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Interactions between *Paramoeba perurans*, the causative agent of amoebic gill disease, and the blue mussel, *Mytilus edulis*

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ABSTRACT

Amoebic gill disease (AGD) is caused by the ectoparasite *Paramoeba perurans* found free-living in seawater. In recent years outbreaks of AGD have occurred in most salmon farming countries causing significant economic losses. Mussels co-cultured with salmon in integrated multi-trophic aquaculture (IMTA) systems may change pathogen dynamics on sites by acting as reservoirs or biological controls. Through the use of an 18S rRNA gene quantitative real-time PCR we tested the interactions between *P. perurans* and blue mussels (*Mytilus edulis*) under experimental conditions by means of water-borne transmission. Quantification of DNA from water samples revealed a rapid decrease in *P. perurans* DNA over two weeks in the presence of mussels under experimental conditions. *P. perurans* was detected on swabs from mussel shells up to 48 h post-exposure. Additionally, no *P. perurans* were detected in mussels collected from natural mussel beds and fish farms. These results indicate that mussels are not a likely reservoir host for *P. perurans* but may in fact actively remove water-borne *P. perurans*.
Statement of relevance: The blue mussel does not appear to pose a biosecurity risk as a vector for the pathogen responsible for amoebic gill disease in salmon (*Paramoeba perurans*), instead the presence of blue mussels in experimental challenges led to a rapid removal of the parasite. The findings provide valuable information for how mussels may modulate pathogen densities on finfish-mussel farms.

1. Introduction

Amoebic gill disease (AGD) is an economically significant disease of Atlantic salmon (*Salmo salar*) caused by an amoeboid protozoan parasite *Paramoeba perurans* (synonymous to *Neoparamoeba perurans*, according to Feehan et al., 2013) (Shinn et al., 2015). In Tasmania, AGD has long been one of the main problems in salmonid aquaculture, with typical losses of 10–20% of production costs (Munday et al., 2001). In Scotland typical mortalities on Atlantic salmon farms range between 10 and 20% but have been reported to be as high as 70% at some sites (Marine Scotland Science, 2012). In recent years a global emergence of this disease has been documented with frequent outbreaks across the Northern and Southern Hemispheres *i.e.* Ireland, Scotland, Norway, Spain, France, North America, Japan, Chile and South Africa (Rodger and McArdle, 1996; Palmer et al., 1997; Findlay and Munday, 1998; Steinum et al., 2008; Crosbie et al., 2010; Bustos et al., 2011; Mouton et al., 2014). *P. perurans* is a free-living amoeba, however, when it colonises Atlantic salmon gills it can cause white swollen lesions, epithelial hyperplasia, and increased mucus production leading to lethargy, apoxia and eventually death (Munday et al., 2001).

Very little is known about the origin of *P. perurans* responsible for disease outbreaks. Fish import and subsequent spread between farms is not generally thought to be a feasible explanation for emergence of this disease in Europe (Steinum et al., 2008). On the other hand, prolonged periods of increased seawater temperature and/or high salinities combined with densities of fish in aquaculture sites most likely provided an advantage to *P. perurans* naturally present in marine environment (Nowak, 2007; Bridle et al., 2010; Wright et al., 2015). In aquaculture, infections with *P. perurans* were reported from a range of farmed fish in addition to Atlantic salmon (Crosbie et al., 2010; Karlsbakk et al., 2013; Mouton et al., 2014) while wild fish collected in the proximity of infected Atlantic salmon farms in Australia were tested negative (Douglas-Helders et al., 2002).

Diversification of aquaculture, reduction of potential adverse environmental impacts of Atlantic salmon farm systems and expansion of the shellfish industry are major aspirations intrinsic to the successful expansion of the aquaculture sector in Scotland. Integrated multi-trophic aquaculture (IMTA) systems are being developed in Atlantic salmon farming countries to test the concept of integration of Atlantic salmon, blue mussels and/or seaweed production. The aim is to deliver a diverse aquaculture system with a parallel reduction in organic waste from fish and dissolved inorganic nutrients (Barrington et al., 2009;

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Chopin et al., 2012; Wang et al., 2012). Co-culturing different species can change disease dynamics on farm sites and create new risks which need to be addressed to ensure good biosecurity in the production systems. Mussels are filter-feeders and have the potential to digest and inactivate some pathogens such as sea lice (*Lepeophtheirus salmonis*) (Molloy et al., 2011; Webb et al., 2013) or infectious salmon anaemia virus (ISAV) (Molloy et al., 2012). On the other hand *Vibrio anguillarum* (Pietrak et al., 2012), *Loma salmonae* (McConnachie et al., 2013) or infectious pancreatic necrosis virus (IPNV) (Molloy et al., 2013) may persist and accumulate in shellfish posing a threat of disease transmission to farmed fish.

The aim of the study was to assess mussels as a potential wild reservoir of *P. perurans* and investigate interactions between *Mytilus* species and *P. perurans* in terms of pathogen accumulation and transmission. Previously published molecular tests (real-time PCR) (Fringuelli et al., 2012) were applied to (1) examine the persistence of *P. perurans* in water, on shell surfaces and in mussel tissues after experimental exposure and (2) test for the presence of *P. perurans* in mussel tissues from wild mussel individuals and from bio-fouling mussels collected on fish farms in Scotland.

2. Materials and methods

2.1. P. perurans culture maintenance and dilution curve for quantification

P. perurans, maintained at the Marine Scotland Science (Aberdeen, UK), originated from gills of infected farmed Atlantic salmon (west coast of Scotland) collected in 2012. A culture was established following the protocol of Crosbie et al. (2012). The *P. perurans* culture was maintained routinely at 15–18 °C on malt yeast agar (MYA) (0.1% malt, 0.1% yeast, 2% Bacteriological agar, 35‰ 0.22 μm filtered seawater) overlaid with 7 ml of 0.22 μl filtered seawater (SSW, 35 ‰) collected offshore of Stonehaven (Aberdeenshire, UK). The culture was subcultured every two weeks by transferring the seawater overlay containing a free-floating population of amoebae to a new MYA plate. The old MYA plate containing a population of attached amoebae received a new seawater overlay. The culture of *P. perurans* was first isolated in November 2012 and used in experimental challenges described in the present study in May and June 2014. The pathogenicity of this culture has been confirmed under experimental conditions by challenging naïve Atlantic salmon at 7, 12 and 24 months post-isolation (Marine Scotland Science, unpublished data).

P. perurans were harvested from culture plates by centrifugation of culture supernatants at 6000 ×*g* for 5 min. Pellets were pooled and re-suspended in SSW. Before harvesting, quadruplicate $\log_{10}$ dilutions were made in 96-well plates for enumeration of the total number of amoebae harvested under light microscope. After centrifugation, $\log_{10}$ dilutions were made in duplicate from a starting cell density of *P. perurans* of 7×10^3 cells. Then, homogenised mantle-gill tissue was spiked with the prepared dilutions and total DNA was extracted using two different DNA extraction protocols: automated extraction using the QIAsymphony DNA DSP mini kit (Qiagen) or manual extraction using the MasterPure DNA and RNA Purification kit (Epicentre). Negative controls representing mussel tissue homogenate without *P. perurans* and blank controls were set up. *P. perurans* DNA was quantified using the quantitative real-time PCR (qPCR) (see Section 2.4.) and C_t values recorded to create a standard dilution curve in R′ statistical package (version 3.0.2, R Core Team, 2013).

2.2. Experimental exposure of mussels to P. perurans culture

2.2.1. Mussel maintenance

Depurated market-size mussels were bought from a commercial supplier or vendor and kept in outdoor covered seawater tanks at 9 °C. The seawater that was from Nigg Bay (Aberdeen, Scotland) was filtered (through a 1 mm wedge wire screen and 15 μm particle filter) before pumping to storage tanks. This water was also used in experimental beakers and tanks. Mussels were naturally feeding on nanophytoplankton present in the sea water, considered as one of the significant dietary components for bivalves (Shumway et al., 1985) and no additional food was added to the holding tanks prior to the experiments.

2.2.2. Experiment 1: pilot experiment to establish the time frame for P. perurans ingestion by mussels and distribution in mussel tissues

Forty eight beakers containing 500 ml of 35 ‰ seawater (10 °C) with a small air stone were placed in a temperature-controlled room (18 °C). By the end of the experiment (144 h after exposure) the water temperature had risen to 14 °C. Five mussels were placed in each duplicate treatment beaker and allowed to acclimatise before adding *P. perurans*. Mussels were exposed to *P. perurans* (10^4 cells L^{-1} in suspension) in 500 ml and sampled at 1, 2, 3, 4, 6, 24, 48 and 144 h post-exposure (hpe). The mussel filtering activity was monitored throughout the experiment.

At each time point, four beakers were sampled without replacement: two treatment beakers (containing *P. perurans* and mussels), one positive control beaker (*P. perurans* only) and one negative control beaker (mussels only). At each time point, all mussels were removed and seawater from the entire beaker was filtered through 1.0 μm cellulose nitrate membrane filters (25 mm diameter, GE Healthcare Life Sciences) which were stored dry in 2 ml Safelock tubes (Eppendorf). The shell of each individual mussel was swabbed with a sterile cotton-tip swab on one side using four strokes lengthwise. Additionally, the bottom inside edge of each beaker was swabbed using two circular strokes to test adhesion and replication of *P. perurans* on plastic surfaces. All swabs were stored in RNA*later* (VWR). The whole digestive gland and a section of mantle and gill tissue were sampled from each mussel and placed in separate tubes containing 100% ethanol (Sigma). All samples were frozen at −20 °C immediately after sampling.

2.2.3. Experiment 2: tank experiment to quantify removal rates of P. perurans from the water column

Mussels were exposed to *P. perurans* (3×0^4 cells L^{-1} in suspension) in tanks containing 20 L of 35 ‰ seawater. These tanks were placed within a larger tank connected to a flow-through seawater system to maintain the experimental tanks at 12 °C throughout the experiment. Thirty mussels were placed in four tanks: three treatment tanks (containing *P. perurans* and mussels), one positive control tank (*P. perurans* only) and one negative control tank (mussels only). The mussels were fed with algae (Shellfish diet 1800 from Reed Mariculture Inc.) according to the manufacturer's instructions, when a reduced filtering capacity was observed (day 7 post-exposure).

Water samples and shell swabs were taken at 1, 4, 7, 24, 48, 168 and 384 hpe. A 500 ml water sample was taken from each tank and filtered using the method described above. The entire shell of three mussels in each tank was swabbed thoroughly; sampled mussels were placed in a net (3 mm diameter nylon mesh), within the tank to avoid repeated swabbing at different time points. The swabs were placed in a dry microcentrifuge tube (opposed to RNA*later* as in experiment 1), immediately stored at −20 °C and processed within 48 h. Finally, a section of net (1 × 1 cm) (retaining sampled mussels) from each tank was placed in a dry tube to test *P. perurans* adhesion. Mussels were checked daily and filtering activity and mortalities were recorded.

2.3. Sampling of wild and biofouling mussels

To assess the presence of *P. perurans* in wild mussels fifty individuals were collected from each of three sampling points on the west coast of Scotland (Lunderston Bay, Loch Long (Ardgarten) and Rascarrel Bay) in 2013. To assess the presence of *P. perurans* in mussels in aquaculture farm environment, a sample of 150 mussels, settled directly on the