 301-307.

MURPHY, M.P. 2012. Modulating mitochondrial intracellular location as a redox signal. Sci Signal, 5(242), pe39.

MÜLLER, J. AND HEMPHILL, A. 2013. In vitro culture systems for the study of apicomplexan parasites in farm animals. Int J Parasitol,43(2), 115-24.

NAKAGAKI, K.Y.R., ABREU, C.C., COSTA, R.C., ORLANDO, D.R., FREIRE, L.R., BRUHN, F.R.P., PECONICK, A.P., WOUTERS, F., WOUTERS, A.T.B., RAYMUNDO, D.L. AND VARASCHIN, M.S. 2016. Lesions and distribution of *Neospora caninum* in tissues of naturally infected female goats. Small Ruminant Research, 140, 57-62.

NASIR, A., ASHRAF, M., KHAN, M.S., JAVEED, A., YAQUB, T., AVAIS, M. AND REICHEL, M.P. 2012. Prevalence of *Neospora caninum* antibodies in sheep and goats in Pakistan. J Parasitol, 98(1), 213-5.

NATHAN, C. 2006. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol, 6(3), 173-82.

NAVARRO, H.D. 1983. Bioeconomic evaluation of semi-comercial goat production systems in Costa Rica. Thesis. Universidad de Costa Rica.

NETO, A.F.A., BANDINI, L.A., NISHI, S.M., SOARES, R.M., DRIEMEIER, D., ANTONIASSI, N.A.B., SCHARES, G. AND GENNARI, S.M. 2011. Viability of sporulated oocysts of *Neospora caninum* after exposure to different physical and chemical treatments. Journal of Parasitology, 97(1), 135-139.

NEWTON, K. AND DIXIT, V.M. 2012. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol, 4(3).

OGINO, H., WATANABE, E., WATANABE, S., AGAWA, H., NARITA, M., HARITANI, M. AND KAWASHIMA, K. 1992. Neosporosis in the aborted fetus and newborn calf. J Comp Pathol, 107(2), 231-7.

OMATA, Y., UMESHITA, Y., WATARAI, M., TACHIBANA, M., SASAKI, M., MURATA, K., YAMADA, T.K., 2006. Investigation for presence of *Neospora caninum, Toxoplasma gondii* and *Brucella*-species infection in killer whales (*Orcinus orca*) mass stranded on the coast of Shiretoko, Hokkaido. Jpn. J. Vet. Med. Sci. 68, 523e526

ORTEGA-MORA, L.M., FERNANDEZ-GARCIA, A. AND GOMEZ-BAUTISTA, M. 2006. Diagnosis of bovine neosporosis: Recent advances and perspectives. Acta Parasitol, 51, 1-14.

ORTUÑO, A., CASTELLÁ, J. AND ALMERÍA, S. 2002. Seroprevalence of antibodies to *Neospora caninum* in dogs from Spain. J Parasitol, 88(6), 1263-6.

OSAWA, T., WASTLING, J., MALEY, S., BUXTON, D. AND INNES, E.A. 1998. A multiple antigen ELISA to detect *Neospora*-specific antibodies in bovine sera, bovine foetal fluids, ovine and caprine sera. Vet Parasitol, 79(1), 19-34.

OSORO, K., ORTEGA-MORA, L.M., MARTÍNEZ, A., SERRANO-MARTÍNEZ, E. AND FERRE, I. 2009. Natural breeding with bulls experimentally infected with *Neospora caninum* failed to induce seroconversion in dams. Theriogenology, 71(4), 639-42.

ÖNNHEIM, K., CHRISTENSON, K., GABL, M., BURBIEL, J.C., MÜLLER, C.E., OPREA, T.I., BYLUND, J., DAHLGREN, C. AND FORSMAN, H. 2014. A novel receptor cross-talk between the ATP receptor P2Y2 and formyl peptide receptors reactivates desensitized neutrophils to produce superoxide. Exp Cell Res, 323(1), 209-17.

PABÓN, M., LÓPEZ-GATIUS, F., GARCÍA-ISPIERTO, I., BECH-SÀBAT, G., NOGAREDA, C. AND ALMERÍA, S. 2007. Chronic *Neospora caninum* infection and repeat abortion in dairy cows: a 3-year study. Vet Parasitol, 147(1-2), 40-6.

PALAVICINI, P., ROMERO, J.J., DOLZ, G., JIMÉNEZ, A.E., HILL, D.E.AND DUBEY, J.P. 2007. Fecal and serological survey of *Neospora caninum* in farm dogs in Costa Rica. Vet Parasitol, 149(3-4), 265-70.

PALIĆ, D., OSTOJIĆ, J., ANDREASEN, C.B. AND ROTH, J.A. 2007. Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol. 31(8), 805-16.

PARSEGHIAN, M.H. AND LUHRS, K.A. 2006. Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity. Biochem Cell Biol, 84(4), 589-604.

PORTO, W.J., REGIDOR-CERRILLO, J., KIM, P.C.P., BENAVIDES, J., SILVA, A.C., HORCAJO, P.,OLIVEIRA, A.A.,FERRE, I., MOTA, R.A.AND ORTEGA-MORA, L.M. 2016. Experimental caprine neosporosis: the influence of gestational stage on the outcome of infection. Vet Res, 47, 29.

PORTO, W.J., HORCAJO, P., KIM, P.C.P., REGIDOR-CERRILLO, J., ROMÃO, E.A., ÁLVAREZ-GARCÍA, G., MESQUITA, E.P., MOTA, R.A.AND ORTEGA-MORA, L.M. 2017. Peripheral and placental immune responses in goats after primoinfection with *Neospora caninum* at early, mid and late gestation. Vet Parasitol, 242, 38-43.

PARÉ, J., HIETALA, S.K. AND THURMOND, M.C. 1995. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora sp.* infection in cattle. J Vet Diagn Invest, 7(3), 352-9.

PARÉ, J., THURMOND, M.C. ANDHIETALA, S.K.1996. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can J Vet Res, 60,133-139.

PARKER, H., DRAGUNOW, M., HAMPTON, M.B., KETTLE, A.J., WINTERBOURN, C.C. 2012. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. J Leukoc Biol, 92(4), 841-9.

PÉREZ, E., GONZALEZ, O., DOLZ, G., MORALES, J.A., BARR, B. AND CONRAD, P.A. 1998. First report of bovine neosporosis in dairy cattle in Costa Rica. Vet Rec, 142(19), 520-1.

PÉREZ, D., MUÑOZ, M.C., MOLINA, J.M., MUÑOZ-CARO, T., SILVA, L.M., TAUBERT, A., HERMOSILLA, C. AND RUIZ, A. 2016. *Eimeria ninakohlyakimovae* induces NADPH oxidase-dependent monocyte extracellular trap formation and upregulates IL-12 and TNF-α, IL-6 and CCL2 gene transcription. Vet Parasitol, 227, 143-50.

PETERS, M., LÜTKEFELS, E., HECKEROTH, A.R. AND SCHARES, G. 2001. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. Int J Parasitol, 31(10), 1144-8.

PLONECZKA, K. AND MAZURKIEWICZ, M. 2008. Seroprevalence of *Neospora caninum* in dogs in south-western Poland. Vet Parasitol, 153(1-2),168-71.

RAMOS-KICHIK, V., MONDRAGÓN-FLORES, R., MONDRAGÓN-CASTELÁN, M., GONZALEZ-POZOS, S., MUÑIZ-HERNANDEZ, S., ROJAS-ESPINOSA, O., CHACÓN-SALINAS, R., ESTRADA-PARRA, S. AND ESTRADA-GARCÍA, I. 2009. Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis.*Tuberculosis (Edinb), 89(1), 29-37.

RASMUSSEN, K. AND JENSEN, AL. 1996. Some epidemiologic features of canine neosporosis in Denmark. Vet Parasitol, 62(3-4), 345-9.

RAUSCH, P.G. AND MOORE, T.G. 1975. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. Blood. 46(6), 913-9.

REICHEL, M.P. AND ELLIS, J.T. 2002. Control options for *Neospora caninum* infections in cattle-current state of knowledge. N Z Vet J, 50(3), 86-92.

REICHEL, M.P. AND ELLIS, J.T. 2006. If control of *Neospora caninum* infection is technically feasible does it make economic sense? Vet Parasitol, 142(1-2), 23-34.

REICHEL, M.P., ELLIS, J.T. AND DUBEY, J.P. 2007. Neosporosis and hammondiosis in dogs. J Small Anim Pract, 48(6), 308-12.

REICHEL, M.P., AYANEGUI-ALCÉRRECA, M.A., GONDIM, L.F. AND ELLIS, J.T. 2013. What is the global economic impact of *Neospora caninum* in cattle–the billion dollar question. Int J Parasitol, 43:133-142.

REICHEL, M.P., McALLISTER, M.M., POMROY, W.E., CAMPERO, C., ORTEGA-MORA, L.M. AND ELLIS, JT. 2014. Control options for *Neospora caninum* is there anything new or are we going backwards? Parasitology, 141(11), 1455-70.

REPNIK, U., STARR, A.E., OVERALL, C.M. AND TURK, B. 2015. Cysteine cathepsins activate ELR chemokines and inactivate non-ELR chemokines. J Biol Chem, 290(22), 13800-11.

RIGANTI, C., GAZZANO, E., POLIMENI, M., COSTAMANGA, C., BOSIA, A. AND GHIGO, D. 2004. Diphenyleneiodonium inhibits the cell redox metabolism and induces oxidative stress. J Biol Chem, 279, 47726-47731.

RODRIGUES, A.A., GENNARI, S.M., Aguiar, D.M., SREEKUMAR, C., HILL, D.E., MISKA, K.B., VIANNA, M.C. AND DUBEY, J.P. 2004. Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Vet Parasitol, 124(3-4), 139-50.

RODRÍGUEZ-PONCE, E., CONDE, M., CORBERA, J.A., JABER, J.R., VENTURA, M.R. AND GUTIÉRREZ, C. 2016. Serological survey of antibodies to *Toxoplasma gondii* and *Neospora caninum* in goat population in Canary Islands (*Macaronesia Archipelago*, Spain). Small Rumin Res, 147, 73-76.

ROMERO, J.J., PEREZ, E., DOLZ, G. AND FRANKENA, K. 2002. Factors associated with *Neospora caninum* serostatus in cattle of 20 specialised Costa Rican dairy herds. Prev Vet Med 53, 263-73.

ROMERO, J.J. AND FRANKENA, K. 2003. The effect of the dam-calf relationship on serostatus to *Neospora caninum* on 20 Costa Rican dairy farms. Vet Parasitol, 114, 159-71.

ROMERO, J.J., BREDA, S.V., VARGAS, B., DOLZ, G. AND FRANKENA, K. 2005. Effect of neosporosis on productive and reproductive performance of dairy cattle in Costa Rica. Theriogenology, 64(9), 1928-39.

RUIZ, A., MUÑOZ, M.C., MOLINA, J.M., HERMOSILLA, C., ANDRADA, M., LARA, P., BORDÓN, E., PÉREZ, D., LÓPEZ, A.M., MATOS, L., GUEDES, A.C, FALCÓN, S., FALCÓN, Y., MARTÍN, S. AND TAUBERT, A. 2014. Immunization with *Eimeria ninakohlyakimovae*-live attenuated oocysts protect goat kids from clinical coccidiosis. Vet Parasitol, 199(1-2), 8-17.

SAHOO, G., MORE, T. AND SINGH, V.K. 1998. A comparative study on certain enzymes of the granulocyte from different ruminant species. Comp Immunol Microbiol Infect Dis, 21(4), 319-25.

SANDOVAL, A.J., RIQUELME, J.P., CARRETTA, M.D., HANCKE, J.L., HIDALGO, M.A. AND BURGOS, R.A. 2007. Store-operated calcium entry mediates intracellular alkalinization, ERK1/2, and Akt/PKB phosphorylation in bovine neutrophils. J Leukoc Biol, 82(5), 1266-77.

SANTOLARIA, P., LÓPEZ-GATIUS, F., YÁNIZ, J., GARCÍA-ISPIERTO, I., NOGAREDA, C., BECH-SÁBAT, G., SERRANO, B. AND ALMERÍA, S. 2009. Early postabortion recovery of *Neospora*-infected lactating dairy cows. Theriogenology, 72(6), 798-802.

SANTOLARIA, P., LÓPEZ-GATIUS, F., GARCÍA-ISPIERTO, I., BECH-SÁBAT, G., ANGULO, E., CARRETERO, T., SÁNCHEZ-NADAL, J.A. AND YÁNIZ, J. 2010. Effects of cumulative stressful and acute variation episodes of farm climate conditions on late embryo/early fetal loss in high producing dairy cows. Int J Biometeorol, 54(1), 93-8.

SCHARES, G., PETERS, M., WURM, R., TACKMANN, K., HENNING, K. AND CONRATHS, F.J. 1997. *Neospora caninum* causes abortions in a cattle herd in North Rhine Westphalia. Dtsch Tierarztl Wochenschr, 104(6), 208-12.

SCHARES, G., PETERS, M., WURM, R., BÄRWALD, A.AND CONRATHS, F.J. 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet Parasitol, 80(2), 87-98.

SCHARES, G., DUBREMETZ, J.F., DUBEY, J.P., BÄRWALD, A., LOYENS, A. AND CONRATHS, F.J. 1999. *Neospora caninum*: identification of 19-, 38-, and 40-kDa surface antigens and a 33-kDa dense granule antigen using monoclonal antibodies. Exp Parasitol, 92(2), 109-19.

SCHARES, G., RAUSER, M., SÖNDGEN, P., REHBERG, P., BÄRWALD, A., DUBEY, J.P., EDELHOFER, R. AND CONRATHS, F.J. 2000. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. Int J Parasitol, 30(10), 1123-30.

SCHARES, G., HEYDORN, A.O., CÜPPERS, A., MEHLHORN, H., GEUE, L., PETERS, M AND CONRATHS, F.J. 2002a. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed *Neospora caninum* upon feeding of intermediate host tissues. Parasitol Res, 88(1). 44-52.

SCHARES, G., BÄRWALD, A., STAUBACH, C., SÖNDGEN, P., RAUSER, M., SCHRÖDER, R., PETERS, M., WURM, R., SELHORST, T. AND CONRATHS, FJ. 2002b. p38-avidity-ELISA: examination of herds experiencing epidemic or endemic *Neospora caninum*-associated bovine abortion. Vet Parasitol, 106(4), 293-305.

SCHARES, G., BÄRWALD, A., STAUBACH, C., ZILLER, M., KLÖSS, D., WURM, R., RAUSER, M., LABOHM, R., DRÄGER, K., FASEN, W., HESS, R.G. AND CONRATHS, F.J. 2003. Regional distribution of bovine *Neospora caninum* infection in the German state of Rhineland-Palatinate modelled by logistic regression. Int J Parasitol, 33(14), 1631-40.

SCHARES, G., BÄRWALD, A., STAUBACH, C., ZILLER, M., KLÖSS, D., SCHRÖDER, R., LABOHM, R., DRÄGER, K., FASEN, W., HESS, R.G. AND CONRATHS, F.J. 2004. Potential risk factors for bovine *Neospora caninum* infection in Germany are not under the control of the farmers. Parasitology, 129(Pt 3), 301-9.

SCHRAUFSTATTER, I., REVAK, S.D. AND COCHRANE, C.G. 1984. Biochemical factors in pulmonary inflammatory disease. Fed Proc, 43(13), 2807-10.

SCHWARZ, L., WORLICZEK, H.L., WINKLER, M. AND JOACHIM, A. 2014. Superinfection of sows with *Cystoisospora suis* ante partum leads to a milder course of cystoisosporosis in suckling piglets. Vet Parasitol, 204(3-4), 158-68.

SERRANO-MARTÍNEZ, E., FERRE, I., OSORO, K., ADURIZ, G., MOTA, R.A., MARTÍNEZ, A., DEL-POZO, I., HIDALGO, C.O. AND ORTEGA-MORA, L.M. 2007. Intrauterine *Neospora caninum* inoculation of heifers and cows using contaminated semen with different numbers of tachyzoites. Theriogenology, 67(4), 729-37.

SHARMA, R., KIMMITT, T., TIWARI, K., CHIKWETO, A., THOMAS, D., LANZA PEREA, M., BHAIYAT, M.I. 2015. Serological evidence of antibodies to *Neospora caninum* in stray and owned Grenadian dogs. Trop Biomed, 32(2), 286-90.

SINGH, V.K., MORE, T. AND SINGH, S. 1997. The effect of activation of granulocytes on enzyme release and hydrogen peroxide and superoxide production in buffaloes. Vet Res Commun, 21(4), 241-7.

SILVA, L.M., CARO, T.M., GERSTBERGER, R., VILA-VIÇOSA, M.J., CORTES, H.C., HERMOSILLA, C. AND TAUBERT, A. 2014. The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. Parasitol Res, 113(8), 2797-807.

ŠLAPETA, J. 2013. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol, 43(12-13), 957-70.

SOARES, R.M., LOPES, E.G., KEID, L.B., SERCUNDES, M.K., MARTINS, J. AND RICHTZENHAIN, LJ. 2011. Identification of *Hammondia heydorni* oocysts by a heminested-PCR (hnPCR-AP10) based on the *H. heydorni* RAPD fragment AP10. Vet Parasitol, 175(1-2), 168-72.

STOIBER, W., OBERMAYER, A., STEINBACHER, P. AND KRAUTGARTNER, W.D. 2015. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. Biomolecules, 5(2), 702-23.

SÖNDGEN, P., PETERS, M., BÄRWALD, A., WURM, R., HOLLING, F., CONRATHS, F.J. AND SCHARES, G. 2001. Bovine neosporosis: immunoblot improves foetal serology. Vet Parasitol, 102(4), 279-90.

SÖRGEL, S.C., MÜLLER, M., SCHARES, G., GROSSMANN, E., NEUSS, T., PUCHTA, H., KREUZER, P., EWRINGMANN, T., EHRLEIN, J., BOGNER, K.H. AND SCHMAHL, W. 2009. Beteiligung von *Neospora caninum* bei Rinderaborten in Nordbayern. Tierärztl Umschau, 64, 235-243.

STRIEPEN, B., JORDAN, C.N., REIFF, S., AND VAN DOOREN, G.G. 2007. Building the perfect parasite: cell division in apicomplexa. PLoS Pathog, 3(6), e78.

STYRT, B., WALKER, R.D. AND WHITE, J.C. 1989. Neutrophil oxidative metabolism after exposure to bacterial phospholipase C. J Lab Clin Med, 114(1), 51-7.

SYKES, J.E. 2013.Neosporosis. *In*: Canine and Feline Infectious Diseases. St Louis, USA, Elsevier.

TOPAZIO, J.P., WEBER, A., CAMILLO, G., VOGEL, F.F., MACHADO, G., RIBEIRO, A., MOURA, A.B., LOPES, L.S., TONIN, A.A., SOLDÁ, N.M., BRÄUNIG, P. AND SILVA, A.S. 2014. Seroprevalence and risk factors for *Neospora caninum* in goats in Santa Catarina state, Brazil. Rev Bras Parasitol Vet, 23(3), 360-6.

TREES, A.J., DAVISON, H.C., INNES, E.A. AND WASTLING, J.M. 1999. Towards evaluating the economic impact of bovine neosporosis. Int J Parasitol, 29(8), 1195-200.

TREES, A.J. AND WILLIAMS, D.J. 2005. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii.*Trends Parasitol, 21(12), 558-61.

UGGLA, A., STENLUND, S., HOLMDAHL, O.J., JAKUBEK, E.B., THEBO, P., KINDAHL, H.AND BJÖRKMAN, C. 1998. Oral *Neospora caninum* inoculation of neonatal calves. Int J Parasitol, 28(9), 1467-72.

URBAN, C.F., REINHARDT, U., BRINKMANN, V. AND ZYCHLINKSY, A. 2006. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol, 8(4), 668-76.

URBAN, C.F., ERMERT, D., SCHMID, M., ABU-ABED, U., GOOSMANN, C., NACKEN, W., BRINKMANN, V., JUNGBLUT, P.R. AND ZYCHLINKSY, A. 2009. Neutrophil

extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. PLoS Pathog, 5(10), e1000639.

UZEDA, R.S., COSTA, Kde. S., SANTOS, S.L., PINHEIRO, A.M., De ALMEIDA, M.A., McALLISTER, M.M. AND GONDIM, L.F. 2007. Loss of infectivity of *Neospora caninum* oocysts maintained for a prolonged time. Korean J Parasitol, 45(4),295-9.

VÁCLAVEK, P., SEDLÁK, K., HURKOVÁ, L., VODRÁZKA, P, SEBESTA, R. AND KOUDELA, B. 2007. Serological survey of *Neospora caninum* in dogs in the Czech Republic and a long-term study of dynamics of antibodies. Vet Parasitol, 143(1), 35-41.

VALLEJO, M., ROSALES, R., MÜLLER, E. AND ARAGON, A. 1989. Evaluación de índices productivos y reproductivos de varios rebaños de cabras en el Valle Central de Costa Rica. Agron. Costarric. 13(2), 153-158.

VANDERCAPPELLEN, J., VAN DAMME, J. AND STRUYF, S. 2008. The role of CXC chemokines and their receptors in cancer. Cancer Lett, 267(2), 226-44.

VARASCHIN, M.S., HIRSCH, C., WOUTERS, F., NAKAGAKI, K.Y., GUIMARÃES, A.M., SANTOS, D.S., BEZERRA, P.S. Jr., COSTA, R.C., PECONICK, A.P. AND LANGOHR, M. 2012. Congenital neosporosis in goats from the State of Minas Gerais, Brazil. Korean J Parasitol, 50(1), 63-7.

VILLAGRA-BLANCO, R., SILVA, L.M.R., GÄRTNER, U., WAGNER, H., FAILING, K., WEHREND, A., TAUBERT, A. AND HERMOSILLA, C. 2017a. Molecular analyses

on *Neospora caninum*-triggered NETosis in the caprine system. Dev Comp Immunol, 72, 119-127.

VILLAGRA-BLANCO, R., SILVA, L.M.R., MUÑOZ-CARO, T., YANG, Z., LI, J., GÄRTNER, U., TAUBERT, A., ZHANG, X. AND HERMOSILLA, C. 2017b. Bovine polymorphonuclear neutrophils cast neutrophil extracellular traps against the abortive parasite *Neospora caninum*. Front Immunol. 29, 8:606.

VILLAGRA-BLANCO, R., WAGNER, H., DOLZ, G., ROMERO, J.J., TAUBERT, A., WEHREND, A. AND HERMOSILLA, C. 2017c. First report on the seroprevalence of *Neospora caninum* in goats from the Federal State of Hesse, Germany. Berl Münch Tierärztl Wochenschr 130, 11/12, 517-522.

VILLAGRA-BLANCO, R., ESQUIVEL-SUAREZ, A., ROMERO, J.J., TAUBERT, A., WEHREND, A., HERMOSILLA, C. AND DOLZ, G. 2018a. Seroprevalence and factors associated with *Toxoplasma gondii-, Neospora caninum-* and *Coxiella burnetii*-infections in dairy goat flocks from Costa Rica. (Submitted data).

VILLAGRA-BLANCO, R., ANGELOVA, L., CONZE, T., SCHARES, G., BÄRWALD, A., TAUBERT, A., HERMOSILLA, C. AND WEHREND, A. 2018b. Seroprevalence of *Neospora caninum*-specific antibodies in German breeding bitches. Parasit Vectors, 11:96.

VON KÖCKRITZ-BLICKWEDE, M., GOLDMANN, O., THULIN, P., HEINEMANN, K., NORRBY-TEGLUND, A., ROHDE, M.AND MEDINA, E. 2008. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood, 111(6), 3070-80.

VON KÖCKRITZ-BLICKWEDE, M. AND NIZET, V. 2009. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. J Mol Med (Berl), 87(8), 775-83.

WALTER, K., STEINWEDE, K., ALY, S., REINHECKEL, T., BOHLING, J., MAUS, U.A.AND EHLERS, S. 2015. Cathepsin G in experimental tuberculosis: relevance for antibacterial protection and potential for immunotherapy. J Immunol, 195(7), 3325-33.

WANDERLEY, F.S., PORTO, W.J.N., CAMARA, D.R., DE OLIVEIRA, V.V.G., GARCIA, J.L., DE ALBUQUERQUE, P.P.F., OLIVEIRA, A.A.F., MOTA, R.A. 2015. Venereal transmission of *Toxoplasma gondii* in goats after a buck was experimentally infected. Small Rumin Res, 123, 2-3, 301-305.

WANG, Y., CHEN, Y., XIN, L., BEVERLEY, S.M., CARLSEN, E.D., POPOV, V., CHANG, K.P., WANG, M. AND SOONG, L. 2011. Differential microbicidal effects of human histone proteins H2A and H2B on *Leishmania* promastigotes and amastigotes. Infect Immun, 79(3), 1124-33.

WANHA, K., EDELHOFER, R., GABLER-EDUARDO, C. AND PROSL, H. 2005. Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. Vet Parasitol, 128(3-4), 189-93.

WARDINI, A.B., GUIMARÃES-COSTA, A.B., NASCIMENTO, M.T., NADAES, N.R., DANELLI, M.G., MAZUR, C., BENJAMIM, C.F., SARAIVA, E.M. AND PINTO-DA-SILVA, L.H. 2010. Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J Gen Virol, 91(Pt 1), 259-64.

WARTHA, F., BEITER, K., NORMARK, S. AND HENRIGUES-NORMARK, B. 2007. Neutrophil extracellular traps: casting the NET over pathogenesis. Curr Opin Microbiol, 10(1), 52-6.

WEI, Z., HERMOSILLA, C., TAUBERT, A., HE, X., WANG, X., GONG, P., LI, J., YANG, Z. AND ZHANG, X. 2016. Canine neutrophil extracellular traps release induced by the apicomplexan parasite *Neospora caninum in vitro.* Front Immunol, 7, 436.

WEISS, D.J. AND WARDROP, K.J. 2011. Schalm's Veterinary Hematology, Iowa, USA. John Wiley-Blackwell.

WESTON, J.F., HEUER, C. AND WILLIAMSON, NB. 2012. Efficacy of a *Neospora caninum* killed tachyzoite vaccine in preventing abortion and vertical transmission in dairy cattle. Prev Vet Med, 103(2-3), 136-44.

WHYTE, M.K., MEAGHER, L.C., MacDERMOT, J. AND HASLETT, C. 1993. Impairment of function in aging neutrophils is associated with apoptosis. J Immunol, 150 (11), 5124-5134.

WILLIAMS, D.J., McGARRY, J., GUY, F., BARBER, J. AND TREES, A.J. 1997. Novel ELISA for detection of *Neospora*-specific antibodies in cattle. Vet Rec, 140(13), 328-31.

WILLIAMS, J.H., ESPIE, I., VAN WILPE, E. AND MATTHEE, A. 2002. Neosporosis in a white rhinoceros (*Ceratotherium simum*) calf. J S Afr Vet Assoc, 73(1), 38-43.

WILLIAMS, D.J., GUY, C.S., SMITH, R.F., ELLIS, J., BJÖRKMAN, C., REICHEL, M.P. AND TREES, A.J. 2007. Immunization of cattle with live tachyzoites

of *Neospora caninum* confers protection against fetal death. Infect Immun, 75(3), 1343-8.

WILLIAMS, D.J., HARTLEY, C.S., BJÖRKMAN, C. AND TREES, A.J. 2009. Endogenous and exogenous transplacental transmission of *Neospora caninum* - how the route of transmission impacts on epidemiology and control of disease. Parasitol,136(14), 1895-900.

WOLF, D., SCHARES G., CARDENAS O., HUANCA, W., CORDERO, A., BÄRWALD, A., CONRATHS, F.J., GAULY, M., ZAHNER H. AND BAUER, C. 2005. Detection of specific antibodies to *Neospora caninum* and *Toxoplasma gondii* in naturally infected alpacas (*Lama pacos*), llamas (*Lama glama*) and vicunas (*Lama vicugna*) from Peru and Germany. Vet Parasitol, 130, 81-7.

WOLFE, A., HOGAN, S., MAGUIRE, D., FITZPATRICK, C., VAUGHAN, L., WALL, D., HAYDEN, T.J. AND MULCAHY, G. 2001. Red foxes (*Vulpes vulpes*) in Ireland as hosts for parasites of potential zoonotic and veterinary significance. Vet Rec, 149(25), 759-63.

YAKHCHALI, M., JAVADI, S. AND MORSHEDI, A. 2010. Prevalence of antibodies to *Neospora caninum* in stray dogs of Urmia, Iran. Parasitol Res, 106(6), 1455-8.

YILDIZ, K., GOKPINAR, S., GAZYAGCI, A.N., BABUR, C., SURSAL, N. AND AZKUR, A.K. 2017. Role of NETs in the difference in host susceptibility to *Toxoplasma gondii* between sheep and cattle. Vet Immunol Immunopathol, 189, 1-10.

YIPP, B.G., PETRI, B., SALINA, D., JENNE, C.N., SCOTT, B.N., ZBYTNUIK, L.D., PITTMAN, K., ASADUZZAMAN, M., WU, K., MEIJNDERT, H.C., MALAWISTA, S.E., DE BOISFLEURY CHEVANCE, A., ZHANG, K., CONLY, J.AND KUBES, P. 2012. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. Nat Med, 18(9),1386-93.

YOUSEFI, S., GOLD, J.A., ANDINA, N., LEE, J.J., KELLY, A.M., KOZLOWSKI, E., SCHMID, I., STRAUMANN, A., REICHENBACH, J., GLEICH, G.J.AND SIMON, H.U. 2008. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med, 14(9), 949-53.

YOUSEFI, S., MIHALACHE, C., KOZLOWSKI, E., SCHMID, I. AND SIMON, H.U. 2009. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ, 16(11), 1438-44.

11. SUPPLEMENTARY DATA

BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) DO ALSO CAST NEUTROPHIL EXTRACELLULAR TRAPS AGAINST THE APICOMPLEXAN PARASITE *NEOSPORA CANINUM*

This supplementary data is based on the following published paper:

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Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

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Brief report

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Neospora caninum.

ABSTRACT

Neutrophil extracellular traps (NETs) are web-like structures composed of nuclear DNA decorated with histones and cytoplasmic peptides which antiparasitic properties have not previously been investigated in cetaceans. Polymorphonuclear neutrophils (PMN) were isolated from healthy bottlenose dolphins (*Tursiops truncatus*), and stimulated with Neospora caninum tachyzoites and the NETs-agonist zymosan. In vitro interactions of PMN with the tachyzoites resulted in rapid extrusion of NETs. For the demonstration and quantification of cetacean NETs, extracellular DNA was stained by using either Sytox Orange® or Pico Green®. Scanning electron microscopy (SEM) and fluorescence analyses demonstrated PMN-derived release of NETs upon exposure to tachyzoites of N. caninum. Co-localization studies of N. caninum induced cetacean NETs proved the presence of DNA adorned with histones (H1, H2A/H2B, H3, H4), neutrophil elastase (NE), myeloperoxidase (MPO) and pentraxin (PTX) confirming the molecular properties of mammalian NETosis. Dolphin-derived N. caninum-NETosis were efficiently suppressed by DNase I and diphenyleneiodonium (DPI) treatments. Our results indicate that cetacean-derived NETs represent an ancient, conserved and relevant defense effector mechanism of the host innate immune system against N. caninum and probably other related neozoan parasites circulating in the marine environment.

Introduction

Bottlenose dolphins (*Tursiops truncatus*) are the most common and well-known members of the family Delphinidae (Cetacea). Female bottlenose dolphins live in groups composed of 10e30 members, but group sizes can vary up to more than hundred specimens. In contrast, adult males live mostly in small groups joining female dolphin pods strictly for mating purposes for short periods of time. Bottlenose dolphins are known to inhabit warm as well as temperate ocean seas worldwide and to be present in all oceans except for the Antarctic and Arctic Circle areas.

Investigations on the dolphin adaptive immune system are quite abundant in literature (Romano et al., 1992; De Guise et al., 2002; Mancia et al., 2007; Beineke et al., 2010; Sitt et al., 2010; Zafra et al., 2015; White et al., 2017). Conversely, investigations on the cetacean innate immune system are less commonly found (Kato and Perrin, 2009; Schwacke et al., 2010; Keogh et al., 2011) despite the fact that PMN are at the forefront of defense against infection (Brinkmann and Zychlinsky, 2012; Silva et al., 2016), resolution of inflammation and wound healing (Rodríguez-Espinosa et al., 2015). Some cetacean PMN data are available on the impact of heavy metals (Cámara Pellissó et al., 2008) and fungi infections (Reif et al., 2009) but any data existing on cetacean PMN effector mechanisms against invasive parasites are still missed.

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Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

Human activities and domestic animal industry clearly impact the ocean health system (Dubey, 2003; Conrad et al., 2005) and recently identified neozoan parasite infections in free-ranging marine mammals, such as *Neospora caninum, Toxoplasma gondii, Giardia intestinalis, Cryptosporidium parvum, Cryptosporidium hominis, Sarcocystis neurona, Entamoeba sp.* and *Balantidium coli* may all originate from human and animal waste/sewage or their related activities (Buergelt and Bonde, 1983; Olsen et al., 1997; Parveen et al., 1997; Johnson et al., 1998; LaPointe et al., 1998; Dubey, 2003, Conrad et al., 2005; Kleinertz et al., 2014; Hermosilla et al., 2016). Consistently to these observations, antibodies against abortive and neurotropic parasites, such as *N. caninum, T. gondii* and *S. neurona*, have been reported to occur around the world, particularly in dolphins (Inskeep et al., 1990; Lapointe et al., 1998; Jardine and Dubey, 2002; Bowater et al., 2003; Cabezón et al, 2004; Santos et al., 2011), whales (Mikaelian et al., 2000; Omata et al., 2006), sea otters (Cole et al., 2000; Conrad et al., 2005) and seals (Dubey, 2003; Fujii et al., 2007), demonstrating the circulation of these typically terrestrial parasitoses in the marine ecosystem.

Commonly in terrestrial susceptible hosts, such as cattle, goats, sheep, horses and dogs, infections of *N. caninum* underlie complex cellular as well as molecular immunological regulations (see Gazzinelli et al., 1998; Boysen et al., 2006; Taubert et al., 2006, 2010; Klevar et al., 2007; Wei et al., 2016; Villagra-Blanco et al., 2017a, b). In bottlenose dolphins, the innate immune system comprehends also PMN and monocytes, which comprise between 22-72% and 0–11% of the circulating leukocytes, respectively (Goldstein et al., 2006; Hall et al., 2007; Venn-Watson et al., 2007; Schwacke et al., 2009). Consistently, PMN are known to play a key role in host innate immunity against apicomplexan protozoan infections (Behrendt et al., 2010; Muñoz-Caro et al., 2014, 2015a; Reichel et al., 2015; Silva et al., 2016; Wei et al., 2016; Villagra-Blanco et al., 2017a, b), since they are the most abundant leukocytes and the first ones that reach apicomplexan parasite infection in vivo (Baker et al., 2008; Abi Abdallah et al., 2012; Muñoz-Caro et al., 2016). Mammalian PMN elicit several effector mechanisms to combat protozoan parasites, such as phagocytosis, production of reactive oxygen species (ROS), the excretion of anti-parasitic peptides/proteins and the release of neutrophil extracellular traps (NETs) (for reviews see Brinkmann and Zychlinsky, 2012; Hermosilla et al., 2014; Silva et al., 2016). Accordingly, marine mammalian PMN are also capable of ROS production and to perform phagocytic activities in dolphins (Itou et al., 2001; Noda et al., 2006). Moreover, harbour seal (*Phoca vitulina*) PMN and monocytes have probed to trigger extracellular traps (ETs) against vital T. gondii-tachyzoites as an efficient host effector mechanism (Reichel et al., 2015). NETs are generally released via a novel PMN cell death process known as NETosis (Fuchs et al., 2007; Brinkmann and Zychlinsky, 2012). Invasive parasites may either be immobilized within NETs (Behrendt et al., 2010; Muñoz-Caro et al., 2014, 2015a,b, 2016; Silva et al., 2016; Wei et al., 2016; Villagra-Blanco et al., 2017a, b) or killed via locally high concentrations of antimicrobial histones, peptides and proteases as postulated elsewhere (Brinkmann et al., 2004; Von Köhritz-Blickwede and Nizet, 2009; Cheng and Palaniyar, 2013).

NETosis is known as a NADPH oxidase (NOX)-dependent mechanism (Behrendt et al., 2010; Von Köhritz-Blickwede et al., 2010; Brinkmann and Zychlinsky, 2012; Muñoz-Caro et al., 2015a, b), which leads to extrusion of DNA-enriched fibers adorned with histones and granular proteins, e. g. neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin, lactoferrin, cathepsin, bacterial permeability-increasing protein (BPI), peptidoglycan recognition proteins (PGRPs) and other PMN granular components (for reviews see Von Köhritz-Blickwede and Nizet, 2009; Brinkmann and Zychlinsky, 2012; Hermosilla et al., 2014; Silva et al., 2016). Currently, different protozoan parasites have been described to produce NETosis in humans as well as in wild and domestic animals, such as *Plasmodium falciparum* (Baker et al., 2008), *Leishmania spp.* (Guimarães-Costa et al., 2009; Wang et al., 2011), *Eimeria bovis* (Behrendt et al., 2010; Muñoz-Caro et al., 2015a), *E. arloingi* (Silva et al., 2014), *E. ninakohlyakimovae* (Pérez et al., 2016), *T. gondii* (Abi Abdallah et al., 2012), *Besnoitia besnoiti* (Muñoz-Caro et al., 2014), *C. parvum* (Muñoz-Caro et al., 2015b), *Trypanosoma cruzi* (Sousa-Rocha et al., 2015), *Entamoeba histolytica* (Ventura-Juárez et al., 2016), *Naegleria fowleri* (Contis-Montes de Oca et al., 2016) and more recently *N. caninum* (Wei et al., 2016; Villagra-Blanco et al., 2017a, b).

To the best our current knowledge there is only one report focusing on NETosis occurring in marine mammals, namely in pinniped-derived PMN and monocytes casting ETs against *T. gondii* (Reichel et al., 2015). Thus, aim of the herein work was to confirm that cetacean PMN can also cast NETs against neozoan apicomplexan parasites. Therefore isolated PMN from bottlenose dolphins (*T. truncatus*) were exposed to vital N. caninum-tachyzoites and further analyzed in detail to describe molecules as well as signaling pathways implicated in this ancient host innate immune effector mechanism.

Materials and methods

Ethic statement

All animal procedures were performed according to the dolphinarium Mundomar (Benidorm, Spain) Animal Care Committee guidelines, and approved by the Bioethical Committee of Murcia University (Murcia, Spain) and the local Committees for animal research (REGA ES300305440012), and in accordance to the current European Animal Welfare Legislation: ART13TFEU.

Parasites

All NET-related experiments were performed with tachyzoites of *N. caninum* (strain Nc1) which were cultivated in vitro as described elsewhere (Dubey et al., 1988; Taubert et al., 2006; Villagra-Blanco et al., 2017a, b). In brief, N. caninum tachyzoites were maintained by several passages in permanent African green monkey kidney epithelial cells (MARC-145) according to methods described before by Taubert et al. (2006) and Muñoz-Caro et al. (2014). Vital *N. caninum*-tachyzoites were collected in supernatants of infected host cell monolayers, filtered with 5 μm sterile syringe filters (Sartorius AG) to removed cell debris, washed thrice with sterile PBS (400 × g, 12 min), counted using a Neubauer haemocytometer chamber (Marienfeld) and re-suspended in sterile RPMI 1640 medium without phenol red (Gibco) until further experimental use as recently reported elsewhere (Villagra-Blanco et al., 2017a, b).

Host cells

MARC-145 cell monolayers were cultured in DMEM (Sigma-Aldrich) cell culture medium supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (500 mg/ml; Sigma-Aldrich) and 2% fetal calf serum (FCS; Gibco) and incubated at 37 °C and 5% CO_2 until confluency. Then, MARC-145 monolayers were infected with viable N. caninum tachyzoites and cultured at 37 °C and 5% CO_2 atmosphere until release of new vital tachyzoites. Cell medium was changed every second day.

Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

Dolphin blood collection and PMN isolation

Healthy adult male bottlenose dolphins (*T. truncatus*; n ¼ 3) kept at the dolphinarium of the Marine Animal Park Mundomar (Benidorm, Spain) were bled by puncture of the ventral superficial fluke plexus from the caudal peduncle bundle (see Fig. 1a). To reduce stress of blood donor animals, professional trainers stayed with the animals to facilitate the physical restraint by using whistles to give them instructions, associated with a positive reinforcement (see Fig. 1b). All dolphin blood extractions were performed during the morning-feeding daily routines of the dolphinarium, when animals were accustomed to periodically medical procedures, such as extraction of urine-, faeces-, semen-, milk- and blood samples.

The blood was collected in 5 ml sterile plastic tubes containing lithium heparin as anticoagulant (BD Vacutainer®) and thereafter immediately transported using cold ice-packs to the Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain. Heparinized blood of each dolphin (20 ml) was diluted in 30 ml of sterile PBS containing 0.02% EDTA (Sigma-Aldrich), layered on Biocoll Separating Solution® (Biochrom AG) and centrifuged (800 × g, 45 min at 4 °C) as recently described for other marine mammals (Reichel et al., 2015). After the removal of plasma, two different protocols of PMBC extraction were herein tested: i) the pellet containing erythrocytes and PMN was re-suspended in 15 ml RPMI medium 1640 without phenol red, treated with Red Blood Cell Lysis® buffer (1 ml, Sigma-Aldrich) to remove erythrocytes and centrifuged (500 × g, 7 min). Alternatively, ii) the pellet containing the PMN and erythrocytes was suspended in 25 ml distilled water and shaken for 40 s to lyse erythrocytes according to Muñoz-Caro et al. (2014). Osmolarity was immediately re-adjusted by adding the appropriate amount of Hanks salt solution (4 ml, HBSS 10x, Biochrom AG). This procedure was repeated twice to wash the dolphin PMN. In both protocols, the pellets were re-suspended in RPMI medium (Gibco) and PMN were counted in a Neubauer haemocytometer chamber. Finally, cetacean-derived PMN were incubated for 30 min to allow repose at 37 °C and 5% CO_2 atmosphere until use. In our hands, the second PMN isolation protocol achieved better results due to the fact that dolphin erythrocytes revealed to be quite resistant to applied Blood Cell Lysis® buffer (Sigma-Aldrich).

Quantification of dolphin NETs

Dolphin-derived NET formation was quantified by using Pico Green® (Invitrogen), an extracellular DNA-binding fluorescent dye, as reported elsewhere (Muñoz-Caro et al., 2015a,b; Villagra-Blanco et al., 2017a, b). Therefore, cetacean PMN (n ¼ 3) were re-suspended in serum-free cell culture medium RPMI 1640 lacking phenol red and incubated in duplicates with vital *N. caninum*-tachyzoites (37 °C, 60 min, 3:1 ratio: 7,5 × 10^5 N. caninum tachyzoites versus 2,5 × 10^5 dolphin PMN/200 ml). For NET blockage, PMN were pre-treated with the NOX-inhibitor [DPI, 10 mM, Sigma-Aldrich, according to O'Donnell et al. (1993)] for 30 min at 37 °C prior to exposure to N. caninum tachyzoites and DNase I (90 U/well, Roche Diagnostics, addition was performed 15 min before the end of the incubation period). For NET quantification, the samples were treated with micrococcal nuclease (0.1 U/ml, New England Biolabs, 15 min, 37 °C) and centrifuged (300 × g, 5 min). The supernatant was transferred into a 96-well flat-bottom plate (100 ml per well in duplicates). Then, Pico Green® (50 ml/sample, diluted 1:200 in 10 nMTris/1 mM EDTA buffer, in the dark) was added. NET formation was determined by spectrofluorometric analysis at an excitation wavelength of 484 nm and an emission wavelength of 520 nm using an automated plate monochrome reader (FLUOstarOmega, BMG Labtech). NETs were quantified by fluorescence intensity analyses. For negative controls, PMN alone in plain medium were used and for positive controls served PMN stimulated with zymosan (1 mg/ml; Invitrogen) according to Muñoz-Caro et al. (2015a, b).

In order to evaluate parasite dose-dependent effects, different PMN: tachyzoites ratios were applied (1:1, 1:2, 1:3) during 90 min of incubation and processed as described earlier. For NET-kinetic analyses, PMN and parasites were co-cultured with parasites for different time periods (i. e. 30, 60, 90 min).

Visualization of extracellular DNA adorned with histones, neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin (PTX) in Neospora caninum-induced dolphin NETs.

After the co-culture of cetacean PMN with tachyzoites (ratio 1:3, 90 min) on pre-coated poly-L-lysine coverslips, fixation of the samples (4% paraformaldehyde, Merck) and three PBS washings, the samples were carefully transported in a flat-bottom cell culture 6-well plates to the Institute of Parasitology (Justus Liebig University Giessen, Germany) and thereafter blocked with BSA (2%, Sigma-Aldrich, 15 min, RT), incubated in antibody solutions [1 h, RT] and finally mounted in ProLongGold® Antifade (Invitrogen) stained with Sytox Orange® [Invitrogen, 5 mM Sytox Orange®, 10 min, RT, in dark, according to Martinelli et al. (2004)]. For the detection of dolphin histones, MPO, NE and PTX in NETs the following antibodies were used: anti-histone (H1, H2A/H2B, H3, H4) monoclonal (mouse clone H11-4, 1:1000, Merck Millipore), anti-MPO (Alexa Fluor 488, 1:1000, ABIN906866, Antibodies-online.com), anti-NE (AB68672, 1:1000, Abcam) and anti-PTX (SAB2104614-50UG, 1:1000, Sigma-Aldrich) antibodies. NETs-

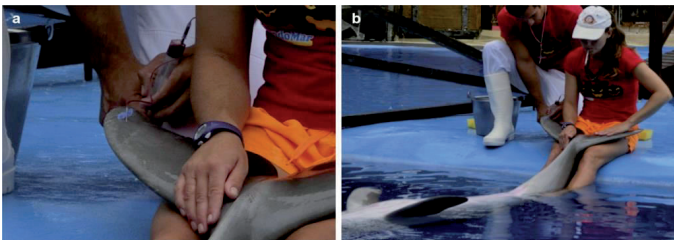

Fig. 1. Minimally-invasive blood extraction method for cetaceans. (a) Puncture of the ventral superficial fluke plexus with a fine needle attached to infusion system and o syringe to create a vacuum for blood extraction. (b) Professional trainers performed physical restraint of one dolphin using whistle to give a positive reinforcement during sampling.

Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

visualization and illustration were achieved by using an inverted Olympus® IX81 fluorescence microscope equipped with a digital camera.

Scanning electron microscopy (SEM)

Dolphin PMN were co-cultured with vital N. caninum-tachyzoites (ratio: 1:3) for 60 min on poly-L-lysine (Sigma-Aldrich) precoated coverslips (10 mm of diameter; Nunc). Cells were fixed in 2.5% glutaraldehyde (Merck), post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold particles. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology of the Justus Liebig University Giessen, Germany.

Statistical analysis

Statistical analyses were performed by using Graph Pad Prism® 6 software. One- or two-factorial analyses of variance (ANOVA) with repeated measures were applied to compare co-culture/stimulation conditions using a normal distribution of data. Differences were regarded as significant at a level of $p :;; 0.05$ (*); $p :;; 0.01$ (**); $p :;; 0.001$ (***) and $p :;; 0.0001$ (****).

Results and discussion

Cetacean PMN, likewise terrestrial mammalian hosts, are well-known to elicit phagocytic and respiratory burst activities resulting in the production of ROS (Keogh et al., 2011). PMN extrusion of NETs is nowadays considered one of the main effector mechanisms of this leukocyte population to combat infectious agents (Brinkmann and Zychlinsky, 2012; Hahn et al., 2013), including protozoan and metazoan parasites (for reviews see Hermosilla et al., 2014; Silva et al., 2016). Since the first description of NETs by Brinkmann et al. (2004), very little has been investigated on NETosis in marine mammals, with the exception of one report on *T. gondii*-triggered NETs in exposed harbour seal PMN (Reichel et al., 2015). Both, T. gondii and N. caninum, are considered terrestrial neozoan parasites which were recently identified within the marine environment (Fujii et al., 2007; Dubey et al., 2008), and have emerged as important neozoan parasitic pathogens for dolphins (Dubey et al., 2008), pinnipeds (Cabezón et al., 2011), whales (Mazzariol et al., 2012) and sea otters (Conrad et al., 2005; Miller et al., 2008). Alongside, *N. caninum* has recently been identified as potent NET inducer of PMN in dogs (Wei et al., 2016), goats (Villagra-Blanco et al., 2017a) and cattle (Villagra-Blanco et al., 2017b). To our best knowledge, the present study represents the first description of cetacean-extruded NETs against *N. caninum* tachyzoites.

Dolphin PMN-mediated NETosis in presence of N. caninum *tachyzoites*

SEM analyses unveiled that exposure of dolphin-derived PMN to N. caninum tachyzoites resulted in the formation of a fine network and slimmer strands of fibers originating from PMN and being attached to the parasites, seemingly entrapping them (Fig. 2a). During NETosis, some dolphin PMN still showed the morphology of intact vital cells (Fig. 2b, 60 min) and others demonstrated the morphological features of PMN activation. Later on, *N. caninum* tachyzoites were observed entrapped in meshworks of PMN-released filaments (Fig. 2c and, 60 min) or even entangled by a single activated PMN (Fig. 2d, 60 min). Accordingly to these SEM findings, NETosis is most probably a relevant innate effector mechanism by which dolphin PMN confirmly attach and subsequently entrap N. caninum tachyzoites, as reported for other apicomplexan parasites in vitro and in vivo (see Behrendt et al., 2010; Muñoz-Caro et al., 2014, 2015b, 2016; Reichel et al., 2015; Silva et al., 2016).

Immunofluorescence analyses of N. caninum*-induced dolphin NETs*

Sytox Orange® staining-based fluorescence investigations further proved the presence of dolphin-derived NETs containing DNA (Fig. 2e–e1). *N. caninum* tachyzoites were located in close proximity to extruded cetacean-triggered NETs and often being trapped within these extracellular structures. Extruded dolphin NETs demonstrated classical NETs components, i. e. histones (H1, H2B/H2B, H3, H4) (Fig. 2e and i), NE (Fig. 2f and j), MPO (Fig. 2g and k) and PTX (Fig. 2h, l), as proofed by co-localization of extracellular DNA adorned with these molecules in parasite-entrapping structures (Fig. 2i, j, 2k, 2l). Co-localization assays of dolphin-derived NETs demonstrated the concomitant existence of histones (H1, H2A/H2B, H3, H4), NE, MPO and PTX, confirming typical molecular characteristics of NETs (Brinkmann and Zychlinsky, 2012), and agreed with previous reports on other apicomplexan-triggered NETosis (Baker et al., 2008; Behrendt et al., 2010; Abi Abdallah et al., 2012; Silva et al., 2014; Muñoz-Caro et al., 2014, 2015a, b; 2016; Reichel et al., 2015; Maksimov et al., 2016). In this context, the pivotal role of MPO and NE in *N. caninum*-triggered NETs has been recently proven by functional inhibition assays, leading to the reduction of tachyzoite-mediated NETosis in the canine (Wei et al., 2016), the goat (Villagra-Blanco et al., 2017a) and the bovine systems (Villagra-Blanco et al., 2017b). The presence of PTX in dolphin-derived NETs is in accordance to recent PTX findings obtained from extruded NETs against the same parasite in goats (Villagra-Blanco et al., 2017a) and cattle (Villagra-Blanco et al., 2017b). PTX is known to be stored in PMN granules and relevant in early host innate immune reactions. In common with proteinase 3 and MPO, NE is expressed on the apoptotic PMN surface as reported elsewhere (Bottazzi et al., 2009). In dolphin-derived NET formation, it can be speculated that PTX might therefore participate in *N. caninum* recognition thereby facilitating the entrapment of tachyzoites as previously demonstrated for bacterial pathogens (Bottazzi et al., 2009). More importantly, whole PMN proteome analysis unveiled that PTX forms a complex with other NETs-related molecules in human PMN and appears to have a crucial role in boosting the actions of different NETs components (Daigo and Hamakudo, 2012). Similar synergistic properties have been described for other PMN-excreted pro-inflammatory cytokines/chemokines in response to *N. caninum*-, *T. gondii*- (Taubert et al., 2006) and *C. parvum*-exposed bovine and human PMN (Muñoz-Caro et al., 2016).

Neospora caninum*-induced dolphin NETosis at different tachyzoite ratios*

Quantification of dolphin NET induction through tachyzoites of N. caninum confirmed a strong dose-dependent NET formation, as increasing amounts of N. caninum tachyzoites led to significantly enhanced Pico Green®-derived fluorescence intensities ($p :;; 0.05$) when compared to negative controls, coinciding to published data on T. gondii-mediated NETosis in pinniped PMN (Reichel et al., 2015). However, time dependency was not observed. As expected, DNase I treatments leading to NET disintegration reduced NETosis ($p :;; 0.05$) under the basal levels of the negative controls (Fig. 3a), which confirmed the DNA backbone nature of *N. caninum*-triggered cetacean NETosis.

Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

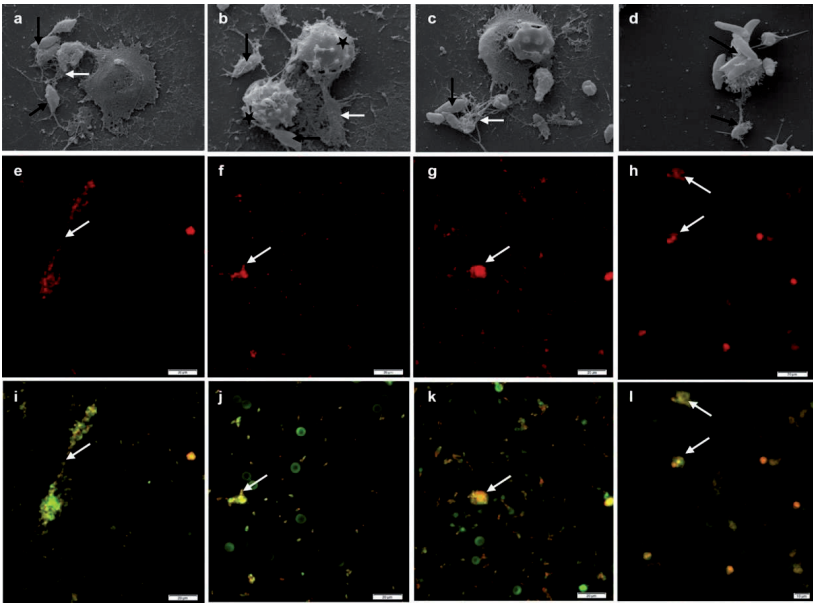

Fig. 2. *Neospora caninum* tachyzoite-triggered dolphin NET structures (SEM) and co-localization of extracellular DNA with histones (H1, H2A/H2B, H3 and H4), NE, MPO and PTX. (a€d) Scanning electron microscopy (SEM) analyses revealed NETs being formed by dolphin PMN after co-culture with N. caninum tachyzoites. (a) Mesh of DNA-structures (white arrow) derived from dolphin PMN attached to N. caninum-tachyzoites (black arrows). (b) Intact cetacean-PMN (black stars) derived a fine filaroid structure (white arrow) being attached to tachyzoites (black arrows). (c) Conglomerates of several tachyzoites (black arrow) being entrapped in a rather chunky meshwork of cetacean-PMN-released thicker extracellular filaments (white arrow) (d) Dolphin PMN activated (black star) entrapping diverse N. caninum-tachyzoites (black arrows). (e€l) Co-cultures of dolphin PMN and N. caninum tachyzoites were fixed, permeabilized, stained for analysis of co-localization (i-l; merge, white arrows) of extracellular DNA (e-h; red; Sytox Orange®) and classical NETs components (all green, white arrows) such as histones (i), NE (j), MPO (k) and pentraxin (l). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Neospora caninum-*triggered dolphin NETosis is a NOX-dependent effector mechanism*

Kinetic-related studies revealed a fast parasite-triggered NET induction in exposed dolphin PMN. As such, N. caninum tachyzoites induced NETosis was detected as fast as 30 min of exposure, i. e. at the earliest time point measured in this assay (until 90 min of exposure, as the latest time point). In contrast, tachyzoite-free negative controls of dolphin PMN contributed very low to extracellular DNA extrusion ($p :::; 0.05$, Fig. 3b) when compared to parasite-exposed PMN (ratio 1:3). Besides, functional inhibition experiment performed with DPI, a potent inhibitor of NOX, resulted in a significant reduction of tachyzoite-triggered dolphin NET formation ($p :::; 0.05$, Fig. 3b). Regarding signal pathways, the NOX pathway clearly participates in *N. caninum*-induced dolphin NETosis, since DPI treatment assays resulted in significant reduction of NET formation. NOX-dependent NETosis findings, were recently reported for other closely related apicomplexan-related NETosis investigations, i. e. *E. bovis* (Muñoz-Caro et al., 2015a), *T. gondii* (Abi Abdallah et al., 2012; Reichel et al., 2015), *B. besnoiti* (Muñoz-Caro et al., 2014), *N. caninum* (Wei et al., 2016) and C. parvum (Muñoz-Caro et al., 2015b), highlighting the relevance of NOX in protozoan-mediated NETosis (Silva et al., 2016). Nonetheless, also NOX-independent parasite-induced NETosis has recently been reported for N. caninum in the caprine system (Villagra-Blanco et al., 2017a), showing differences depending on the parasite species as well as the origin of the PMN donor host species.

Nowadays, there is growing evidence on the crucial role of NETosis as efficient defense mechanism in diverse terrestrial vertebrate host species against several protozoan (Silva et al., 2016) and metazoan parasites (Chuah et al., 2013; Bonne-Année et al., 2014; Muñoz-Caro et al., 2015a,b; Lange et al., 2017). Nonetheless, with respect to NETosis research still scarce data are available for marine mammals and whether this efficient defense mechanism might actively participate in vivo against neozoan parasites, needs to be addressed in the near future, but in vitro evidence here

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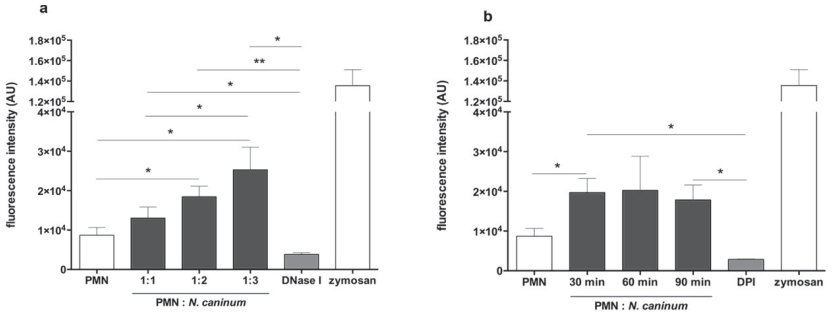

Fig. 3. Dose, kinetic and functional inhibition assays of *N. caninum* tachyzoites-triggered NET formation in dolphins. PMN were incubated with tachyzoites, zymosan (1 mg/ml, positive control) or plain medium (negative control) at different ratios (a; PMN: tachyzoites ¼ 1:1, 1:2, 1:3) and time periods (b; 30, 60 and 90 min). To prove the DNA nature of NETs, the samples were treated with DNase I (a; 15 min). Moreover, cetacean PMN cells were pre-treated with NOX-inhibitor (b; DPI, 10 mM) for 30 min prior to *N. caninum* stimulation (1:3 ratio; 90 min). After incubation, all samples were analyzed for extracellular DNA by quantifying Pico Green®-derived fluorescence intensities. Each condition was performed in duplicates. Geometric means of three PMN donors. Differences were regarded as significant at a level of $p < 0.05$ (*) and $p < 0.01$ (**).

presented strongly suggest that this is most probably occurring. Presented results clearly evidence that N. caninum is a competent parasite species capable to trigger NETs in the cetacean immune system, and consistent to previous *N. caninum* observations of NETs in terrestrial host species (Wei et al., 2016; Villagra-Blanco et al., 2017a, b). Considering the biology of *N. caninum*, which include obligate intracellular parasite stages, the entrapment/immobilization of extracellular tachyzoites through NETosis might have a significant impact on the outcome of the disease as already demonstrated for other related apicomplexan protozoa *in vitro* and *in vivo* (Baker et al., 2008; Behrendt et al., 2010; Abi Abdallah et al., 2012; Hermosilla et al., 2014; Muñoz-Caro et al., 2014, 2016; Silva et al., 2014, 2016).

Conclusion

The current study describes for the first time the ability of bottlenose-dolphin PMN to cast NETs against the abortive protozoan parasite *N. caninum* providing evidence of the importance of this ancient and well conserved effector mechanism of the host innate immune system of marine cetacean species.

Declaration

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests in the present study.

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References

Abi Abdallah, D.S., Lin, C., Ball, C.J., King, M.R., Duhamel, G.E., Denkers, E.Y., 2012. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect. Immun. 80, 768–777.

Baker, V.S., Imade, G.E., Molta, N.B., Tawde, P., Pam, S.D., Obadofin, M.O., Sagay, S.A., Egah, D.Z., Iya, D., Afolabi, B.B., Baker, M., Ford, K., Ford, R., Roux, K.H., Keller 3rd, T.C., 2008. Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malar. J. 7, 41.

Behrendt, J.H., Ruiz, A., Zahner, H., Taubert, A., Hermosilla, C., 2010. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite Eimeria bovis. Vet. Immunol. Immunopathol. 133, 168.

Beineke, A., Siebert, U., Wohlsein, P., Baumgärtner, W., 2010. Immunology of whales and dolphins. Vet. Immunol. Immunopathol. 133, 81–94.

Bonne-Année, S., Kerepesi, L.A., Hess, J.A., Wesolowski, J., Paumet, F., Lok, J.B., Nolan, T.J., Abraham, D., 2014. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval Strongyloides stercoralis. Microbes. Infect. 16 (6), 502–511.

Bottazzi, B., Garlanda, C., Cotena, A., Moalli, F., Jaillon, S., Deban, L., Montavani, A., 2009. The long pentraxin PTX3 as a phototypic humoral pattern recognition receptor: interplay with cellular innate immunity. Immunol. Rev. 227, 9–18.

Bowater, R.O., Norton, J., Johnson, S., Hill, B., O'Donoghue, P., Prior, H., 2003. Toxoplasmosis in indo-pacific humpbacked dolphins (Sousa chinensis), from

Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

queensland. Aust. Vet. J. 81, 627e632.
Boysen, P., Klevar, S., Olsen, I., Storset, A.K., 2006. The protozoan Neospora caninum directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. Infect. Immun. 74, 953e960. http://dx.doi.org/10.1128/IAI.74. 2.953-960.2006.
Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. Science 303, 1532e1535.
Brinkmann, V., Zychlinsky, A., 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? J. Cell. Biol. 198, 773e783.
Buergelt, C.D., Bonde, R.K., 1983. Toxoplasmic meningoencephalitis in a west indian manatee. J. Am. Vet. Med. Assoc. 183 (11), 1294e1296.
Cabezón, O., Hall, A.J., Vincent, C., Pabon, M., García-Bocanegra, I., Dubey, J.P., Almería, S., 2011. Seroprevalence of Toxoplasma gondii in North-eastern Atlantic harbor seal (Phoca vitulina) and grey seal (Halichoerus grypus). Vet. Parasitol. 179 (1e3), 253e256.
Cabezon, O., Resendes, A.R., Domingo, M., Raga, J.A., Agustí, C., Alegre, F., Mons, J.L., Dubey, J.P., Almería, S., 2004. Seroprevalence of Toxoplasma gondii antibodies in wild dolphins from the Spanish Mediterranean coast. J. Parasitol. 90 (3), 643e644. http://dx.doi.org/10.1645/GE-257R.
Cámara Pellissó S., Muñoz, M.J., Carballo, M., Sánchez-Vizcaíno, J.M., 2008. Determination of the immunotoxic potential of heavy metals on the functional activity of bottlenose dolphin leukocytes in vitro. Vet. Immunol. Immunopathol. 121, 189e198.
Cheng, O.Z., Palaniyar, N., 2013. NET balancing: a problem in inflammatory lung diseases. Front. Immunol. 4, 1.
Chuah, C., Jones, M.K., Burke, M.L., Owen, H.C., Anthony, B.J., McManus, D.P., Ramm, G.A., Gobert, G.N., 2013. Spatial and temporal transcriptomics of Schistosoma japonicum-induced hepatic granuloma formation reveals novel roles for neutrophils. J. Leukoc. Biol. 94 (2), 353e365.
Cole, R.A., Lindsay, D.S., Howe, D.K., Roderick, C.L., Dubey, J.P., Thomas, N.J., Baeten, L.A., 2000. Biological and molecular characterizations of Toxoplasma gondii strains obtained from southern sea otters (Enhydra lutris nereis). J. Parasitol. 86, 526e530.
Conrad, P.A., Miller, M.A., Kreuder, C., James, E.R., Mazet, J., Dabritz, H., Jessup, D.A., Gulland, F., Grigg, M.E., 2005. Transmission of Toxoplasma: clues from the study of sea otters as sentinels of Toxoplasma gondii flow into the marine environment. Int. J. Parasitol. 35, 1155e1168.
Contis-Montes de Oca, A., Carrasco-Yépez, M., Campos-Rodríguez, R., Pacheco-Yépez, J., Bonilla-Lemus, P., Pérez-López, J., Rojas-Hernández, S., 2016. Neutrophil extracellular traps damage Naegleria fowleri trophozoites opsonized with human IgG. Parasite Immunol. 38 (8), 481e495.
Daigo, K., Hamakudo, T., 2012. Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. Front. Immun. 3, 378.
De Guise, S., Erickson, K., Blanchard, M., DiMolfetto, L., Lepper, H., Wang, J., Stott, J., Ferrick, D., 2002. Monoclonal antibodies to lymphocyte surface antigens for cetacean homologues to CD2, CD19 and CD21. Vet. Immunol. Immunopathol. 84, 209e221.
Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal Neospora caninum infection in dogs: isolation of the causative agent and experimental transmission. J. Amer. Vet. Med. Assn 193, 1259e1263.
Dubey, J.P., 2003. Review of Neospora caninum and neosporosis in animals. Korean J. Parasitol. 41, 1e16.
Dubey, J.P., Fair, P.A., Sundar, N., Velmurugan, G., Kwok, O.C., McFee, W.E., Majumdar, D., Su, C., 2008. Isolation of Toxoplasma gondii from bottlenose dolphins (Tursiops truncatus). J. Parasitol. 94 (4), 821e823.
Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., Zychlinsky, A., 2007. Novel cell death program leads to neutrophil extracellular traps. J. Cell. Biol. 176, 231e241.
Fujii, K., Kakumoto, C., Kobayashi, M., Saito, S., Kariya, T., Watanabe, Y., Xuan, X., Igarashi, I., Suzuki, M., 2007. Seroepidemiology of Toxoplasma gondii and Neospora caninum in seals around Hokkaido, Japan. J. Vet. Med. Sci. 69 (4), 393e398.
Gazzinelli, R.T., Talvani, A., Camargo, M.M., Santiago, H.C., Oliveira, M.A., Vieira, L.Q., Martins, G.A., Aliberti, J.C., Silva, J.S., 1998. Induction of cell-mediated immunity during early stages of infection with intracellular protozoa. Braz. J. Med. Biol. Res. 31, 89e104.
Goldstein, J.D., Reese, E., Reif, J.S., Varela, R.A., Mcculloch, S.D., Dfran, R.H., Fair, P.A., Bossart, G.D., 2006. Hematologic, biochemical, and cytologic findings from apparently healthy Atlantic bottlenose dolphins (Tursiops truncatus) inhabiting the India River Lagoon, Florida, USA. J. Wildl. Dis. 42, 447e454.
Guimarães-Costa, A.B., Nascimento, M.T., Froment, G.S., Soares, R.P., Morgado, F.N., Conceição-Silva, F., Saraiva, E.M., 2009. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc. Natl. Acad. Sci. U. S. A. 106, 6748e6753.
Hahn, S., Giaglis, S., Chowdhury, C.S., Hösli, I., Hasler, P., 2013. Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin. Immunopathol. 35, 439e453.
Hall, A.J., Wells, R.S., Sweeney, J.C., Townsend, F.I., Balmer, B.C., Hohn, A.A., Rhinehart, H.L., 2007. Annual, seasonal and individual variation in hematology and clinical blood chemistry profiles in bottlenose dolphins (Tursiops truncatus) from Sarasota Bay, Florida. Comp. Biochem. Physiol. A 148, 266e277.
Hermosilla, C., Muñoz-Caro, T., Silva, L.M.R., Ruiz, A., Taubert, A., 2014. The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology 141, 1489e1498.
Hermosilla, C., Silva, L.M.R., Navarro, M., Taubert, A., 2016. Anthropozoonotic endoparasites in free-ranging "urban" South American sea lions (Otaria flavescens). J. Vet. Med. 2016, 7507145. http://dx.doi.org/10.1155/2016/7507145.
Inskeep II, W., Gardiner, C.H., Harris, R.K., Dubey, J.P., Goldston, R.T., 1990. Toxoplasmosis in atlantic bottle-nosed dolphins (Tursiops truncatus). J. Wildl. Dis. 26, 377e382.
Itou, T., Sugisawa, H., Inoue, Y., Jinbo, T., Sakai, T., 2001. Oxygen radical generation and expression of NADPH oxidase genes in bottlenose dolphin (Tursiops truncatus) neutrophils. Dev. Comp. Immunol. 25, 47e53.
Jardine, J.E., Dubey, J.P., 2002. Congenital toxoplasmosis in a indo-pacific bottlenose dolphin (Tursiops aduncus). J. Parasitol. 88, 197e199.
Johnson, S.P., Nolan, S., Gulland, F.M.D., 1998. Antimicrobial susceptibility of bacteria isolated from pinnipeds stranded in central and northern California. J. Zoo. Wildl. Med. 29, 288e294.
Kato, H., Perrin, W.F., 2009. Bryde's whale: balaenoptera edeni/brydei. In: Perrin, W.F., Würsig, B., Thewissen, J.G.M. (Eds.), Encyclopedia of Marine Mammals, second ed. Academic Press, San Diego, pp. 158e163.
Keogh, M.J., Spoon, T., Ridgway, S.H., Jensen, E., Van Bonn, W., Romano, T.A., 2011. Simultaneous measurement of phagocytosis and respiratory burst of leukocytes in whole blood from bottlenose dolphins (Tursiops truncatus) utilizing flow cytometry. Vet. Immunol. Immunopathol. 144, 468e475.
Klevar, S., Kulberg, S., Boysen, P., Storset, A.K., Modal, T., Björkman, C., Olsen, I., 2007. Natural killer cells act as early responders in an experimental infection with Neospora caninum in calves. Int. J. Parasitol. 37 (3e4), 329e339. http://dx.doi.org/10.1016/j.ijpara.2006.11.002.
Kleinertz, S., Hermosilla, C., Ziltener, A., Kreicker, S., Hirzmann, J., Abdel-Ghaffar, F., Taubert, A., 2014. Gastrointestinal parasites of free-living indo-pacific bottlenose dolphins (Tursiops aduncus) in the northern red sea, Egypt. Parasitol. Res. 113, 1405e1415.
Lange, M., Penagos-Tabares, F., Muñoz-Caro, T., Gärtner, U., Mejer, H., Schaper, R., Hermosilla, C., Taubert, A., 2017. Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of Angiostrongylus vasorum, Aelurostrongylus Abstrusus and Troglostrongylus Brevior. Parasit. Vectors 10, 50.
LaPointe, J.M., Duignan, P.J., Marsh, A.E., Gulland, F.M., Barr, B.C., Naydan, D.K., King, D.P., Farman, C.A., Huntingdon, K.A.B., Lowenstine, L.J., 1998. Meningoencephalitis due to a Sarcocystis neurona-like protozoan in Pacific harbor seals (Phoca vitulina richardsi). J. Parasitol. 84, 1184e1189.
Maksimov, P., Hermosilla, C., Kleinertz, S., Hirzmann, J., Taubert, A., 2016. Besnoitia besnoiti infections activate primary bovine endothelial cells and promote PMN adhesion and NET formation under physiological flow conditions. Parasitol. Res. 115, 1991e2001.
Mancia, A., Lundqvist, M.L., Romano, T.A., Peden-Adams, M.M., Fair, P.A., Kindy, M.S., Ellis, B.C., Gattoni-Celli, S., McKillen, D.J., Trent, H.F., Chen, Y.A., Almeida, J.S., Gross, P.S., Chapman, R.W., Warr, G.W., 2007. A dolphin peripheral blood leukocyte cDNA microarray for studies of immune function and stress reaction. Dev. Comp. Immunol. 31 (5), 520e529.
Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P.A., Baumann, C., Fey, M.F., Dummer, R., Simon, H.U., Yousefi, S., 2004. Induction of genes mediating interferon dependent extracellular trap formation during neutrophil differentiation. J. Biol. Chem. 279, 44123e44132.
Mazzariol, S., Marker, F., Mignone, W., Serracca, L., Goria, M., Marsili, L., DiGuardo, G., Casalone, C., 2012. Dolphin Morbillivirus and Toxoplasma gondii co-infection in a Mediterranean fin whale (Balaenoptera physalus). BMC Vet. Res. 8, 20.
Mikaelian, I., Troisclair, J., Dubey, J.P., Kennedy, S., Martineau, D., 2000. Toxoplasmosis in beluga whales from the St. Lawrence estuary: two case reports and a serological survey. J. Comp. Pathol. 122, 73e76.
Miller, M.A., Miller, W.A., Conrad, P.A., James, E.R., Melli, A.C., Leutenegger, C.M., Dabritz, H.A., Packham, A.E., Paradies, D., Harris, M., Ames, J., Jessup, D.A., Worcester, K., Grigg, M.E., 2008. Type X Toxoplasma gondii in wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. Int. J. Parasitol. 38 (11), 1319e1328.
Muñoz-Caro, T., Hermosilla, C., Silva, L.M.R., Cortes, H., Taubert, A., 2014. Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite Besnoitia besnoiti. PLoS One 9 (3), e91415.
Muñoz-Caro, T., Mena Huertas, J.S., Conejeros, I., Alarcón, P., Hidalgo, M.A., Burgos, R.A., Taubert, A., Hermosilla, C., 2015a. CD11b-, ERK/MAP kinase- and SOCE-dependent Eimeria bovis-triggered neutrophil extracellular trap formation. Vet. Res. 46, 23.
Muñoz-Caro, T., Lendner, M., Daugschies, A., Hermosilla, C., Taubert, A., 2015b. NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca2þ influx are essential for Cryptosporidium parvum-induced NET formation. Dev. Comp. Immunol. 52, 245e254.
Muñoz-Caro, T., Silva, M.R.L., Rentería-Solís, Z., Taubert, A., Hermosilla, C., 2016. Neutrophil extracellular traps in the intestinal mucosa of Eimeria-infected animals. Asian Pac. J. Trop. Biomed. 6, 301e307.
Noda, K., Akiyoshi, H., Aoki, M., Shimada, T., Ohashi, F., 2006. Relationship between transportation stress and polymorphonuclear cell functions of bottlenose dolphins, Tursiops truncatus. J. Vet. Med. Sci. 69, 379e383.
O'Donnell, B.V., Tew, D.G., Jones, O.T., England, P.J., 1993. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem. J. 290, 41e49.
Olsen, M.E., Roch, P.D., Stabler, M., Chan, W., 1997. Giardiasis in ringed seals from the

Bottlenose dolphins (*Tursiops truncatus*) do also cast neutrophil extracellular traps against the apicomplexan parasite *Neospora caninum*

western Arctic. J. Wildl. Dis. 33, 646e648.
Omata, Y., Umeshita, Y., Watarai, M., Tachibana, M., Sasaki, M., Murata, K., Yamada, T.K., 2006. Investigation for presence of Neospora caninum, Toxoplasma gondii and Brucella-species infection in killer whales (Orcinus orca) mass-stranded on the coast of Shiretoko, Hokkaido, Jpn. J. Vet. Med. Sci. 68, 523e526.
Parveen, S.R., Murphree, L., Edmiston, L., Kaspar, C.W., Portier, K.M., Tamplin, M.L., 1997. Association of multiple-antibiotic-resistance profiles with point and non-point sources of Escherichia coli in Apalachicola Bay. Appl. Environ. Microbiol. 63, 2607e2612.
Pérez, D., Muñoz, M.C., Molina, J.M., Muñoz-Caro, T., Silva, L.M.R., Taubert, A., Hermosilla, C., Ruiz, A., 2016. Eimeria ninakohlyakimovae induces NADPH oxidase-dependent monocyte extracellular trap formation and upregulates IL-12 and TGF-β, IL-6 and CCL2 gene transcription. Vet. Parasitol. 227, 143e150.
Reichel, M., Muñoz-Caro, T., Sánchez-Contreras, G., Rubio-García, A., Magdowski, G., Gärtner, U., Hermosilla, C., Taubert, A., 2015. Harbour seal (Phoca vitulina) PMN and monocytes release extracellular traps to capture the apicomplexan parasite Toxoplasma gondii. Dev. Comp. Immunol. 50, 106e115.
Reif, J.S., Peden-Adams, M.M., Romano, T.A., Rice, C.D., Fair, P.A., Bossart, G.D., 2009. Immune dysfunction in Atlantic bottlenose dolphins (Tursiops truncatus) with lobomycosis. Med. Mycol. 47 (2), 125e135.
Rodríguez-Espinosa, O., Rojas-Espinosa, O., Moreno-Altamirano, M.M., López-Villegas, E.O., Sánchez-García, F.J., 2015. Metabolic requirements for neutrophil traps formation. Immunol 145 (2), 213e224.
Romano, T.A., Ridgway, S.H., Quaranta, V.J., 1992. MHC class II molecules and immunoglobulins on peripheric blood lymphocytes of the bottlenosed dolphin, Tursiops truncatus. J. Exp. Zool. 263, 96e104.
Santos, P.S., Albuquerque, G.R., da Silva, V.M., Martin, A.R., Marvulo, M.F., Souza, S.L., Ragozo, A.M., Nascimento, C.C., Gennari, S.M., Dubey, J.P., Silva, J.C., 2011. Seroprevalence of Toxoplasma gondii in free-living amazon river dolphins (Inia geoffrensis) from central amazon, Brazil. Vet. Parasitol. 183, 171e173.
Schwacke, L.H., Hall, A.J., Townsend, F.I., Wells, R.S., Hansen, L.J., Hohn, A.A., Bossart, G.D., Fair, P.A., Rowles, T.K., 2009. Hematologic and serum biochemical reference intervals for free-ranging common bottlenose dolphins (Tursiops truncatus) and variation in the distributions of clinicopathologic values related to geographic sampling site. Am. J. Vet. Res. 70, 973e985.
Schwacke, L.H., Twiner, M.J., De Guise, S., Balmer, B.C., Wells, R.S., Townsend, F.I., Rotstein, D.C., Varela, R.A., Hansen, L.J., Zolman, E.S., Spradlin, T.R., Levin, M., Leibrecht, H., Wang, Z., Rowles, T.K., 2010. Eosinophilia and bioxin exposure in bottlenose dolphins (Tursiops truncatus) from a coastal area impacted by repeated mortality events. Environ. Res. 110 (6), 548e555.
Silva, L.M.R., Muñoz-Caro, T., Rüdiger, G., Vila-Viçosa, M.J.M., Cortes, H.C.E., Hermosilla, C., Taubert, A., 2014. The apicomplexan parasite Eimeria arloingi induces caprine neutrophil extracellular traps. Parasitol. Res. 113, 2797e2807.
Silva, L.M.R., Muñoz-Caro, T., Burgos, R.A., Hidalgo, M.A., Taubert, A., Hermosilla, C., 2016. Far beyond phagocytosis: phagocyte-derived extracellular traps act efficiently against protozoan parasites in vitro and in vivo. Mediat. Inflamm. 2016, 5898074.
Sitt, T., Bowen, L., Blanchard, M.T., Gershwin, L.J., Byrne, Ba, Dold, C., McBain, J., Stott, J.L., 2010. Cellular immune responses in cetaceans immunized with a porcine erysipelas vaccine. Vet. Immunol. Immunopathol. 137, 181e189.
Sousa-Rocha, D., Thomaz-Tobias, M., Diniz, L.F.A., Souza, P.S.S., Pinge-Filho, P., Toledo, K.A., 2015. Trypanosoma cruzi and its soluble antigens induce NET release by stimulating toll-like receptors. PLoS One 10, e0139569.
Taubert, A., Krüll, M., Zahner, H., Hermosilla, C., 2006. Toxoplasma gondii and Neospora caninum infection of bovine endothelial cells induce endothelial adhesion molecule gene transcription and subsequent PMN adhesion. Vet. Immunol. Immunopathol. 112, 214e222.
Taubert, A., Wimmers, K., Ponksuksili, S., Jimenez, C.A., Zahner, H., Hermosilla, C., 2010. Microarray-based transcriptional profiling of Eimeria bovis-infected bovine endothelial host cells. Vet. Res. 41, 70.
Venn-Watson, S., Jensen, E.D., Ridgway, S.H., 2007. Effects of age and sex on clinic-pathologic reference ranges in a healthy managed Atlantic bottlenose dolphin population. J. Am. Vet. Med. Assoc. 231, 596e601.
Ventura-Juárez, J., Campos-Esparza, M.R., Pacheco-Yepez, J., López-Blanco, J.A., Adabache-Ortíz, A., Silva-Briano, M., Campos-Rodríguez, R., 2016. Entamoeba histolytica induces human neutrophils to form NETs. Parasite Immunol. 38, 503e509.
Villagra-Blanco, R., Silva, L.M.R., Gärtner, U., Wagner, H., Failing, K., Wehrend, A., Taubert, A., Hermosilla, C., 2017a. Molecular analyses on Neospora caninum-triggered NETosis in the caprine system. Dev. Comp. Immunol. 72, 119e127.
Villagra-Blanco, R., Silva, L.M.R., Muñoz-Caro, T., Yang, Z., Li, J., Gärtner, U., Taubert, A., Zhang, X., Hermosilla, C., 2017b. Bovine PMN cast neutrophil extracellular traps against the abortive parasite Neospora caninum. Front. Immunol. 8, 606. http://dx.doi.org/10.3389/fimmu.2017.00606.
Von Köckritz-Blickwede, M., Nizet, V., 2009. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. J. Mol. Med. 87, 775e783.
Von Köckritz-Blickwede, M., Chow, O., Ghochani, M., Nizet, V., 2010. Visualization and functional evaluation of phagocyte extracellular traps. Methods Microbiol. 37, 139e160.
Wang, Y., Chen, Y., Xin, L., Beverley, S.M., Carlsen, E.D., Popov, V., Chang, K.P., Wang, M., Soong, L., 2011. Differential microbicidal effects of human histone proteins H2A and H2B on Leishmania promastigotes and amastigotes. Infect. Immun. 79, 1124e1133.
Wei, Z., Hermosilla, C., Taubert, A., He, X., Wang, X., Gong, P., Li, J., Yang, Z., Zhang, X., 2016. Canine neutrophil extracellular traps release induced by the apicomplexan parasite Neospora caninum in vitro. Front. Immunol. 7, 436.
White, N.D., Godard-Codding, C., Webb, S.J., Bossart, G.D., Fair, P.A., 2017. Immunotoxic effects of in vitro exposure of dolphin lymphocytes to Louisiana sweet crude oil and Corexit™. J. Appl. Toxicol. 37, 676e682.
Zafra, R., Jaber, J.R., Pérez, J., de la Fuente, R., Arbelo, M., Andrada, M., Fernández, A., 2015. Immunohistochemical characterisation of parasitic pneumonias of dolphins stranded in the Canary Islands. Res. Vet. Sci. 100, 207e212.

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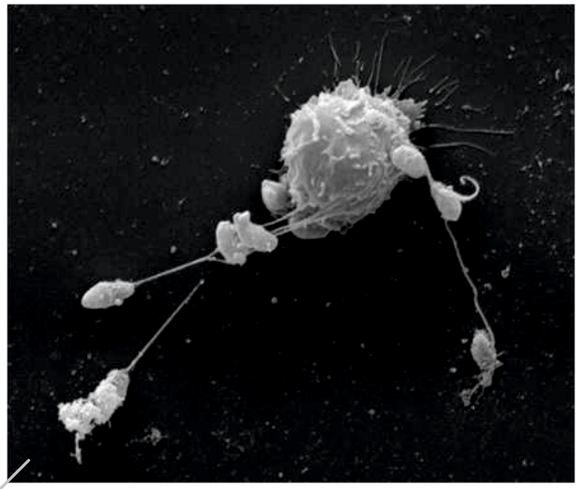
13. DECLARATION

“I hereby declare that I have completed the submitted doctoral thesis independently and without any unauthorised outside help and with only those financial forms of support mentioned in this work. There are no other people who satisfied the criteria for authorship.

I wish to confirm that there are no known conflicts of interest associated with these publications. All texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such. All the analyses conducted in this thesis, followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice.”



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