Experimental exposure to low concentrations of *Neoparamoeba perurans* induces amoebic gill disease in Atlantic salmon

Short running title: low concentrations of *N. perurans* induce AGD

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Data Availability Statement

The data are available by request from the authors.

Conflict of interest statement

The authors declare no conflict of interest.

Abstract

Amoebic gill disease is a significant issue in Atlantic salmon mariculture. Research on the development of treatments or vaccines uses experimental challenges where salmon is exposed to amoebae concentrations ranging from 500 to 5000 L^{-1}. However, the water concentrations of *N. perurans* on affected salmon farms are much lower. The lowest concentration of *N. perurans* previously reported to cause AGD was 10 L^{-1}. Here we report that concentrations as low as 0.1 L^{-1} of *N. perurans* can cause amoebic gill disease. We propose that concentrations of *N. perurans* that reflect those measured on salmon farms should be used for future experimental challenges.

Keywords

Amoebic gill disease, experimental challenge, risk prediction

Introduction

Amoebic gill disease (AGD) is a prevalent infectious disease primarily affecting Atlantic salmon in commercial mariculture (for review see Nowak 2012, Oldham et. al. 2016, Nowak and Archibald 2018). The disease is caused by colonisation of host gills by the marine amoeba *Neoparamoeba perurans* (see Young *et. al.* 2007, Young *et. al.* 2008, Crosbie *et al.* 2012), which initiates a localised host response resulting in the formation of hyperplastic lesions and an overall reduction in the functional gill surface area (Adams & Nowak 2001). Freshwater bathing is the current industry standard treatment to control AGD in Tasmanian salmonid aquaculture (Nowak, 2012). Hydrogen peroxide bathing has been shown to be a relatively effective alternative in the regions where access to large quantities of fresh water is limited, rendering freshwater bathing logistically impossible, (Adams et. al. 2012). Without therapeutic intervention, AGD outbreaks in Atlantic salmon mariculture can result in mortality rates exceeding 50% (Munday *et. al.* 1990). While prominent in Australia, AGD is now of global concern to commercial mariculture operations, with cases reported in the United States, Ireland, Spain, France, South Africa, Norway, Chile, Scotland, New Zealand and Japan (Oldham et al 2016, Marcos-López and Rodger 2020). Although primarily affecting farmed salmonids, AGD has been confirmed in other marine-farmed fish species including ayu, turbot, seabass and Ballan wrasse (Crosbie et al 2010a, Mouton *et. al.* 2014, Karlsbakk et al. 2013, Kim et al 2017). When histology is used for AGD case definition a fish is considered AGD positive if paramoebae (recognisable in histological sections due to their characteristic morphology) are present in an association with epithelial hyperplasia (Nowak, 2012).

Initially, experimental AGD infections were induced using cohabitation of naïve Atlantic salmon with known carriers of the disease (Howard *et al.* 1993, Findlay, *et al.* 1995). However, this method resulted in unreproducible results and highly variable disease severity (Findlay *et al.* 2000). To address these issues, a challenge method was developed which used gill isolated amoebae to infect AGD-naïve Atlantic salmon (Zilberg et al 2001). Since then, more standardised experimental infections have been facilitated through improvements in amoebae isolation and purification techniques. Current AGD challenges are induced either by adding a suspension of *N. perurans* trophozoites (230-500 *N. perurans* L^{-1}) directly to experimental tanks (Morrison *et al.* 2004, Crosbie *et al.* 2010; Adams *et al.* 2012, Benedicenti *et al.* 2019) or by a bath challenge, where fish are immersed in a high concentration of the amoebae, for example 1800 *N. perurans* L^{-1} (Marcos-López *et al*. 2018) or 5000 *N. perurans* L^{-1} (Crosbie *et al.* 2012) for several hours before being returned to the experimental tanks. While initially the minimum infective concentration was determined as 230 *N. perurans* L^{-1} with exposure to 23 *N. perurans* L^{-1} giving negative results 7 days post infection (Zilberg *et al*. 2001), the development of a partial purification technique using amoebic adherence resulted in the induction of AGD in naïve Atlantic salmon 14 days post-exposure to 10 *N. perurans* L^{-1} (Morrison *et al.* 2004).

Concentrations of *N. perurans* measured in sea water in and around Atlantic salmon sea cages, where the presence of AGD was confirmed, ranged from 0-62 *N. perurans* L^{-1}, most often reported as approximately 1 *N. perurans* L^{-1} (Bridle *et al*. 2010; Wright *et al*. 2015; Wright *et al*. 2017) and were much lower than those used in experimental challenges. Given the discrepancy between the typically low seawater concentrations of *N. perurans* and the high concentrations used in experimental challenges, current experimental models may be exerting unrealistically high infection pressures on challenged Atlantic salmon. This may create an environment where the immune system of challenged fish rapidly becomes overwhelmed with the high amoebae concentrations, rendering experimental prophylactic or therapeutic intervention ineffective. An experimental model capable of closely replicating natural disease progression would be more useful to study vaccine or treatment efficacy and host pathogen interactions than currently used challenge models. The aim of this study was to investigate if it was possible to induce AGD experimentally using amoebae concentrations relevant to those found on salmon farms.

2. Materials and Methods

2.1 Fish husbandry

Atlantic salmon smolts (approximately 150 g) were obtained from a commercial hatchery and acclimated to sea water at the University of Tasmania Aquaculture Centre. The salmon were then stocked (n=15) into 6 x 280 L independent recirculating systems, each consisting of a tank with an external biofilter and a protein skimmer and filled with 0.2 µm (nominal) filtered sea water. The fish were acclimated to the system for 20 days and water quality (temperature, pH, ammonia, nitrate, nitrite, salinity) was monitored throughout the experiment. The water was maintained at 15°C to 16.5°C, 35 ppt salinity, and a pH of 8.0. Negative control fish (uninfected) were kept in sea water in separate 4000 L recirculation tanks.

2.2 Amoebae isolation and challenge

N. perurans trophozoites were isolated post-mortem from gills of salmon with AGD from an ongoing infection tank maintained at the University of Tasmania as previously described (Morrison *et. al.* 2004). After enumeration, the freshly isolated amoebae were diluted in 5 L of sea water and added individually to each of the treatment tank using a dispersion vessel to ensure uniform distribution throughout the tank. As each tank was a separate recirculation system there was no water exchange between the tanks and thus transfer of amoebae between tanks was not possible. The infective doses used were 0.1, 1 and 10 *N. perurans* L^{-1} with two replicate 280 L tanks per treatment. All procedures were approved by the University of Tasmania Animal Ethics Committee (A13938).

2.3 Sample collection

During the experiment, gill swabs were taken from 5 fish from each tank starting from day 9 post challenge, repeated every 10 days up until day 49. Past this point, sampling occurred every 5 days until 64 days post challenge. The fish were anaesthetised in a separate container with clove oil (20 mg L^{-1}) and, when not responsive to a mechanical stimulus, they were laid flat on their right side and the left anterior surface of the gill was uniformly swabbed using a clean cotton swab. The tip of the gill swab was placed directly into a 1.5 mL Eppendorf™ tube containing 500 µL of tissue and cell lysis solution (4 M urea, 0.5% SDS, 10% glycerol, 0.2 M NaCl) and frozen at -80°C. After the gill swab was performed, the fish was recovered and returned to its original tank.

Water samples were collected on the same days as gill swabs. Water was sampled from each tank using 1 L glass bottles inverted and submerged at approximately 0.3 m under the surface. The sample was filtered through glass fibre filter under vacuum. The filter was placed in an Eppendorf™ tube containing 500 µL of tissue and cell lysis solution and stored frozen at -80°C.

The experiment was terminated 68 days post challenge when significant gross signs of disease (white patches on the gills) were observed. All fish were euthanised with a lethal dose of clove oil (40 mg L^{-1}), and the second left gill arch was then excised and placed into sea water Davidson's fixative for 24h before being transferred to 70% ethanol. Although 15 fish were initially stocked into each tank, there were some misadventure-related losses which reduced fish numbers across all tanks (Table 1).

2.4 Histology

Gill samples were processed for histology and 5 µm sections stained with haematoxylin eosin were examined using Olympus BX40 microscope. Fish were confirmed as AGD positive by observation of at least one paramoeba associated with hyperplastic lesions. Severity of infection was estimated using percentage affected filaments and size of lesions (number of interlamellar units involved in a lesion). Number of interlamellar units was counted in 10 lesions from each section. If fewer than 10 lesions were present, all of them were included in the analysis. Only well oriented filaments were used for assessment of severity of infection. One section from the second left gill arch was examined for each fish.

2.5 Molecular analysis

Quantitative PCR (qPCR) analyses were conducted using previously published methods (Bridle et al., 2015). In brief, total nucleic acid (TNA) was extracted from gill swabs and water filter samples using a DNA precipitation technique. All qPCR analyses were conducted using a CFX connect Real-Time PCR detection system (Bio-Rad). Forward primers, reverse primers and the Hex probe sequence used in the current study were developed and tested for intra-species specificity in previously published research (Wright et al., 2015). *N. perurans* numbers were estimated based on the 2880 18S rRNA copies cell^{-1} as previously determined (Bridle et al. 2010). Samples were considered positive for the presence of *N. perurans* when duplicate wells were both successfully amplified. Assay results were quantified by analysis of background subtracted raw fluorescence unit (RFU) data from cycles 5 to 45 using a mechanistic model known as 'cm3' developed by Carr and Moore (2012) included in the qpcR package (Spiess and Ritz, 2014) for R studio statistical computing software (R CoreTeam, 2013).

2.6 Statistical analysis

Nested ANOVA with Satterthwaite approximation (due to the unbalanced number of fish in tanks at the end of the trial) was used to analyse the effect of amoebae concentration on AGD prevalence and the number of ILU in a lesion. The percentage data were arcsine transformed before analyses. The data fulfilled assumptions of ANOVA. The analyses were done using the Excel spreadsheet for Nested ANOVA with Satterthwaite approximation (http://www.biostathandbook.com/nestedanova.html, McDonald 2014).

Logistic regression analysis, which models the relationship between a binary response variable and independent predictor variables, was used to predict the probability of *N. perurans* presence on the gills of Atlantic salmon and in the water column, given the initial nominal concentration of *N. perurans*. A sample was considered positive for *N. perurans* if *N. perurans* 18S ribosomal DNA was amplified during qPCR for a given gill swab or water sample. Initial nominal concentration of *N. perurans* was considered a fixed factor with three categories: 0.1, 1 and 10 *N. perurans* L^{-1}.

All generalised linear model analyses were performed in R 4.0.2 (R Core Team), using the package 'lme4'. The probability of *N. perurans* presence on a gill swab was modelled using 240 samples (80 samples per treatment). Backward stepwise variable selection was used using the ‘drop1’ function (library ‘lme4’ in R v.4.0.2) to test all possible single fixed-effect terms and potential two-way interactions, to restrict the number of predictors to only the essential. The model’s fit was assessed using a Hosmer-Lemeshow-le Cessie test via the ‘residuals.lrm’ function (library ‘rms’ in R v.4.0.2), where a larger *p* value suggests a more reliable fit, and predictive accuracy was assessed via receiver operating characteristic curve (ROC) area under curve (AUC) in R.

3. Results

Histology results confirmed that all fish exposed to *N. perurans* were positive and had AGD lesions by the end of the trial. The percentage of filaments affected ranged from 8.1% (an individual exposed to 0.1 *N. perurans* L^{-1}) to 98.5% (an individual exposed to 1 *N. perurans* L^{-1}). The nominal concentration of *N. perurans* in the water column did not have a statistically significant effect on the percentage of filaments affected by *N. perurans* ($P = 0.214$) at end of the trial, however tank had a significant effect ($P = 0.0027$). There was a high individual variability within tanks, with 21.3% of variance in the percentage of affected filaments explained by this effect. Nominal amoeba concentration in the water column at the beginning of the experiment did not have a statistically significant effect of on the size of lesions caused by *N. perurans* ($P = 0.538$). Lesion size varied from just 2 to 74 interlamellar units within one gill arch (an individual exposed to 1 *N. perurans* L^{-1} with 22.2% of filaments affected). Tank had a significant effect on the lesion size ($P = 0.0388$), with 18.7% of the variance in the lesion size explained by this effect.

While the size of AGD lesions was not affected by the nominal concentration of amoebae in water, there were some differences in the morphology of the lesions (Figure 1). In fish exposed to the lowest concentration of *N. perurans*, AGD lesions ranged from mostly small inflammatory nodules with some lamellar synechiae to larger plaque-like lesions (Figure 1A). Some lesion showed oedema (Figure 1B). In the fish exposed to higher concentrations of *N. perurans* the lesions were mostly hyperplastic with lamellar fusion covered by squamous epithelium (Figure 1C). Mucous cells were numerous, particularly in the larger lesions (Figure 1B). Interlamellar vesicles (ILVs), sometimes with amoebae or their remains, were common in the large hyperplastic lesions (Figure 1D). Amoebae were associated with some of the lesions, sometimes in large numbers (Figure 1D).

DNA of *N. perurans* was detected at every sampling point in the gills of fish exposed to 10 *N. perurans* L^{-1}, whereas it was first found on day 29 in the fish exposed to 1 *N. perurans* L^{-1} and from day 39 in the fish exposed to 0.1 *N. perurans* L^{-1} (Table S1). Gill swabs from fish exposed to *N. perurans* were positive for all exposed fish tested from day 59 post challenge (Table S1). However, there was a high variability in amoeba load on the gills of infected fish both between individuals and between tanks (Table 1). As no swabs were taken at the final sampling, the relationship between amoebae load and gill histological changes could not be evaluated. However, the fish from the tanks with higher mean amoebae load on gill swabs at day 64 typically had a greater mean percentage of filaments affected by the end of the trial (Table 1). All gill swabs from the control salmon were negative for *N. perurans*.

A logistic regression was used to model the probability of the presence of *N. perurans* on the gills of Atlantic salmon post *N. perurans* challenge. The likelihood of *N. perurans* presence on the gills of Atlantic salmon was positively related to initial concentration (LRT: $G_1 = 33.1$, $P \leq 0.001$) and the number of days post challenge (LRT: $G_1 = 168$, $P \leq 0.001$). The likelihood of *N. perurans* presence was predicted to increase with initial concentration of *N. perurans* and the number of days post challenge (See Figure 2). Specifically, every 10-day increase post *N. perurans* challenge was associated with a 28.5% (confidence interval, 21.5% to 37.7%) increase in the odds of *N. perurans* being present on the gills of challenged Atlantic salmon. Holding days post challenge at a fixed value, the odds of *N. perurans* being present on the gills of Atlantic salmon increased by a multiplicative factor of 19.5 (confidence interval, 6.27 to 71.3) for those challenged at 10 amoebae L^{-1} and 2.16 for 1 amoebae L^{-1} (confidence interval, 0.799 to 6.10) compared to fish challenged at 0.1 amoebae L^{-1}. The model was deemed reliable (Hosmer-Lemeshow-le Cessie test, $p = 0.610$) and accurate (AUC = 0.941).

A separate logistic regression was used to model the probability of the presence of *N. perurans* in the water column of experimental tanks post *N. perurans* challenge. The likelihood of *N. perurans* presence in the water column was positively related to the number of days post-challenge (LRT: $G_1 = 45.1$, $P \leq 0.001$), but there was no statistical evidence of differences between initial nominal concentrations (LRT: $G_1 = 4.23$ $P = 0.120$). The likelihood of *N. perurans* presence in the water column was predicted to increase with the number of days post-challenge (See Figure 3). Every 10-day increase post-challenge was associated with a 16.4 % (confidence interval, 10.7 % to 23.6 %) increase in the odds of *N. perurans* being present in the water in the experimental tanks. The model was deemed reliable (Hosmer-Lemeshow-le Cessie test, $p = 0.626$) and accurate (AUC = 0.884).

4.Discussion

AGD developed after an experimental exposure of Atlantic salmon to 0.1 *N. perurans* L^{-1}, which was the lowest concentration tested in this experiment and to the best of our knowledge the lowest ever reported to induce AGD. This concentration is consistent with the levels of *N. perurans* observed on the salmon farms affected by AGD in Tasmania and Norway (Bridle et al 2010; Wright et al 2015; Wright et al 2017; Hellebø et al 2017). This means that current experimental models are likely unrealistic and that it is possible to use an experimental model which better reflects conditions during AGD outbreaks in mariculture. Furthermore, the results suggest that any treatment which leaves even a small number of viable *N. perurans* on the gills of treated fish is unlikely to succeed as we have shown that low concentrations of *N. perurans* can result in overt AGD. Therefore, even very low concentrations of *N. perurans* in the marine environment should be considered a risk factor for AGD.

AGD lesions found in the salmon from our experiment belonged to the three phases ranging from epithelial desquamation and oedema associated with primary interactions with amoebae and inflammation and initial focal epithelial hyperplasia to large hyperplastic lesions with squamation-stratification of epithelia at lesion surfaces with variable presence of mucous cells (Adams and Nowak 2003). Fish exposed to higher concentrations of amoebae showed mostly the third phase characterised by the large hyperplastic lesions with squamous epithelium covering the lesion surfaces while the fish exposed to the lower concentration had oedema and inflammatory lesions as well as large hyperplastic lesions. Small AGD lesions in the fish exposed to the lowest concentration of *N. perurans* were consistent with nodules and plaques described in Atlantic salmon gills after transfer sea

water (Nowak and Munday 1994). Together, all these lesion characteristics and the timing of the detection of *N. perurans* in the gill swabs suggest that at the end of the experiment the fish exposed to the lowest concentrations of amoebae were in an earlier stage of AGD than the fish exposed to higher concentrations of amoebae.
The likelihood of *N. perurans* being present in the water column was positively correlated to the number of days post-challenge, but not to the initial concentration of the amoebae supporting the importance of lesion development, severity and *N. perurans* shedding from developed AGD lesions. Once each tank had been inoculated *N. perurans* was not detected in the water until 30 days post-exposure at the earliest during which time it is presumed the initial inoculum of amoebae had either attached to the host gills and initiated the development of lesions or had been removed by the recirculating water filtration. The lag time before *N. perurans* were shed from lesions in numbers great enough to be detected is the likely reason that no statistically significant relationship was found between the initial inoculation concentration and the concentration of *N. perurans* measured in the water. *N. perurans* numbers in duplicate samples of water were highly variable during an experimental challenge (González et al 2016), suggesting uneven distribution of the amoebae in aquatic environment.

The likelihood of *N. perurans* present on the gills was predicted to increase with initial nominal concentration of *N. perurans* and the number of days post-challenge, suggesting that there was a relationship between the number of *N. perurans* on the gills and the initial concentration of *N. perurans* in the water. Similarly, *N. perurans* load 18S rRNA (Cp values) was significantly affected by initial concentration of the amoebae in water 21 days after exposure of salmon to 500 or 5000 amoebae L^{-1} for 21 days (Benedicenti et al 2015).

In the current experiment the gill samples were positive for *N. perurans* later than in challenges where higher concentrations of amoebae were applied (Benedicenti et al 2015, Collins et al 2017, Oldham et al 2020). There was a high variability in the severity of AGD lesions both within and between individuals and the variable size of lesions within the second left gill arch suggested that the lesions were induced at different time points or that there was high individual variability in host response to the amoeba. Nonetheless, the number of *N. perurans* on the gill swabs while variable increased with greater exposure concentrations. Shedding of *N. perurans* during AGD progression explains many of these findings and highlights that once the amoebae colonise fish gills, the gills become the main reservoir of the pathogen, both for the infected individual and for other fish in the same tank or seapen.

There was no relationship between the exposure concentration (0.1 *N. perurans* L^{-1}, 1 *N. perurans* L^{-1}, 10 *N. perurans* L^{-1}) and severity of lesions at 68 days post-infection. While a relationship between the initial concentration of amoebae in the water and lesion severity was previously reported (Zilberg et al 2001, Morrison et al 2004), even then it was not obvious for lower concentrations tested (10, 25 and 50 *N. perurans* L^{-1}) (Morrison *et al* 2004). It is possible that this relationship is only apparent during earlier stages of infection and that by 68 days post-infection lesion severity had plateaued, or when fish are exposed for a short time to higher concentrations of *N. perurans* (>50 *N. perurans* L^{-1}). Our study and the levels of *N. perurans* observed on salmon farms (Bridle et al 2015; Wright et al 2015) suggest that while related, the severity of AGD (lesions) is difficult to predict from concentrations of *N. perurans* detected in the aquatic environment.

Due to sampling logistics the numbers of *N. perurans* on the gills were not available for the last sampling point when the severity of AGD lesions was determined based on histology. However, fish from the tanks with higher *N. perurans* gill loads on day 64 had a greater percentage of filaments affected at the end of the trial (day 68). The relationship between *N. perurans* numbers in gill swabs and severity of lesions has been previously reported in farmed Atlantic salmon (Bridle et al 2010). Furthermore, a positive relationship between the amoeba gill load and score for gill pathology based on number and size of AGD lesions was reported 21 days after infection with clonal *N. perurans* at two different nominal concentrations of 500 or 5000 amoebae L^{-1} (Collins et al 2017).

Tank had a significant effect on severity of AGD, possibly the result of differences between tanks concerning the *N. perurans* loads on the gills and *N. perurans* in water. While care was taken to add the same number of *N. perurans* to the duplicate tanks there was a high variability in qPCR results for water samples and there was no statistical evidence of an effect of the initial concentration of *N. perurans* in water and the presence of *N. perurans* in the water during the experiment. In fact, *N. perurans* were not detected in the water until 30 days post-exposure where it is presumed infected fish were beginning to shed *N. perurans* into the water. Whenever possible more replicate tanks should be used in AGD challenges.

In summary, we show that AGD could be induced using as low concentration as 0.1 *N. perurans* L^{-1}. This, together with the concentrations of *N. perurans* recorded on salmon farms suggests that previous testing of treatments and vaccines using current experimental challenge protocols which apply much higher concentrations could result in an excessive challenge pressure. The low concentrations tested here are similar to those reported on salmon farms affected by AGD meaning that any vaccines or treatments tested under these challenge conditions are likely to represent a realistic host-pathogen interaction.

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Figure and Tables Legends

Table 1. AGD severity 68 days post-challenge measured as the percentage of filaments affected by AGD lesions and the size of AGD lesions (number of interlamellar units/lesion - ILU/lesion). Number of *N. perurans* on a gill swab for 5 fish from each tank on day 64 shown as a measure of infection intensity.

Figure captions

Figure 1. AGD lesions, A – an inflammatory nodule and a larger plaque-like lesions, most of filaments normal, B – lesion showing oedema (asterisk) and numerous mucous cells (arrow), C- lamellar fusion due to hyperplastic epithelium with interlamellar vesicles (arrow), D – lamellar fusion due to hyperplastic epithelium covered by squamous epithelium (arrow left-right), paramoebae, characterised by the presence of the parasome (line arrow) present on the outside of the lesion.

Figure 2. Observed relationship between initial *N. perurans* concentration, days post-challenge and presence of *N. perurans* on the gills of Atlantic salmon. Predicted probability of *N. perurans* presence using a model that incorporated initial nominal concentration, shown as 0.1, 1 and 10 *N. perurans* L^{-1}, and days post-challenge are indicated by separate lines, and the 95% confidence intervals of the predicted probabilities are indicated by the areas of shading.

Figure 3. Observed relationship between days post challenge, initial *N. perurans* concentration and presence of *N. perurans* in the water column. Predicted probability of *N. perurans* presence using a model that incorporated initial nominal concentration, shown as 0.1, 1 and 10 *N. perurans* L^{-1}, and days post challenge are indicated by separate lines, and the 95% confidence intervals of the predicted probabilities are indicated by areas of shading.

Tables

Nominal exposure concentration (*N. perurans* L^{-1}) (fish number)	Filaments affected (%) Mean (SD)	ILU/lesion Mean (SD)	Mean *N. perurans* number on gill swab on day 64 (SE)
0.1 (8)	17.7 (6.6)	15.7 (5.4)	833 (294)
0.1 (10)	32.4 (9.1)	17.1 (5.0)	982 (732)
1 (4)	68.4 (32.5)	17.7 (6.1)	6510 (3895)
1 (11)	35.1 (11.2)	18.5 (3.9)	749 (1958)
10 (11)	58.5 (16.9)	24.6 (6.7)	3151 (589)
10 (12)	60.4 (20.8)	17.3 (7.3)	1515 (556)

Figures

Figure 1.

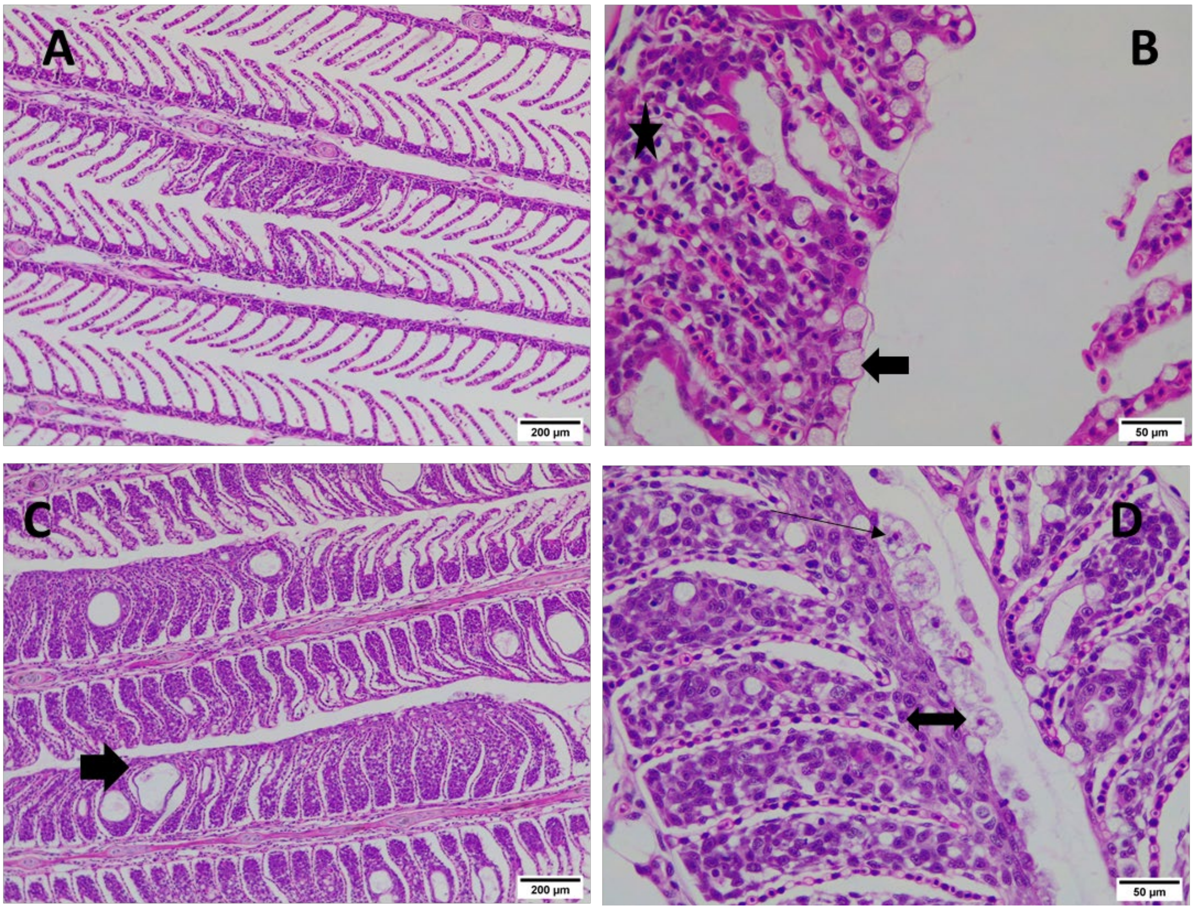

Figure 2

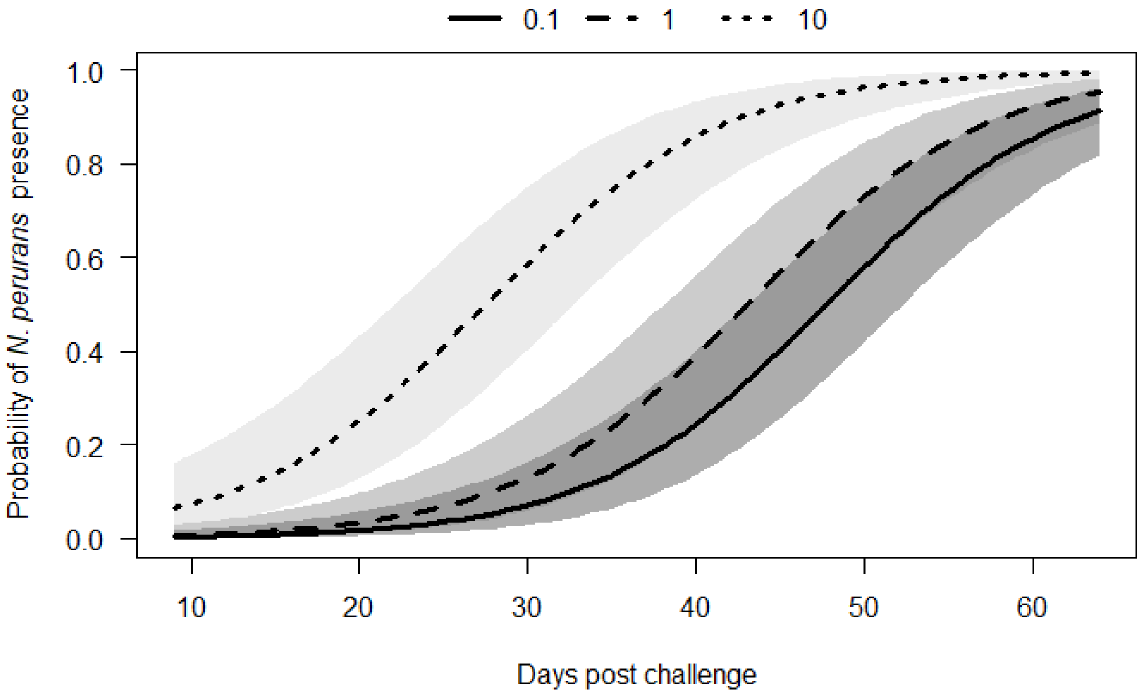

Figure 3