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Antimicrobial peptide gene expression in Atlantic salmon (*Salmo salar*) seven days post-challenge with *Neoparamoeba perurans*

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ABSTRACT

Amoebic gill disease in teleost fish is caused by the marine parasite *Neoparamoeba perurans*. To date, the role of antimicrobial peptides β-defensins and cathelicidins in this infection have not been explored. Using a high-throughput microfluidics quantitative polymerase chain reaction system (Biomark HD™ by Fluidigm), this study aimed to: firstly, to investigate organ-specific expression of antimicrobial peptide genes β-defensin-1, -3 and -4 and cathelicidin 2 in healthy Atlantic salmon; secondly, to compare the expression of these antimicrobial peptide genes in healthy versus asymptomatic Atlantic salmon seven days post-challenge with *Neoparamoeba perurans*.

Results from this study indicate expression of the β-defensin and cathelicidin genes in the selected organs from healthy Atlantic salmon. Furthermore, a statistically significant upregulation of β-defensins −3 and −4 and cathelicidin 2 was detected in gill of parasite-challenged salmon. The upregulated cathelicidin and β-defensin genes in gill could indicate novel potential roles in innate immune responses to *Neoparamoeba perurans*.

1. Introduction

Amoebic gill disease (AGD) is an infection of Atlantic salmon and other teleost fish caused by the ectoparasite *Neoparamoeba perurans* (*N. perurans*) (Young et al., 2007). Hallmarks of the disease include macroscopic white mucoid patches on infected gills, and microscopic evidence of epithelial hyperplasia (Taylor et al., 2009). Advanced progression of AGD has become a significant contributing factor to fish mortality in commercial settings (Shinn et al., 2015). AGD is a disease of global economic importance affecting sites of Atlantic salmon farming in European countries such as Ireland, Scotland and Norway (Rodger 2014). Treatment for AGD includes hydrogen peroxide therapy (Rodger 2014) and freshwater bathing to remove mucus and *N. perurans* (Clark et al., 2003). While experimental vaccines have been proposed (Valdenegro-Vega et al., 2015), many are unsuccessful given that parasites elicit chronic infections and are capable of evading or modulating host immune responses (Crampton and Vanniasinkam 2007).

To date, AGD studies describe a robust cytokine response in Atlantic salmon and rainbow trout, highlighting the importance of innate immunity in protecting the host from *N. perurans* (Bridle et al., 2006; Pennacchi et al., 2014; Marcos-López et al., 2018). Antimicrobial peptides (AMPs) are essential mediators of innate immune responses in vertebrates and invertebrates (Masso-Silva and Diamond 2014). They are low molecular weight peptides which exhibit potent antimicrobial and immunomodulatory activity and whose expression can be constitutive or inducible (Katzenback 2015). Teleost AMPs such as piscidins (Browne et al., 2011), hepcidin (Douglas et al., 2003) and histone derivatives (Fernandes et al., 2002) have been researched extensively in terms of constitutive and pathogen-induced expression (Cuesta et al., 2008; Browne et al., 2011).

Two families of AMPs that warrant more immediate attention in the context of fish health, particularly in relation to parasitic infections, are the β-defensins and cathelicidins. These two AMP families have been identified as mediators of early innate immune responses (Katzenback 2015). The majority of studies, to date, pertaining to the genetic expression of teleost β-defensin and cathelicidin AMPs have reported upregulated expression of these genes in response to bacterial and viral infections (Chang et al., 2005; Bridle et al., 2011; Guo et al., 2012; Chen et al., 2013). Although previous teleost cathelicidin expression has been explored in two parasitic infections (Heinecke and Buchmann 2013; Chettri et al., 2014), neither Atlantic salmon β-defensin or cathelicidin expression profiles have been examined in relation to AGD. Previously, two Atlantic salmon cathelicidin genes (Chang et al., 2006) have been confirmed and more recently, five β-defensin subfamilies have been identified (Harte et al., 2020). These findings

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reinforce the need to fully explore their roles in immune responses to a range of microbial infections, including parasitic infections such as AGD. Both cathelicidins and β-defensins exert antimicrobial activity through electrostatic membrane interactions with invading pathogens (Falanga et al., 2016). Furthermore, mammalian β-defensins and cathelicidin are well characterized as cytokine stimulators, chemoattractants and inducers of immune cell differentiation and maturation (Semple and Dorin 2012; van Harten et al., 2018). Despite this information, there is insufficient knowledge on the specific roles of Atlantic salmon β-defensin and cathelicidin AMPs in early innate immune responses.

Recently, a trial was conducted whereby AGD was established in Atlantic salmon over a three-week period (Ní Dhufaigh et al. 2021). During the trial, AGD-affected fish were sampled once a week until the trial reached its endpoint at 21 days post-infection, at which stage the infected fish displayed AGD-typical mucoid patches on their gills. The aims of the current study are to extend the finding of this trial, primarily to investigate the expression of selected Atlantic salmon β-defensin and cathelicidin 2 AMP genes, in the context of very early asymptomatic AGD. Secondly, while it has been established that AGD elicits an immune response in primary immunological organs such as the liver, head kidney and spleen (Bridle et al., 2006; Botwright et al., 2021), the expression of AMP genes in organs with ancillary immunological function – namely, swim bladder, intestine and gill – has not yet been explored. Gill was selected as this is the site of AGD infection and thus the local AMP response was of primary interest. Similar to gill, the intestine is a mucosal barrier exposed constantly to marine pathogens (Yu et al., 2020). Given the dense population of B- and T-cells, epithelial cells (Muniz et al., 2012) and the presence of gut-associated lymphoid tissue (GALT) (Yu et al., 2020), it was of interest to investigate if AGD affected AMP expression in this immunologically active mucosal barrier. To date, the immune function of fish swim bladder has been suggested (Cui et al., 2014) and its functional importance in the context of AGD is not yet defined. Therefore, for this study, swim bladder, gill and intestine from healthy and *N. perurans*-infected salmon were assessed for β-defensin and cathelicidin 2 AMP genes expression in order to elucidate the functional role that these AMP genes play in local and systemic responses to *N. perurans* infection. Quantification of gene expression was performed at day 7 post infection, using the Biomark HD™ system (Fluidigm) high-throughput microfluidics quantitative polymerase chain reaction (qPCR) platform. Within the first seven days post-infection with *N. perurans*, there is evidence of an upregulation in the genes of important mediators of innate immunity such as complement C3, C-type lectins and chemokines prior to the appearance of macroscopic lesions (Morrison et al. 2006a, 2006b). Given their importance in immunity, a study investigating the gene expression of AMPs in early innate immune responses in AGD is warranted.

2. Materials and methods

2.1. Animal husbandry and in-vivo AGD infection trial

All protocols described here adhere to the relevant guidelines. The Health Products Regulatory Authority (HPRA) approved the *in-vivo* infection challenge and assigned it the project authorization number AE 19137/P002, following the Animals Scientific Procedures Act 1986 (Directive 2010/63/EU transposed into Irish law by S.I. No. 543 of 2012).

Details of this trial have been previously published (Ní Dhufaigh et al. 2021). Briefly, naïve Atlantic salmon smolts (average weight of 120 g) were obtained from a commercial hatchery setting in Ireland and transported to the fish housing facility in GMIT. Smolts were divided into two cohorts of 40 fish each, namely Control and *N. perurans* Infected. All fish were placed directly into 1,000 L tanks of seawater, with 20 fish per tank (see Fig. 1 for distribution of fish from each cohort) and were now deemed post-smolts. The conditions of the seawater were maintained as follows for the entirety of the trial; water temperature at 16 °C, oxygen saturation at 80–120% and salinity at 30 ppt. Prior to exposure to *N. perurans*, the fish were given a two-week acclimatization period.

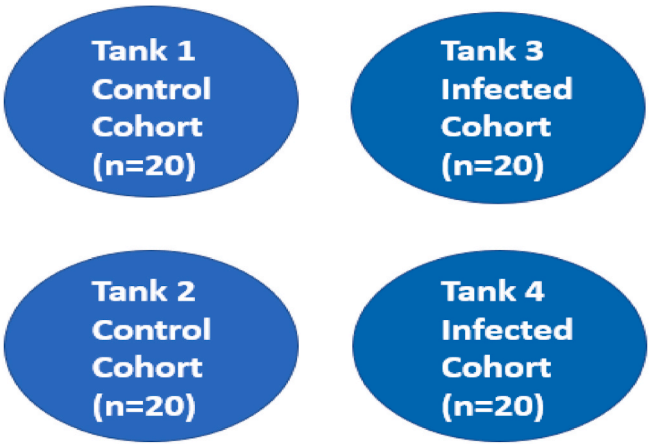

Fig. 1. Distribution of Control and N. perurans Infected fish. The 40 fish from each cohort were evenly distributed between four tanks, with 20 fish per tank.

For the inoculation protocol, the amoeba selected were originally obtained from AGD-affected fish and were cultured in-house for one year prior to the commencement of the trial as previously detailed (Ní Dhufaigh et al. 2021). Fish from the *N. perurans* Infected cohort (n = 40) were transferred to a 300 L tank containing fresh clean seawater and infected with *N. perurans* (concentration 2000 amoeba/L (Pennacchi et al., 2014); this count was achieved by using a Sedgewick cell counting chamber (API Supplies) to count amoebic cells). After addition of the amoeba to the 300 L tank, the water was gently mixed and agitated to ensure even distribution of *N. perurans* throughout the tank. After 4-h in the 300 L tanks, the fish were moved back to their original tanks. The Control cohort were transferred to a 300 L tank containing clean seawater for a period of 4 h before being transferred back to their original 1,000 L tanks.

2.2. Organ sampling of control and N. perurans infected cohorts

One day prior to the inoculation protocol (time point 0, T_0), 3 fish from each Control cohort tank (n = 6) were sacrificed using a lethal dose of the anaesthetic tricaine methanesulfonate (MS-222) (Sigma). These fish were examined for macroscopic signs of AGD and a gill score, adapted from an established protocol (Taylor et al., 2009), was recorded for each fish. Organs sampled from these fish included whole swim bladder, whole intestine and the third left gill arch. All samples were stored overnight in 500–1000 μl of RNALater® at 4 °C before being stored at −80 °C. At seven days post-inoculation (7dpi), 3 fish from each *N. perurans* Infected tank (n = 6) were euthanized with MS-222 (time point 1, T_1). The assignment of a gill score and the sampling of select organs was repeated for the sampled fish. A gill from each fish sampled at T_0 and T_1 was sent to the Marine Institute (Oranmore, Co. Galway) for qPCR analysis to detect the presence of *N. perurans* as previously described (Downes et al., 2017).

As this study was interested in the early phase immune response to AGD, samples were not taken from the remaining fish in each cohort. However, the trial in its entirety continued from a total of 21 days, with 6 fish from each cohort sampled at T_2 (14dpi) and T_3 (21dpi) (Ní Dhufaigh et al. 2021).

2.3. RNA extraction and synthesis of cDNA

RNA was extracted from homogenized samples representing whole swim bladder (n = 12), whole intestine (n = 12) and whole gill (n = 12) excised from Controls at T_0 and the *N. perurans* Infected cohort at T_1

using the RNEasy Mini Kit (QIAGEN), as per the manufacturer's instructions. The starting quantities of each sample type ranged from 37 to 52 mg for swim bladder, 30–44 mg for intestine and 27–39 mg for gill. Extracted RNA was quantified in duplicate using the Epoch™ Microplate Spectrophotometer (Biotek). The absorbance ratios of the extracted RNA at 260 nm and 280 nm were inspected to ensure all values fell between 1.8 and 2.0. Of the RNA extracted from each organ, 2 μg was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN) as per the manufacturer's protocol. Negative controls, which contained RNA and all the cDNA conversion reagents except for reverse transcriptase, were also included.

2.4. Selection of housekeeping and AMP genes

Two housekeeping genes, elongation factor 1α_a (EF1α_a) and elongation factor 1α_b (EF1α_b) were selected as the housekeeping genes against which the expression of the selected AMP genes was normalized. These housekeeping genes were selected as they have proven to be two of the most stable reference genes across a range of organs from Atlantic salmon (Olsvik et al., 2005). Sequences for the forward and reverse primers of EF1α_a and EF1α_b were adapted from Olsvik et al. (2005). The expression of the AMP genes was normalized to the two housekeeping genes to ensure greater accuracy (Vandesompele et al., 2002). Housekeeping gene stability across swim bladder, intestine and gill was verified using three software packages: geNorm (Vandesompele et al., 2002), Best Keeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004).

For the AMP genes, cathelicidin 2 primers were designed to amplify a region of the full cathelicidin 2 gene that corresponds to the mature sequence of the AMP located in exon 4. Cathelicidin 1 was excluded from the study, as prior studies have shown that this gene is not expressed constitutively in gill or intestine. When compared to cathelicidin 2, its expression peaks within the first seven days of infection (Bridle et al., 2011), therefore it did not fit with the timeframe of this early immune response study.

For the β-defensin genes, primers spanning exon-exon boundaries were designed to amplify regions of the β-defensin AMP genes that represent the mature peptides. Primers for the amplification of cathelicidin 2 and β-defensin AMP genes were designed using Primer Quest Tool software (Integrated DNA Technologies). All primer sequences, primer efficiencies (data not shown) and accession numbers for selected genes are listed in Table 1.

Table 1
Forward and reverse sequences of housekeeping and AMP genes and relevant primer efficiencies.

Gene	Accession No.	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Primer Efficiency
Elongation Factor 1α_a (EF1α_a)	AF321836	CCC CTC CAG GAC GTT TAC AAA	CAC ACG GCC CAC TAC A	92.14%
Elongation Factor 1α_b (EF1α_b)	BG933853	TGC CCC TCC AGG ATG TCT AC	CAC GGC CCA CAG GTA CTG	91.43%
β-defensin-1 (asBD1)	LC3879731	GCT GCA TCA TTT CCC TTC TCT T	ACA CAG CAC AAG AAT CCC TTT C	95.22%
β-defensin-3 (asBD3)	LC387975	GTC ATT GCT TGT GGA ATA CAA GAG	GAA GCA AGG CAC AAA CGA AG	90.63%
β-defensin-4 (asBD4)	LC387976	CAC ATG TGA TGT AAA TGA GGC A	TGG TAG TTC TGC TGA CAG AC	90.17%
Cathelicidin 2 (asCath2)	AY360357	AAG CCC AGC GGA GGC TCT AGG	GCC AAA CCC AGG ACG AGA GCC	98.87%

2.5. Standard PCR of housekeeping genes

Standard PCR was performed to amplify the selected housekeeping genes from randomly selected Control and *N. perurans* Infected cohort cDNA samples to check the quality of the RNA extracted and the subsequent cDNA synthesized. Each 20 μl volume PCR reaction contained appropriate volumes of DreamTaq Master Mix 2X (Thermo Scientific), relevant forward and reverse primers, nuclease-free water and 2.5 μl of the relevant cDNA sample. No template controls (NTCs) and no primer controls (NPCs) were also included, with nuclease-free water replacing template DNA and primers, respectively. Housekeeping genes from the selected samples were amplified using the same PCR program on a MiniAmp Thermal Cycler (Applied Biosystems) using the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. All PCR products were assessed using agarose gel electrophoresis (stained with SYBR™ Safe [Invitrogen]). Gels were imaged using the Gel Doc™ EZ Imager (Bio-Rad) after electrophoresis to determine the presence of amplicons representing the selected housekeeping genes.

2.6. Biomark HD™ gene expression analysis

The Biomark HD™ (Fluidigm) microfluidics system was utilized to investigate AMP gene expression between the Control and *N. perurans* Infected cohorts from the *in-vivo* infection trial. The system employs a 48.48 integrated fluidics circuit (IFC) chip that allows for 48 individual cDNA samples and 48 individual primer sets to be assessed in one experiment, using qPCR techniques to amplify target genes from the selected samples.

2.6.1. Pre-amplification of cDNA samples

Pre-amplification of the target genes from the Control and *N. perurans* Infected cohort cDNA samples was performed as per Fluidigm's protocol. Briefly, a single primer pool was created by mixing 2 μl of each target gene primer (forward and reverse) with 372 μl of nuclease-free water to create a primer stock where each primer is at a concentration of 500 nM (primers supplied by Integrated DNA Technology as 100 μM stocks). A master mix was then prepared, containing 112.5 μl of DreamTaq Master Mix 2X (Thermo Scientific), 22.5 μl of the 500 nM primer pool and 33.75 μl of nuclease-free water. In a 96 well PCR plate, 3.75 μl of the master mix was added to the appropriate wells. To the wells, 1.25 μl of the appropriate cDNA samples were added. NTCs were also included. The PCR products were amplified using the MiniAmp Thermal Cycler (Applied Biosciences) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 12 cycles of denaturation at 95 °C for 15 s and annealing at 58 °C for 4 min.

2.6.2. Exonuclease I treatment of pre-amplification samples

PCR products were treated with exonuclease I to remove all components of the pre-amplification reaction. A master mix containing 9 μl exonuclease I 10X reaction buffer (New England Biolabs), 18 μl of exonuclease I (New England Biolabs) and 63 μl of nuclease-free water was prepared. To each well in the 96 well PCR plate containing pre-amplification samples, 2 μl of an exonuclease I master mix was added. The exonuclease digest was performed using the MiniAmp Thermal Cycler as per Fluidigm protocols. After the digest reaction, the PCR plate was placed in the −20 °C freezer for overnight storage.

2.6.3. Biomark HD™ gene expression analysis by 48.48 IFC

The 48.48 IFC chip was primed as per manufacturer's instructions using 300 μl of control line fluid (mineral oil). Primer mixes for each target gene were subsequently prepared by mixing 25 μl of 2X Assay Loading Reagent (Fluidigm), 20 μl of EB buffer (QIAGEN) and 5 μl of a pre-prepared 50 μM primer stock. In total, seven primer mixes, each one 5 μM in concentration, was prepared. A primer mix lacking any primers

acted as the NPC. A master mix was prepared by mixing 210 μl of 2X SSO Fast™ Eva Green qPCR master mix (Bio-rad) with added reference dye (Integrated DNA Technology) and 21 μl of 20X DNA binding dye (Fluidigm).

20 μl of the primer mixes were to the relevant wells of a new sterile 96 well PCR plates. To each well 3.85 μl of the master mix and 3.15 μl exonuclease-treated pre-amplified cDNA samples or nuclease-free water were added. 6 μl of the primer mixes were added to their relevant inlets in the 48.48 IFC chip. Each primer set was tested in duplicate. The same volume of sample mixes was aliquoted to the inlets of the 48.48 IFC chip. By adding each primer mix in duplicate, this allowed each primer set to be tested against each individual cDNA sample six times, creating six technical replicates for each biological replicate. Following manufacturer's protocol, the chip was primed again and loaded onto the instrument to perform the qPCR program, which was set up as follows: hot start at 95 °C for 1 min, followed by 20 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 20 s and then melt curve analysis from 58 to 95 °C with 3 s between each increasing temperature increment.

2.6.4. Data analysis

All retrieved qPCR data were analyzed in the Fluidigm Real Time PCR Analysis software (Fluidigm) and all statistical analysis was performed using GraphPad 8.0 (Prism). The Fluidigm software was instructed to compare the same organs from both Control T_0 and *N. perurans* Infected T_1 cohorts. The housekeeping genes EF1α_a and EF1α_b were assigned as the reference genes in the software, and this provided delta-delta Ct values (ΔΔCt) and fold change values for both the housekeeping genes and the selected AMP genes from both cohorts. Using the averaged fold change values for the AMP genes from each biological replicate, statistical analysis was performed in GraphPad software. A non-parametric one-way ANOVA Kruskal-Wallis test was used to compare housekeeping gene and AMP gene expression in the organs of healthy Control T_0 fish. Furthermore, a non-parametric Mann-Whitney statistical test was performed to investigate differential expression of AMP genes between the Control T_0 and *N. perurans* Infected T_1 cohorts.

3. Results

3.1. Gill score and confirmation of N. perurans infection in infected cohort

The results of gill scoring and confirmation of *N. perurans* infection for the samples examined here have been previously described (Ní Dhufaigh et al. 2021). Based on the AGD gill scoring protocol previously established (Taylor et al., 2009), fish sampled at T_0 and T_1 (7dpi) were all assigned a gill score of 0. On average, the salmon sampled at T_0 weighed 127.67 g and measured 23.07 cm in length. Similarly, fish sampled at T_1 weighed 146 g on average and measured 24.2 cm in length on average. qPCR analysis of gill samples from each fish at timepoints T_0 and T_1 was undertaken at the Marine Institute (Oranmore, Co. Galway) as per the protocol previously described (Downes et al., 2017). The results of the qPCR analysis confirmed that the parasite had established AGD in salmon sampled from the *N. perurans* Infected T_1 cohort without the disease being clinically apparent, a finding that has been reported previously (Downes et al., 2015). This finding was further validated in that fish from the *N. perurans* Infected cohort had achieved a gill score of 1 by T_2 and a gill score of 2 by T_3. Histological analysis of gills from the *N. perurans* cohort also found evidence of epithelial hyperplasia, indicating the amoeba utilized for the trial were virulent (Ní Dhufaigh et al. 2021). Throughout the entirety of the trial, from T_1 to T_3, the Control cohort retained a PCR-negative result for presence of *N. perurans* and displayed no evidence of epithelial hyperplasia.

3.2. RNA extraction and synthesis of cDNA

The 260/280 nm ratio of the extracted RNA ranged from 1.970 to 2.090, indicating relatively pure RNA was isolated from the sampled swim bladder, intestine and gill. The total quantity of RNA extracted from the sampled organs ranged from 7.25 to 63.35 μg.

Standard PCR to confirm the presence of the housekeeping genes EF1α_a and EF1α_b was performed on random cDNA samples representing swim bladder, intestine and gill from both Control T_0 and *N. perurans* Infected T_1 cohorts. The agarose gel electrophoresis (Fig. 2A and B) performed on the PCR products indicated that the selected organs were strongly positive for the expression of the housekeeping genes EF1α_a and EF1α_b. The cDNA formed from the extracted RNA was of good quality as indicated by the gel electrophoresis images.

3.3. Housekeeping gene selection

Three software packages were utilized to establish the stability of the housekeeping genes in the selected organs. Housekeeping gene stability is ranked by the software packages geNorm and NormFinder based on calculated stability values, with the lowest value indicating greatest gene stability. The software Best Keeper ranks housekeeping gene stability based on R^2 values, with those values closest to 1 indicating greatest gene stability. Table 2 outlines the stability and R^2 values of the housekeeping genes as determined by the different software packages.

The criteria outlined by Fluidigm for acceptable Ct values state Ct values must fall between 5 and 25 (Fluidigm n.d.). The Ct values for housekeeping genes EF1α_a and EF1α_b were acceptable based on Fluidigm's criteria. From the R^2 and stability values obtained in Table 2, there was general agreement between the softwares that EF1α_a and EF1α_b are stable housekeeping genes for the selected organs.

3.4. Bio mark HD™ gene expression analysis

To assess differences in gene expression between healthy and AGD-affected salmon, qPCR was performed using the Biomark HD™ 48.48 IFC chip (Fluidigm). Acceptable qPCR reactions were those where Ct values for the target gene fell between 5 and 25, as outlined by Fluidigm (Fluidigm n.d.). The results collected from the qPCR experiment were evaluated to confirm positive amplification of target housekeeping and AMP genes. Outliers were input as 0 in further statistical analysis. For the remaining samples, averaged fold changes from the technical replicates for each biological replicate were analyzed using GraphPad (Prism).

To examine if there is a difference in the expression of housekeeping and AMP genes in swim bladder, intestine and gill sampled from the salmon in the Control T_0 cohort, a non-parametric one-way ANOVA Kruskal-Wallis test was performed. Results of this statistical analysis indicated no statistical difference in the expression of housekeeping or AMP genes between the three organs types sampled in the healthy control fish. Fig. 3A–F represents the expression of the housekeeping and AMP genes in swim bladder, intestine and gill sampled from the Control T_0 cohort.

All organs express the housekeeping genes EF1α_a (Fig. 3 A) and EF1α_b (3 B). Similarly, asBD1 (3C), asBD3 (3D) and asBD4 (3 E) are all present in swim bladder, intestine and gill. Cathelicidin 2 (asCath2) was also expressed in the selected organs (3 F).

For the expression of the AMP genes between the Control T_0 and *N. perurans* Infection T_1 cohorts, AMP gene expression was normalized to both housekeeping genes EF1α_a and EF1α_b. A non-parametric Mann-Whitney test was utilized to identify significant differences in AMP gene expression in swim bladder, intestine and gill. Compared to healthy swim bladder at T_0, small differences in asCath2 and asBD3 expression were identified in swim bladder from the *N. perurans* Infected T_1 cohort. A similar trend was observed in asCath2 and asBD4 expression in the *N. perurans* Infected T_1 cohort intestine compared to intestines sampled

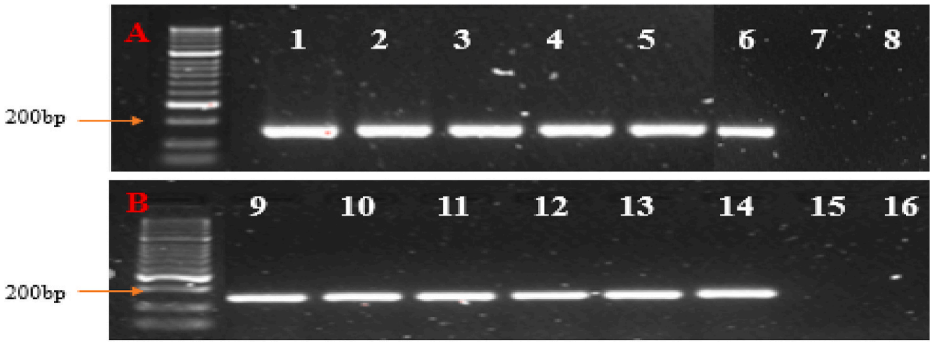

Fig. 2. A–B: **2.5% gel electrophoresis of standard PCR products amplified from random cDNA samples using housekeeping gene primers. 2 A; EF1α$_a$ PCR products amplified from Control T$_0$ cohort swim bladder, intestine and gill (lanes 1–3) and *N. perurans* Infected T$_1$ cohort swim bladder, intestine and gill (lanes 4–6). 2 B; EF1α$_b$ PCR products amplified from Control T$_0$ cohort swim bladder, intestine and gill (lanes 9–11) and *N. perurans* Infected T$_1$ cohort swim bladder, intestine and gill (lanes 12–14). NTC reactions for the housekeeping genes are represent by lanes 7 and 15 while NPC reactions for the housekeeping genes are represented by lanes 8 and 16. The amplicon sizes for EF1α$_a$ and EF1α$_b$ are 146 base pairs and 150 base pairs respectively.**

Table 2
Housekeeping Gene Selection determined by NormFinder, BestKeeper and geNorm Software.

Organ	Software	EF1α$_a$	EF1α$_b$
Swim bladder	*NormFinder*	0.036	0.087
	BestKeeper	0.965	0.949
	geNorm	0.134	0.147
Intestine	*NormFinder*	0.061	0.106
	BestKeeper	0.976	0.960
	geNorm	0.141	0.136
Gill	*NormFinder*	0.156	0.082
	BestKeeper	0.824	0.850
	geNorm	0.158	0.120

from Control T$_0$ fish. However, these differences in AMP gene expression did not reach statistical significance. In gill sampled from the *N. perurans* Infected T$_1$ cohort, a statistically significant upregulation in asCath2 ($p = 0.0022$) and asBD3 and asBD4 ($p = 0.0411$ and 0.0152, respectively) was identified, compared to gill from the Control T$_0$ cohort. The organ-specific expression of these AMP genes is represented by Fig. 4A–C.

4. Discussion

The study described here demonstrates exciting novel findings with respect to AMP gene expression in both healthy and AGD-affected salmon at 7dpi. Results obtained from the study indicate that there is a statistically significant upregulation of the β-defensin (asBD3 and asBD4) and cathelicidin 2 (asCath2) AMP genes in the gill in response to the early phase of the parasitic infection at T$_1$, before clinical presentation is observed. The lack of statistically significant AMP gene upregulation in the intestine and swim bladder would suggest that AGD does not elicit a systemic immune response in these organs at this early stage, but rather it triggers a localized response in the gill as the primary site of the infection. In AGD, non-statistically significant increases in tissue specific AMP gene expression in intestine and swim bladder may be attributed to husbandry conditions or stress. However, it has been shown that at 7dpi there is a peak in the expression of other early immune response genes in organs such as the liver, which could be reflective of the results seen here (Bridle et al., 2006). Moreover, in a previous study, liver sampled from AGD-affected Atlantic salmon also exhibited upregulation of important innate immunity markers such as TNF-α2, MHC-II and IL-1β1, although not statistically significant (Bridle et al., 2006).

In swim bladder, intestine and gill sampled from healthy Control cohort salmon, there was variable, but constitutive expression of housekeeping and each of the AMP genes. In this study, the expression of asBD1, 3, 4 and asCath2 were found in swim bladder of Atlantic salmon for the first time. These findings highlight a possible novel immune role for this organ whose ancillary functions are largely underexplored to date.

The use of the Biomark HD™ by Fluidigm here is one of the first instances in which a high-throughput microfluidics gene expression platform has been employed in the context of fish health. The system has been used in previous studies to monitor microbial populations, such as *N. perurans*, in Pacific salmon in Canada (Miller, 2016). The Biomark HD™ system has been utilized for studies in the context of human health, investigating gene expression profiles from specifically identified cells (Kara et al., 2015); identifying biomarkers in diseases (Mastrokolias et al., 2015) and detecting pathogens (Olwagen et al., 2019). This study illustrates the applicability of microfluidics technology in monitoring marine health too. The Biomark HD™ microfluidics system provides significant advantages over conventional qPCR methods, such as reducing costs and variability introduced by the user, and an increased sensitivity in detecting the selected target genes. The stability of the selected housekeeping genes EF1α$_a$ and EF1α$_b$ were verified using three different software packages – geNorm, BestKeeper and NormFinder – and the results further validate housekeeping gene stability results from a previous study (Olsvik et al., 2005).

The role of teleost β-defensins in bacterial infections has been examined (Casadei et al., 2009), with extensive and species-specific antibacterial and antiviral activity previously identified (Cuesta et al., 2008; Guo et al., 2012). However, β-defensin gene expression has not been explored to -date in parasite-infected teleost fish, nor has any anti-parasitic activity been confirmed (Katzenback 2015). In this study, a statistically significant upregulation of asBD3 and asBD4, but not asBD1, in infected salmon gill compared with healthy gill was confirmed. This suggests a more prominent and previously unexplored role for these two genes in response to localized early phase, preclinical AGD infections. The long-term impact of asBD3 and asBD4 on the sustained innate immune response to AGD, past the early phases of infection, remains unknown and necessitates further study. A recent publication on the proteomic innate immune response to early AGD infection found immunosuppression was evident in the first 7 days of infection (McCormack et al., 2021). The significance of the upregulation of asBD3 and asBD4 in AGD-affected gill, in conjunction with their known antimicrobial activity and immunomodulatory properties, is strongly indicative of a more robust innate immune response to early stage AGD.

Human β-defensins-3 and -4 (hBD3 and hBD4) are known chemoattractants for macrophages (Wu et al., 2003), while hBD3 can interact with Toll-like receptors (TLRs) on antigen presenting cells and influence their differentiation (Funderburg et al., 2007). Human β-defensin-1 (hBD1) has the ability to chemoattract effector immune cells, but its potency is not as pronounced as other human β-defensins (Yang et al., 1999). Furthermore, hBD3 displays anti-parasitic activity against *Plasmodium falciparum* by promoting permeabilization of cellular membranes and subsequent cellular lysis (Terkawi et al., 2017), while intestinal hBD2 expression has been induced by the protozoan parasite

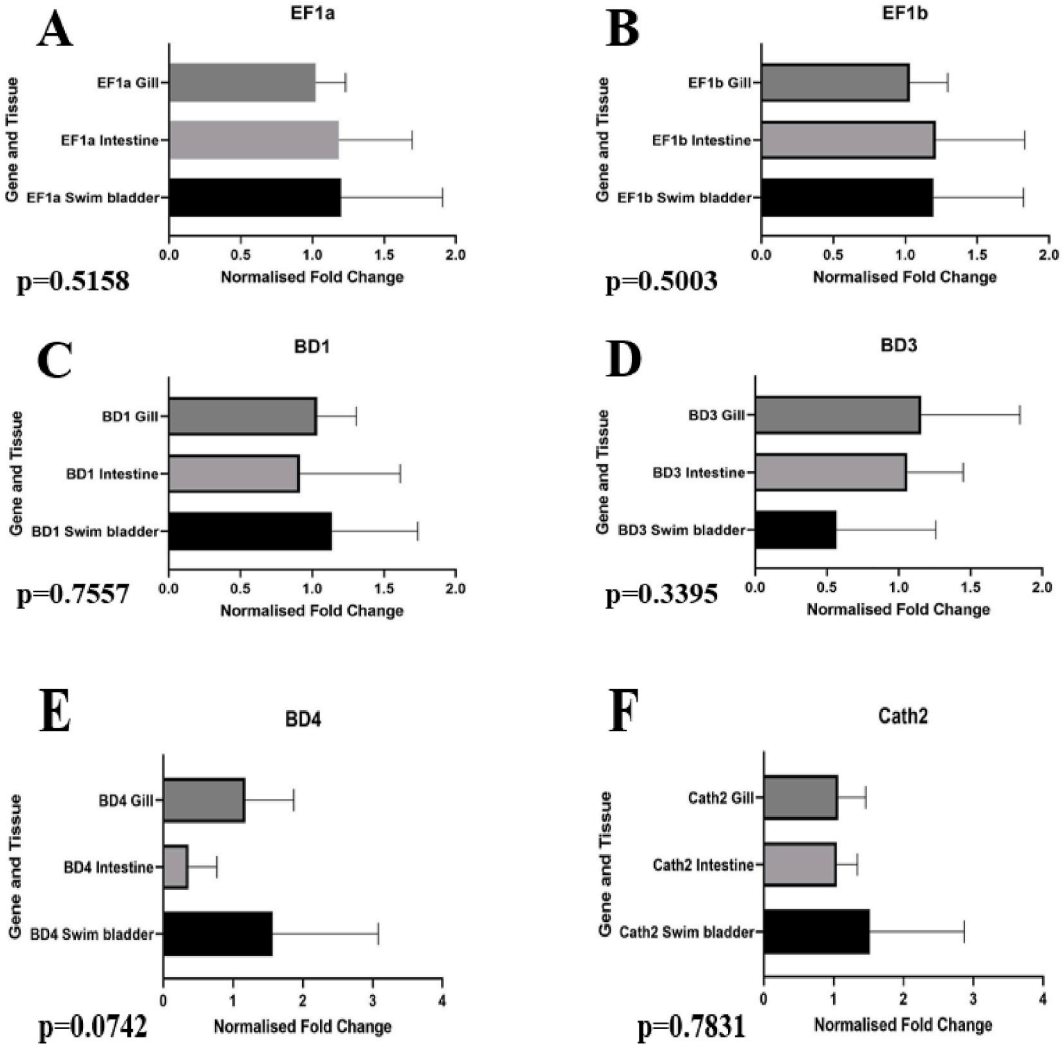

Fig. 3. A–F: **Housekeeping and AMP gene expression in healthy swim bladder, intestine and gill sampled from the Control T_0 cohort. Normalized fold changes for EF1a (A), EF1b (B), asBD1 (C), BD3 (D), BD4 (E), Cath2 (F) are shown**. The p-values (all >0.05) obtained from the non-parametric one-way ANOVA statistical analysis imply there is no statistically significant difference in the expression of housekeeping and AMP genes between the organs sampled. Bars represent mean values ± standard deviation.

Entamoeba histolytica through TLR interactions (Ayala-Sumuano et al., 2013). The strong immunomodulatory capabilities of β-defensins −3 and −4 could imply similar roles in AGD, which could enhance the host's response to the invading pathogen.

In addition to β-defensin AMP gene expression, the current study also demonstrated a statistically significant increase in asCath2 gene expression in gill from AGD-affected salmon. Interestingly, upregulation of cathelicidin antimicrobial peptide gene expression in the spleen of AGD-affected Atlantic salmon has been reported, thus implicating an important role for the gene in host-pathogen interactions (Botwright et al., 2021). The role of asCath2 in AGD has not yet been fully elucidated; although, its upregulation in infected gill signifies a probable significant role in innate immunity and infection. Cathelicidin AMPs, like β-defensins, are known to exhibit species-specific antibacterial (Lu et al., 2011) and antifungal properties (Li et al., 2013). Anti-parasitic activity of cathelicidin AMPs have been previously reported against *Leishmania* and African trypanosomes (Crauwels et al., 2019) (Haines et al., 2003). In teleost fish, cathelicidin 2 gene upregulation has been previously documented in rainbow trout infected with *Ichthyobodo necator* at 9dpi (Chettri et al., 2012) and also in rainbow trout larvae exposed to the parasite *Ichthyophthirius multifiliis* (Heinecke and Buchmann 2013). Atlantic salmon cathelicidin 2 has been linked with inducing the synthesis of interleukin-8 (IL-8) in peripheral blood leucocytes (Bridle et al., 2011). IL-8 is a chemotactic pro-inflammatory chemokine synthesized by neutrophils and T-lymphocytes (Harada et al., 1994). Human cathelicidin LL-37 has been found to induce IL-8 expression in human airway epithelium (Tjabringa et al., 2003), thus elucidating a role of this AMP in augmenting inflammation. The potential role of the cathelicidin 2 in AGD host responses may, in addition to the defensins described above, be immunomodulatory; driving inflammation in the host through direct and indirect immune cell recruitment to the site of infection.

Numerous AGD studies performed to date have highlighted cytokine stimulation as part of the host immune response to *N. perurans*. Earlier AGD studies in salmonids indicated a classical innate pro-inflammatory response in the lesion and non-lesion areas of the gill, with increases in cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) observed within the first 14 days of early infection, particularly in lesion-specific areas (Bridle et al., 2006; Pennacchi et al., 2014). A cytokine of adaptive immunity, interleukin 4/13a, has also been implicated in the host response to *N. perurans* (Benedicenti et al., 2015; Marcos-López et al., 2018). An overlap or synergistic effect commonly occurs between the activity of cytokines and AMPs in immunity (Auvynet and Rosenstein 2009). As described above, AMPs are potent

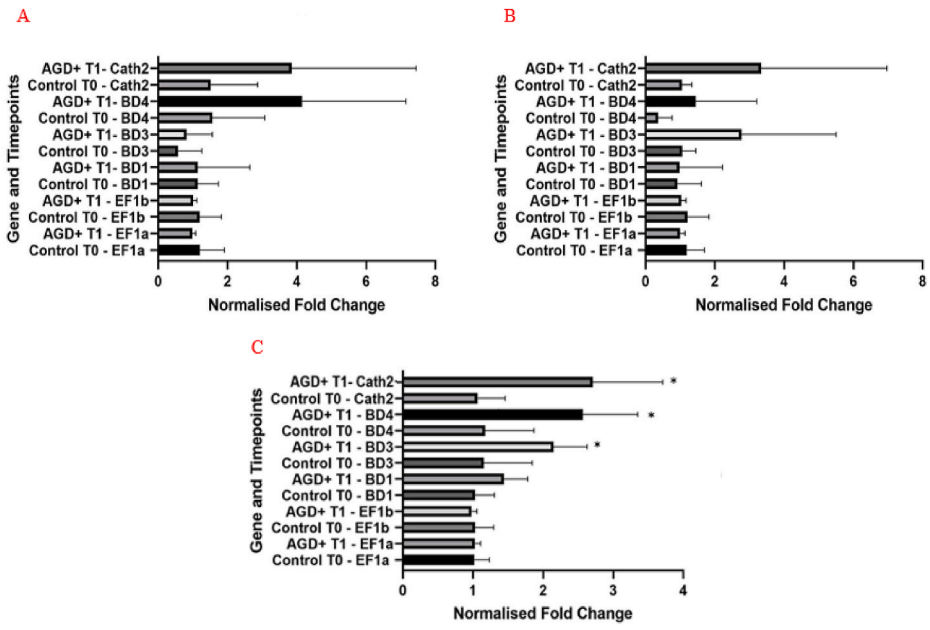

Fig. 4. A–C: **Comparison of AMP gene expression between Control T_0 cohort (Control T0) and *N. perurans* Infected T_1 cohort (AGD + T1) in swim bladder (3A), intestine (3B) and gill (3C). Expression of AMP genes was normalized to housekeeping gene expression in the selected organs.**
Statistical analysis of AMP gene expression was performed using a non-parametric Mann-Whitney test. Statistically significant p-values are denoted* *: (*p-value* $= < 0.05$*). Bars represent mean values ± standard deviation.

chemoattractants and robust inducers of cytokine synthesis in immune cells. Both AMPs and cytokines have the capacity to drive and quell inflammatory processes in innate immunity, to influence T-cell differentiation in adaptive immunity and to modulate the host response to infectious agent (Bals 2000; Zhang and An 2007). The upregulation of AMPs in the present study and the demonstration of cytokine production in previous studies could suggest a synergistic AMP-cytokine response in AGD-affected Atlantic salmon. Further assessment of a combined cytokine-AMP response in AGD could substantiate the proposed immunomodulatory role of asBD3, 4 and asCath2 in gill infected with *N. perurans*.

The AMP gene expression profiles obtained for healthy Atlantic salmon swim bladder, gill and intestine are as noteworthy as their *N. perurans* challenged counterparts. To our knowledge, this is the first instance that β-defensin and cathelicidin 2 AMP gene expression has been reported in healthy swim bladder. The swim bladder functions as a hydrostatic organ involved in buoyancy and gaseous exchange (Stewart and Hughes 2014), and the expression of AMP genes in this organ could be associated with maintenance of immunity within the swim bladder or surrounding organs. A previous study has identified immune genes in swim bladder from *Takifugu rubripes* via KEGG pathway analysis (Cui et al., 2014), thus supporting the concept of an immune role for this organ, but this has not been previously described in the context of innate immunity in teleost fish. Constitutive expression of AMPs in healthy gill and intestine was also observed. The presence of AMPs in these structures may provide low level protection from opportunistic pathogens (Semple and Dorin 2012; Prasad et al., 2019), particularly where microbial colonization is present. The intestine, being a primary innate immune organ in teleost fish (Magnadottir 2006), has been confirmed as a previous source of β-defensin and cathelicidin AMP gene expression, which was further confirmed here.

5. Conclusion

AMPs are versatile indispensable effectors of innate immunity, whose role in AGD has yet to be fully clarified. This study highlights that Atlantic salmon AMP genes play a novel role in early asymptomatic innate immune responses to the parasite *N. perurans*. In this study, high-throughput microfluidics qPCR technology, using minimal cDNA, confirmed the expression of AMP genes cathelicidin 2 and β-defensins −1, −3 and −4 in swim bladder, intestine and gill from both healthy and AGD-infected Atlantic salmon. Furthermore, statistically significant upregulation of cathelicidin 2 and β-defensins −3 and −4 in AGD-infected gill indicate a pro-inflammatory immune response localized specifically at the site of early *N. perurans* infection. Further work is warranted to investigate if the upregulation of these specific AMP genes in response to AGD is related to anti-parasitic activity or immunomodulation. Given the evidence to date, there is potential for these AMPs to have dual functionalities in AGD, supporting the importance of these innate immune defense molecules in response to pathogenic microorganisms.

Authors' contributions

Leisha McGrath: Investigation, Methodology, Formal Analysis, Writing – Original Draft **Joan O'Keeffe:** Conceptualization, Methodology, Resources, Supervision, Writing – Review and Editing, Funding Acquisition **Orla Slattery:** Conceptualization, Methodology, Resources, Supervision, Writing – Review and Editing, Visualisation, Funding Acquisition.

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Declarations of interest

The authors declare no conflicts of interest.

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