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Brief report

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

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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.


## 1. 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 10–30 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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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 Köckritz-Blickwede and Nizet, 2009; Cheng and Palaniyar, 2013).

NETosis is known as a NADPH oxidase (NOX)-dependent mechanism (Behrendt et al., 2010; Von Köckritz-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 Köckritz-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.

## 2. Materials and methods

### 2.1. 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.

### 2.2. 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).

### 2.3. 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.