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Timing and quantifying *Aphanomyces astaci* sporulation from the noble crayfish suffering from the crayfish plague

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ABSTRACT

Aphanomyces astaci sporulation is crucial for the spreading potential of this disease agent. For the first time, we are reporting timing and quantity of *A. astaci* spores released from noble crayfish (*Astacus astacus*) suffering from crayfish plague under practical aquatic conditions. We infected nine noble crayfish with *A. astaci* PsI-genotype and maintained them in individual 8 L tanks. Spores (zoospores and cysts) were quantified from water samples (3 × 1 mL) taken every 12 h over 10 d using *A. astaci* specific qPCR. A clear sporulation trend was found, together with a high individual spore estimate variation. The median spore counts from two days before death to 12 h post mortem were from ~500 to ~2000 spores L^{-1}. A significant sporulation increase occurred after 24 h post mortem (~12,000 spores L^{-1}) and reached a peak after two days (~65,000 spores L^{-1}) before declining to or below pre mortem levels from the fourth day. The single most sporulating crayfish released from ~75,000 to ~400,000 spores L^{-1} during the mass sporulating period, yielding a maximum estimate of ~3,200,000 spores released from a single crayfish if we assume homogeneous spore distribution. The results confirm a mass *A. astaci* spore release from moribund and recently dead infected noble crayfish, with a sporulation peak one to three days post mortem. The acute crayfish mortality only three days after zoospore exposure confirm the lethal potential of the PsI-genotype. The powerful sporulation potential observed here may be one of the key virulence factors of this genotype.

1. Introduction

The crayfish plague (*Aphanomyces astaci)* epidemics are spreading via swimming zoospores, that are released after the sporangia of the oomycete *A. astaci* protrude from the crayfish into the ambient water (Söderhäll and Cerenius, 1999). The mass zoospore release occurs during or immediately after mortality of the infected crayfish (Svensson, 1978; Söderhäll and Cerenius, 1999). After the initial release, the life time of zoospores is prolonged due to encystment and repeated zoospore emergence (Söderhäll and Cerenius, 1999), thus allowing *A. astaci* zoospores a few more chances to find and infect a suitable crayfish host. Zoospore dynamics of *A. astaci* and other aquatic oomycetes have been studied previously (Unestam, 1966, 1969a,b; Svesson and Unestam, 1975; Cerenius and Söderhäll, 1984a,b; Kiryu et al., 2005). However, quantitative studies on zoospore production and release from infected crayfish under laboratory conditions are missing, except for our recent study on chronically infected signal crayfish (*Pacifastacus leniusculus*) (Strand et al., 2012).

The quantity of zoospores released during the sporulation period may determine the potential crayfish mortality rate on population level (Unestam and Weiss, 1970; Söderhäll and Cerenius, 1999). The timing of the mass zoospore release might also be essential, especially if only moribund or dead noble crayfish produce favourable

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conditions (Söderhäll and Cerenius, 1999). The understanding of the rationale behind the chronic or latent crayfish plague infection (Fürst, 1995; Jussila et al., 2011a; Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Svoboda et al., 2012) would benefit from further knowledge on the *A. astaci* sporulation dynamics especially prior to the crayfish mortality.

The purpose of this study was to broaden the understanding of the *A. astaci* sporulation dynamics during an acute crayfish plague outbreak. We present quantitative spore data (mainly zoospores but potentially also cysts) during different phases through the disease development, death and post mortem stages. We demonstrate moderate spore release during disease development and an increase in sporulation 24 h post mortem, with a sporulation peak approximately 48 h post mortem. This knowledge is important for the development of better strategies for crayfishery management, as well as for improved conservation strategies of native European crayfish stocks.

2. Material and methods

2.1. Experimental design

The experiment was carried out at the University of Eastern Finland (UEF) crayfish infection system RapuLatorio (Jussila et al., 2011b). Prior to the experiment, farmed noble crayfish (*Astacus astacus*) (ESeppä) were stored in the UEF fish farm in communal tanks with flow through Lake Kallavesi water, in ambient temperature. Crayfish were acclimated prior to the experiments in the infection system to the rearing temperature of +18 °C for a week.

Filtered Lake Kallavesi water was used in the experiment (5 μm absolute filter; Pleatflow II, Dominic Hunter Technologies Ltd.). Water quality (pH and DO-%) was measured twice a week and crayfish were given peeled peas every other day as food. In the infection trial system, water circulation was stopped in the 8 L individual holding tanks when the infected crayfish in question had died.

The virulent PsI-genotype *A. astaci* isolate UEF8866-2, originating from Lake Puujärvi, Karjalohja (Finland), was used in the experiment in order to produce zoospore inoculums for infection of the noble crayfish. The zoospores were produced according to Jussila et al. (2011b).

2.2. Infection and sampling

The noble crayfish ($n = 9$) were infected in a bath of PsI-genotype *A. astaci* zoospore solution (1820 zoospores mL^{-1}) for 20 h. In order to remove zoospores that were not firmly attached to the hosts the crayfish were transferred to fresh, filtered (spore free) and aerated Lake Kallavesi water for 1 h, then rinsed again with fresh and filtered Lake Kallavesi water before transfer to the RapuLatorio infection system (Jussila et al., 2011b) into fresh and filtered water.

The crayfish were housed individually in three separate systems, each consisting of three small (8 L), adjacent holding tanks and a sump tank, hereafter referred to as systems 1, 2 and 3. The set up was monitored twice a day (9 am and 9 pm) in order to follow the crayfish disease development and to determine their time of death. At the same time, 1 mL water samples were taken from the holding tanks in triplicate every 12 h from the initiation of the experiment until seven days post mortem (hereafter referred to as PoMo) and immediately frozen. The samples were taken approximately 15 cm below the water surface and were expected to contain mainly zoospores. However, we cannot exclude that the sampling also would pick up primary cysts and eventually secondary cysts not yet sedimented, or attached to organic substances in the water column. In the following, we will therefore use the term 'spores' as a collective term for both zoospores and cysts.

2.3. Quantification of spores by qPCR

A quantitative TaqMan® minor groove binder (MGB) real-time PCR assay (qPCR) that specifically detect and quantify a unique sequence motif of *A. astaci* was conducted as previously described (Vrålstad et al., 2009; Strand et al., 2011) with following modifications. Instead of using DNA extracts, eluates of the freeze dried water and putative *A. astaci* spore samples were used directly as templates. The freeze dried samples were eluated in 50 μL of milliQ water and vortexed rigorously before a step of freezing (−80 °C for 30 min) and heating (+65 °C for 2 min) in order to facilitate cell burst and direct release of DNA from the putative *A. astaci* spores (zoospores and cysts). Initial tests demonstrated that qPCR detection directly on the spore eluates gave similar results compared to previous samples where DNA had been extracted with a CTAB protocol (i.e. Strand et al., 2011; see also below). For each sample, 5 μL of non-diluted (1×) and diluted (10×) templates were analysed by qPCR performed on a Mx3005P qPCR system (Stratagene) using the TaqMan® Environmental Master Mix (Applied Biosystems) to reduce PCR inhibition (Strand et al., 2011). The qPCR data analysis was carried out with MxPro software version 4.10 (Stratagene). A negative PCR control was included for each qPCR run.

The content of *A. astaci* DNA was quantified based on PCR forming units (PFU) according to Vrålstad et al. (2009) where one PFU corresponds to one amplifiable target copy of *A. astaci* DNA, and the limit of quantification (LOQ) = 50 PFU. A four-fold dilution series of pure *A. astaci* culture genomic DNA were run together with the unknown samples in order to establish a standard curve based on known PFU values, thus making quantification of *A. astaci* PFUs in unknown samples possible (Vrålstad et al., 2009).

We controlled for putative PCR inhibition by comparing Ct values from concentrated and diluted replicates, i.e. $\Delta Ct = Ct_{10\text{-fold diluted}} - Ct_{concentrated}$, where ideally the $\Delta Ct = 3.32$ in the absence of inhibition. Practical calculation of PFU values for each sample was carried out as described earlier (Kozubiková et al., 2011a; Pârvulescu et al., 2012), where 15% variance in $\Delta Ct = 3.32$ was accepted. This allows for quantification in samples with a ΔCt range from 3.32 ± 0.5 (=2.82–3.82) between 10-fold diluted and the non-diluted replicate. When these criteria were met, the PFU value for each sample was calculated as the mean of PFU values, i.e. $PFU_{mean} = (PFU_{concentrated} + 10 \times PFU_{10\text{-fold diluted}})/2$. If the non-diluted replicate showed signs of inhibition, the PFU