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Nematopsis temporariae (Gregarinasina, Apicomplexa, Alveolata) intracellular infectious agent of tadpole livers

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Running header: Gregarines in tadpole cells

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Abstract:

Amphibians are in decline as a result of habitat destruction, climate change and infectious diseases. Tadpoles are thought susceptible to infections because they are dependent on only an innate immune system (*e.g.* macrophages). This is because the frog adaptive immune system does not function until later stages of the life cycle. In 1920, Nöller described a putative infectious agent of tadpoles named *Nematopsis temporariae*, which he putatively assigned to gregarine protists (Apicomplexa). Here, we identify a gregarine infection of tadpoles using both microscopy and ribosomal DNA sequencing of three different frog species (*Rana temporaria*, *R. dalmatina*, and *Hyla arborea*). We show that this protist lineage belongs to the subclass Gregarinasina Dufour 1828 and is regularly present in macrophages located in liver sinusoids of tadpoles, confirming the only known case of a gregarine infection of a vertebrate.

Introduction:

Amphibian populations are in crisis with 48% of populations reported as declining (Stuart et al., 2004). The emergence of infectious diseases is thought to be a major factor (Daszak et al., 2003; Martel et al., 2013). Amphibian physiology varies considerably during the life cycle. Tadpoles have a weak adaptive immunity with fewer antibody classes, poorer B and T lymphocytes function, no consistent expression of the MHC class I protein and a poor switch from IgM to IgY (Du Pasquier et al., 1989). Tadpoles therefore rely on an innate immune system that provides rapid and non-specific protection. As such tadpoles host a diversity of different microbial organisms, acting as either definitive or intermediate hosts. Specifically, investigation of tadpole livers have identified a diversity of alveolate protists (Jirků et al., 2002; Davis et al., 2007; Jirků et al., 2009; Chambouvet et al., 2015) for which their role as putative parasites in unclear.

One enigmatic group of alveolates are the gregarines. Phylogenetic analyses show gregarines branch within the subphylum Apicomplexa Levine, 1980, emend. Adl *et al.* 2012 (Leander et al., 2003; Adl et al., 2012), which also includes parasites of mammals, e.g. *Plasmodium* spp. All described gregarines are parasites (Leander et al., 2003) and are known to infect many groups of invertebrates, particularly annelids and insects (Leander, 2008). In 1920, Nöller described a gregarine named *Nematopsis temporariae* infecting the liver tissue of the frog *Rana temporaria* (Nöller, 1920). Here we report the identification of an infectious microbe fitting this description from three species of frog tadpoles sampled in the Czech Republic using molecular and microscopy data.

Results and Discussion:

During an amphibian population survey in the Czech Republic we identified a gregarine-like intracellular infection of liver cells from tadpoles of *R. temporaria*, *R. dalmatina* and *H. arborea*. These tadpoles showed no signs of disease or impairment of fitness/function, although livers of some tadpoles appeared slightly enlarged and light coloured, they were not yellowish as previously reported for Perkinsea (Alveolata) infections (Davis et al., 2007). No mortalities of tadpoles or metamorphs were recorded in the field. Dissections of the tadpoles were carried out using standard procedures identifying the protist infection in multiple samples (n=20 *R. damatina*, 20 *R. temporaria* and 15 *H. arborea* and 20 *R. temporaria*) from Zaječí potok, Brno, Czech Republic (49.23765N, 16.60637E) and Raduň, Czech Republic (49.88997N, 17.94375E). All specimens were in Gosner stage 26 or higher (Table S1 - and see below for discussion of sampling for *N. temporariae* beyond metamorphosis). The observed morphological characteristics are consistent with the original description of *N. temporariae*, specifically the protists observed possess monozoic oocysts and are morphologically and morphometrically consistent with the original description of *N. temporariae* (see description below), we therefore assign the gregarine-like oocysts to this species.

Standard light microscopy squash examination of liver, gall bladder, skin, heart, intestine and tail muscle of all examined tadpoles from the two localities revealed the presence of *N. temporariae* oocysts exclusively in host livers, demonstrating the intracellular microbial infection was not present in other host tissues examined. Samples of all examined tissues from each tadpole were fixed in 10% buffered formalin and glutaraldehyde, processed routinely, stained either with haematoxylin and eosin or toluidine blue, and examined by light or transmission

electron microscopy. Each oocyst is ovoid, asymmetrical with one side usually flattened measuring 15.5 (14.0-17.0) × 6.5 (5.0-7.5) µm (Fig. 1A, B). Using light microscopy, sporozoites appeared transversely striated that corresponds to micronemes organized in parallel layers (Fig. 1C). On a few occasions, we observed a free sporozoite, keeping its' overall banana shape during gliding movement, with only apical end appearing fully flexible (Fig. 1D). Oocysts were the only developmental stage of *N. temporariae* consistently sampled, making unclear if the tadpoles serve as definitive or intermediate host of *N. temporariae*.

In most preparations (n = 40), both *N. temporariae* oocysts and *Goussia* oocysts (i.e. protists cell with a fine elastic oocyst wall and four dizoic sporocysts measuring 7.5 (7.0–8.0) × 4.7 (4.0–5.0) (n = 50) - Eimeriorina Léger, 1911, Apicomplexa) were observed to occupy the same cells (Fig. 1B) (Jirků et al., 2009). However, in the *H. arborea* samples inspected (n = 15), this co-infection was not identified. In tadpole liver histological sections stained with Toluidine-Blue, oocysts were readily identified due to their characteristic morphology (Fig. 1E). Similarly as in fresh preparations, some oocysts were empty, sometimes containing residual granules. Interestingly, histological and TEM examinations revealed presence of oocysts exclusively in phagocytic cells in liver sinusoids (Fig. 1E). Both non-pigmented (c.f. Kupffer cells) and pigmented (containing melanosomes) cell types were identified (Fig 1A, B, E, F). The oocysts-containing cells belong to a macrophage lineage as reflected by their amoeboid nature with a notable variability in size and shape, typical filopodia, irregularly shaped nucleus, the presence of various quantities of lysosomes and phagosomes, poorly developed rough endoplasmic

reticulum, Golgi bodies, a well-developed cortical microvacuolar system, small mitochondria, and eventually melanosomes (e.g. (Guida et al., 1998)) (Fig. 1F).

To investigate progression of the *Nematopsis* infection, an additional 45 tadpoles of *Rana dalmatina* (Gosner stages 33-42) were collected at Zaječí potok on the 1st of July 2004. Twenty-five tadpoles were euthanized by pithing and examined as described above for a presence of *Nematopsis* demonstrating presence of the infection in liver tissue. Additionally, tadpoles of *R. dalmatina* were kept in captivity beyond metamorphosis to assess the fate of *Nematopsis* oocysts in metamorphosed animals. A subset of 20 juvenile (and later sub-adult) frogs in total were dissected at intervals of two weeks for the first two months, then every one month for the 3rd and 4th months, and every three months for the rest of the experiment up to the 15th month post-metamorphosis. In both fresh and histological preparations of livers from hosts examined, all tadpoles investigated were *Nematopsis* positive, while for organisms four to six weeks after metamorphosis, only empty oocysts were found.

In parallel to the histology analysis, we selected two liver samples from two different species: *R. temporaria* and *H. arborea* (four in total) and isolated 10-15 cells by mouth pipetting for DNA extraction. Using the eukaryotic forward primer (Euk1F) with the general -non-metazoan- reverse primer (Table S2), we PCR amplified and double strand sequenced (~1000 bp of SSU gene) 10 clones per liver sample. All sequences recovered showed ≥97% identity. A conserved portion of the alignment was selected to design a '*Nematopsis*' specific forward primer. This primer NEM-1F was used in association with the primer 28S-R1 targeting the 5' of the LSU rRNA gene from *R. temporaria* (three samples), *H. arborea* (three samples) and *R. dalmatina*

(two samples - Table S2). For each liver sample, three independent PCR amplifications were mixed and cloned. Three clones per sample were double strand sequenced (see SMM and Table S2).

Currently, there is only one sequence of the complete ribosomal RNA encoding gene belonging to the Gregarinasina Dufour, 1928 available in the Genbank nr database (*Gregarina* sp. JF412715, March 2016). To allow for comprehensive taxon sampling, phylogenetic analysis was therefore based on an alignment of the SSU gene that encompassed the V4 and V9 loops. The sequence alignment included 65 publically available sequences previously used for phylogenetic analysis (Rueckert et al., 2011; Wakeman et al., 2014) and 24 clone sequences recovered here. The ML and Bayesian phylogenies recovered a weakly supported backbone as previously described in phylogenies of the gregarines (Rueckert et al., 2011; Wakeman et al., 2014)(Fig. 2A). However, the SSU rDNA gene sequences recovered from the tadpole tissue form a highly supported clade (1/100/100) and branch with moderate bootstrap values (1/77/100) with the terrestrial gregarine clade 1 sequences (Rueckert et al., 2011; Wakeman et al., 2014) (Fig. 2A). Many alveolate genomes are highly AT rich (Gardner et al., 2002; Kopecna et al., 2006). We conducted Log-Det distance bootstrap analysis to account for differential base composition as a source of artifact (Foster and Hickey, 1999). This phylogenetic method provides strong support for phylogenetic association of *Nematopsis* with the terrestrial gregarines. This clade encompasses gregarine pathogens of a wide range of invertebrates, e.g. damselflies, earthworms, dragonflies, green darners, mosquitoes and sandflies (Fig. 2A). The phylogenetic results show that *N. temporariae* belongs to gregarines and confirms that this is the first example of a member of the subclass Gregarinasina,

Dufour 1828, infecting a vertebrate.

Eukaryotic ribosomal RNA gene clusters (rRNA genes) are typically present in multiple copies within a nuclear genome (Long and Dawid, 1980). The internal transcribed spacers (ITS1 and ITS2) that separate the SSU, 5.8S and LSU genes have a high rate of sequence variation. We generated 24 independent clone sequences from eight liver samples (three clones per sample). These sequences showed between 96% to 99% sequence identities across the SSU-ITS1-5S-ITS2 ribosomal sequences (Fig. 2B, C, and Table S3). Considering only single nucleotide polymorphisms that occurred in at least two independent clone sequences, we identified SNPs that identify variation specific for distinct rDNA-types. The main region of polymorphism was located within the ITS1 region identifying a minimum of two major rDNA-types (Fig. 2C), representing either inter or intra-individual genetic diversity.

This study represents the first molecular and microscopic description of the association between a gregarine and a vertebrate, and importantly shows that the *N. temporariae* oocysts form intracellular infections of tadpole cells. It is unclear whether tadpoles serve as definitive or intermediate hosts. These results provide the molecular tools for studying this infectious agent with regard to wider environmental ecology and specifically distribution in amphibian populations.

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FIGURE LEGEND.

Figure 1. Oocysts and free sporozoite of *Nematopsis temporariae* of *Rana dalmatina* tadpoles using light microscopy; fresh mount NIC (A-D), histological section stained with Toluidine-Blue (E), TEM (F). A. Intracellular oocyst (arrow) with single sporozoite (s) in a macrophage (white arrowheads). **B.** Macrophage containing oocysts of both *N. temporariae* and *G. noelleri* (arrowhead); the macrophage as well as *G. noelleri* oocyst are ruptured by pressure during the squash preparation; *N. temporariae* oocysts are mechanically flattened, making the sporozoites more dispersed than normal; see the pigment granules upper right. **C.** Composite micrograph of oocysts containing sporozoites showing distinct transverse striation. **D.** Composite micrograph of a free sporozoite in gliding motion. **E.** Macrophage containing two oocysts of *N. temporariae* (arrows) in lumen (L) of liver sinusoid. **F.** Macrophage (white arrowheads) containing oocyst of *N. temporariae* (arrow) n - macrophage nucleus, s - sporozoite. A, B, D in the same scale.

Figure 2. A. RAxML tree investigating the phylogenetic placement of *N. temporariae*. The phylogeny is calculated from 89 sequences and 1276 alignment positions. Bayesian posterior probability, ML and Log-Det bootstrap values were notated using the following convention: support values are summarized by black

circles when ≥ 0.9/80%/80% and white circles when this is not the case but all values are ≥ 0.6/50%/50%, actual values are shown for key branching relationships. The double-slashed line represents branches shortened by ½. The identification of the different clades was reported as described in (Rueckert et al., 2011; Wakeman et al., 2014). **B. Unrooted maximum likelihood phylogenetic tree of the ribosomal RNA gene cluster sequences.** The colours of the clone's names identified the tadpole liver tissue samples and the host taxonomy (see key). **C. Representation of the ribosomal gene cluster and the relative position of the different primer used in this study (not to scale).** For each region of the rRNA gene cluster the number of SNPs were indicated in brackets if the mutation is retrieved in at least two independent clones. The ITS1 region where at least two separate nucleotide motifs have been detected is represented using http://weblogo.berkeley.edu.

Supplementary informations:

TS1_ Detail of tadpole taxonomic identification

TS2_ Detail of primers used in this study

TS3_Percentage of similarity between the different clone sequences

SMM_Supplementary Material and Methods

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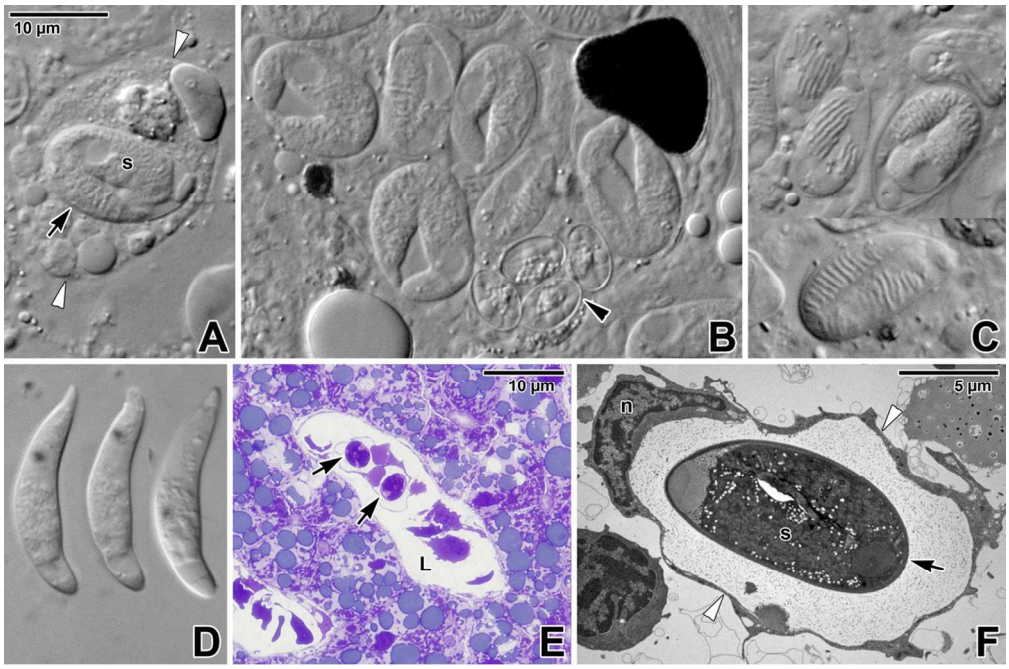

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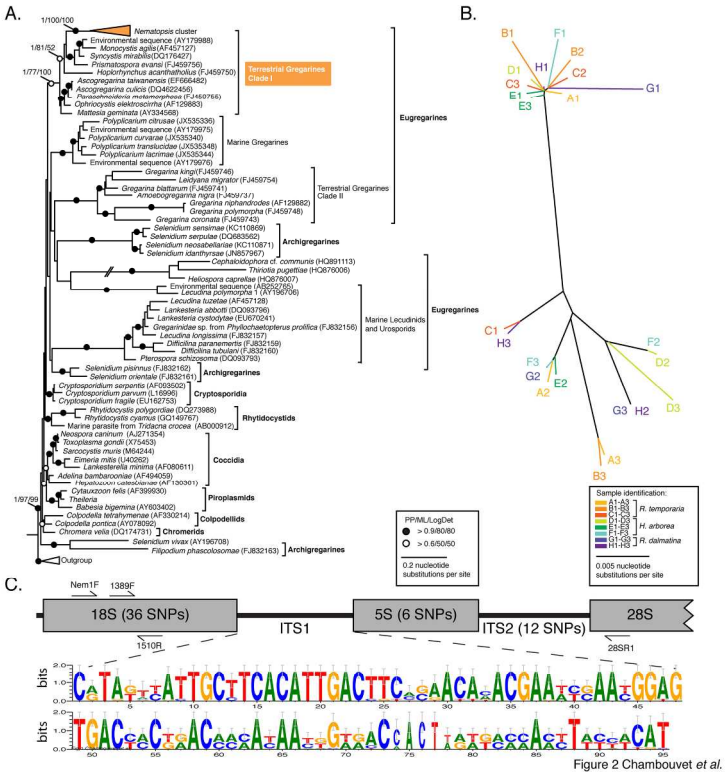

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220x169mm (300 x 300 DPI)