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Nasal responses to elevated temperature and *Francisella noatunensis* infection in Atlantic cod (*Gadus morhua*)

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ABSTRACT

We report the histological and transcriptomic changes in the olfactory organ of Atlantic cod exposed to *Francisella noatunensis*. Experimental infection was performed at either 12 °C or 17 °C. Infected fish presented the classic gross pathologies of francisellosis. Nasal morpho-phenotypic parameters were not significantly affected by elevated temperature and infection, except for the number of mucus cells in the 12 °C group seven weeks after the challenge. A higher number of genes were altered through time in the group reared at 17 °C. At termination, the nasal transcriptome of infected fish in both groups was similar to the control. When both infected groups were compared, 754 DEGs were identified, many of which were involved in signalling, defence, transmembrane and enzymatic processes. In conclusion, the study reveals that elevated temperature could trigger responses in the olfactory organ of Atlantic cod and shape the nasal response to *F. noatunensis* infection.

1. Introduction

The nasal immunity of teleost fish offers compelling insights into the evolution of vertebrate immune system [13,49,59]. The discovery of nasopharynx-associated lymphoid tissue (NALT) in the olfactory organ of teleost fish challenged the standing paradigm that this arm of mucosal immunity is only present in terrestrial vertebrates and provided another perspective into the evolution of mucosal immunity by moving the emergence of vertebrate NALT to at least 100 million years earlier [59]. In terrestrial vertebrates, nasal immunity protects the host from airborne antigens and hazardous chemicals. Similarly, the teleost olfactory organ is an important first line of defence, as it is directly and constantly exposed to biological and chemical threats in the aquatic environment [8,13,31]. It is believed that NALT first appeared as a diffuse lymphoid tissue protecting the olfactory epithelium of aquatic vertebrates against waterborne pathogens and was later developed into an organised structure against airborne stimuli in terrestrial vertebrates [51].

Despite the water-exposed surface area of the olfactory organ being relatively smaller than the exposed surfaces of skin and gills, several pathogens can pass through the nasal mucosa, making the olfactory organ a key portal for several bacteria, parasites and viruses [13,29]. However, we have limited knowledge about the number of pathogens infecting the olfactory organ of fish, and the mechanisms and responses these pathogens trigger are largely unknown. This significant knowledge gap on the nasal mucosa as an entry site is partly attributed to the fact that the olfactory organs are not often considered a target when performing pathological evaluation in fish following infection. Salmonid pathogens, such as *Renibacterium salmoninarum,* infectious hematopoietic necrosis virus (IHNV) and viral haemorrhagic septicemia virus (VHSV), are some examples that have been shown to cause lesions in the nares [13,26,41,59].

Atlantic cod (*Gadus morhua,* hereafter cod) has one of the most intriguing immune systems in the teleost world. The production of antibodies to various antigens following vaccination is a hallmark of the vertebrate immune system. During the evolution of the gadoid lineage, the major histocompatibility complex (MHC) II system, an essential immunological component for antigen presentation and adaptive humoral response, was lost [55]. A fundamental trade-off may be at play in cod as several immune gene families, such as the MHC I and Toll-like receptors (TLRs), have been found to exhibit dramatic expansion and diversification [53,55,58]. The significant loss of the MHC II system may also mean that cod must rely on its immunological artillery at mucosal

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sites. The mucosal immune system of Atlantic cod has been studied to a great extent, enabling the identification of mucosal molecules and mechanisms that have been crucial in protecting cod from various stimuli for millions of years despite the absence of an important component in its immune system [9,21,28,44,48]. However, the nasal immunity of Atlantic cod and how pathogenic and environmental stimuli regulate it is yet to be described.

Francisellosis, an infectious disease caused by the Gram-negative aerobic, facultatively intracellular bacterium *Francisella noatunensis* ssp. *noatunensis* (hereafter *F. noatunensis*) affects several fish species, including cod. Francisellosis remains one of the bacterial diseases which is very difficult to combat because of the intracellular lifestyle of the pathogen [3,17]. Infection often gradually develops where pathological changes characterised by granulomas in different parts of the fish, including skin, mouth cavity, gills, spleen, head, kidney and heart, are exhibited, though sometimes the disease can present no external clinical signs [14]. It is yet to be described whether the olfactory organ is affected by *F. noatunensis*. However, it is known that the olfactory organ is an infection route for neurotropic and respiratory pathogens. In fish, the pathogen can hijack the olfactory organ to gain a direct portal of entry into the central nervous system via the olfactory nerve [13]. Given the neurotrophic nature of the *F. noatunensis* [42], we believe it can target the olfactory organ or, at least, trigger nasal responses, in cod.

This paper presents the first report on the nasal responses of Atlantic cod to *F. noatunensis.* Mortality associated with francisellosis is primarily related to high seawater temperatures (>15 °C) [36,38,39], and thermal stress and compromised immunity are suggested to be important predisposing factors [37,40]. It is known that temperature beyond or below the permissive threshold has a strong physiological impact on the fish because, as ectotherms, they cannot control their body temperatures [2]. The influence of temperature on immunity has been given attention in recent years in light of the thermal stress associated with climate change [50]. Therefore, the present study explored the interaction between temperature and francisellosis by focusing on the local immune responses in the olfactory organ of Atlantic cod. We hypothesised that francisellosis could trigger local immune responses in the olfactory organ that could be reshaped by elevated temperature.

2. Materials and methods

2.1. Ethical considerations

The study complied with the European Union Directive 2010/63/EU guidelines and the National Guidelines for Animal Care and Welfare established by the Norwegian Ministry of Education and Research. The Norwegian Food Safety Authority approved the experiment under FOTS ID 23048. The key personnel involved in the fish trial hold a FELASA C certificate.

2.2. Franciscella noatunensis culture conditions

Franciscella noatunensis (type strain NCIMB 14265) was re-isolated from experimentally infected Atlantic cod and kept frozen in glycerol at −80 °C until use. The culture was revived and grown on Cystine heart agar with blood (CHAB, Difco™, USA) plates for four days at 20 °C. Multiple single colonies were resuspended in saline, and optical density was measured at OD_{600nm}. The colony forming unit (CFU) was determined by plating on CHAB agar plates in duplicate of the serially tenfold diluted bacterial suspension.

2.3. Temperature manipulation and experimental infection

Atlantic cod (*Gadus morhua*) were provided by the National Cod Breeding Program at Nofima, and they were quarantined for four weeks following arrival at the Tromsø Aquaculture Research Station. The fish were allowed to acclimate under the following production conditions: temperature = 12 °C, dissolved oxygen ≥85% saturation, salinity = 33 ppt, photoperiod = continuous illumination, and feeding = continuous feeding (Amber Neptun, Skretting, Norway). These parameters were maintained throughout the whole trial except for temperature. The experimental fish (*n* = 120; ~ 100 g) were evenly distributed into four 200 L tanks connected to a flow-through system (Fig. 1). The fish were allowed to acclimate for two weeks.

The trial had two main phases: 1) temperature increase and 2) infection and disease development at two different temperatures. Following acclimation, the water temperature in the two tanks was increased to 17 °C at an increment of 1 °C per day (Group B). This temperature is the upper thermal limit for cod [47]. The other tanks remained at 12 °C (Group A). Four days after reaching the target temperature in Group B, fish were sedated with Benzoak Vet (ACD Pharmaceuticals AS, Norway; 15 mL/100 L of water) and were tagged by the dorsal fin using visible implant elastomer (VIE, Northwest Marine Technology, USA) to identify their tank history. Six fish were separated from each tank to serve as vectors for the cohabitation trial [14]. Half of these fish (3 per tank) were injected intraperitoneally with 100 μl fresh culture of *F. noatuenensis* at a challenge dose of 10^8 CFU/fish. The other half was injected with 100 μl sterile phosphate-buffered saline. Thereafter, the fish in the two tanks from each temperature group were mixed, divided into 2 and redistributed equally into two 200 L tanks with 27 fish per tank. The sham-injected fish went to one tank, while the *F. noatunensis*-injected went to another tank of each temperature group. Fish were reared for another seven weeks before the trial was terminated. During the 7-week disease development period, the fish were at either 12 °C or 17 °C, and all other parameters were similar in both groups. The whole trial, including both phases, was carried out over nine weeks.

2.4. Sample collection

There were four samplings in the study, as illustrated in Fig. 1. Samples were collected at i) T0 = the day before the temperature was increased/start of the trial, ii) T1 = 4 days after the water temperature reached 17 °C (mean weight = 123.4 ± 39.5 g; mean length = 23.3 ± 2.1 cm), iii) T2 = 3 days after infection (123.3 ± 37.4 g; 23.5 ± 1.9 cm) and iv) T3 = 7 weeks after infection (12 °C group: 232.2 ± 28.0 g; 84.2 ± 3.1 cm; 17 °C group: 174.3 ± 25.5 g; 49.the 8 ± 2.3 cm), which also marked 9th week of the whole trial. Fish (*n* = 6) were dip-netted from each tank and euthanised with an overdose of Benzoak Vet. The right olfactory organ (together with the nostril) was immediately placed in BiopSafe containing 10% formalin (Mermaid Medical, Vedbæk, Denmark) for histological processing. The left olfactory organ was exposed by opening the olfactory cavity via the nostril, and the specimen was suspended in RNAlater® (Ambion, Inc., Connecticut, USA), allowed tissue penetration at 4 °C overnight and stored at -40 °C prior to RNA isolation. Samples collected at T0, T1 and T3 were subjected to RNA Sequencing and histology. Samples collected at T2 were only subjected to histology and qPCR detection of the pathogen. T3 samples were also used for pathogen detection. Additionally, the pathogen was re-isolated from the head kidney of the infected fish by streaking onto the CHAB agar plate.

2.5. Histological processing and morpho-phenotypic evaluation

The formalin-fixed olfactory organs from T0, T1 and T3 were sent to the Norwegian Veterinary Institute for histological processing. Samples were embedded in paraffin, sectioned to 5 μm and stained with Alcian Blue-Periodic Acid Schiff (AB/PAS). The tissue sections were subjected to two kinds of histological evaluation. First, the overall health quality of the tissue was assessed based on a 0-to-3 assessment scale given in Supplementary Material S1. The assessment comprises the structural integrity of the tissue as well as the prevalence of common lesions, i.e., hyperplasia, vacuolisation, necrosis. 0 means the organ was in good

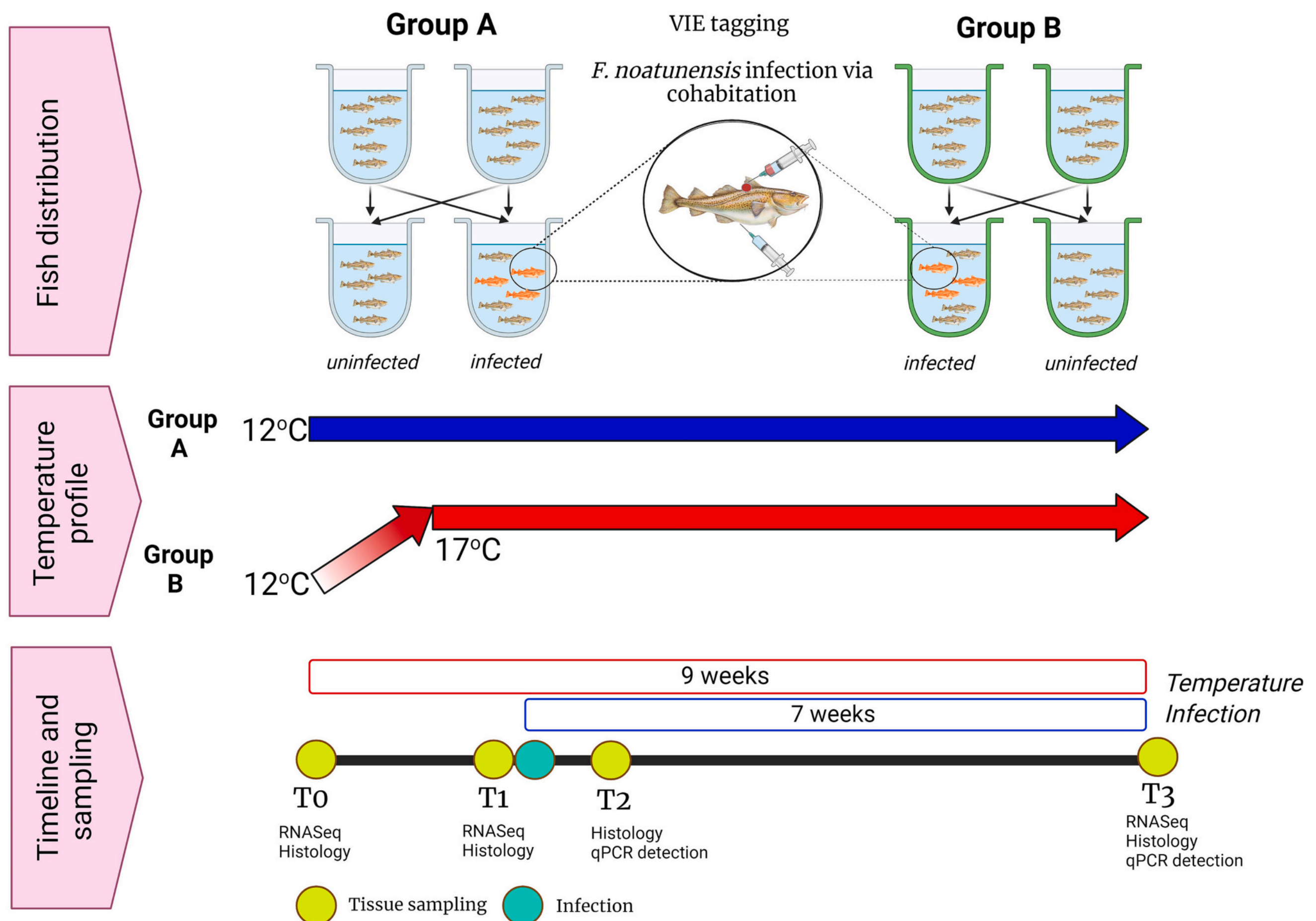

Fig. 1. Overview of the experimental set-up. Sampling time points: T0 = start of the trial, before the temperature was gradually increased to 17 °C in one of the groups; T1 = 4 days after the temperature reached 17 °C in Group B; T2 = 3 days after infection; T3 = 7 weeks after infection. Created with BioRender.com under publication agreement number DY25U97T7X.

health condition, while 3 indicates severely compromised tissue. To limit bias, the scoring was performed on three different occasions by a single evaluator with no prior knowledge of the sample history. Morphometric measurements were also carried out, including the thickness of the olfactory epithelium and *lamina propria*, the number of mucous cells and the number and size of sacciform cells. The measurements were taken from 5 randomly selected olfactory lamella. The number and size of sacciform cells on the external epithelial lining leading to the base of the nostrils were also determined. Measurement was carried out within a 1000 μm distance. The presence of sensory cells and melanocytes was also recorded.

2.6. RNA isolation and quality assessment

Total RNA was isolated from the olfactory organ using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). The purified RNA was quantified using NanoDrop 8000 Spectrophotometer (ThermoFisher Scientific, United States), and integrity was further assessed using the Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples had an RNA Integrity Number (RIN) above 7.0.

2.7. Detection of F. noatunensis by qPCR

The presence of *F. noatunensis* in the olfactory organ samples collected at T2 and T3 was detected by qPCR. Briefly, cDNA (Applied Biosystems, USA) was synthesised from 200 ng of template RNA (Section 2.6) using Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit (Massachusetts, USA). The qPCR reaction consisted of 7 μL diluted cDNA, 1.2 μL 5 μM of each primer (FcF50: AACGACTGTTAA-TACCGCATAATATCT, FcR50:CCTTACCCTACCAACTAGCTAATCCA) [40], 0.6 μl H_2O, and 10 μL PowerUp™, SYBR™ PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA) was run under the following thermocycling conditions: initial incubation at 95 °C, 20 s followed by 40 cycles: 95 °C, 1 s, 60 °C, 20 s, and a melting curve (95 °C, 1 s, 60 °C, 20 s, and 95 °C, 1 s) using QuantStudio 5 (Thermo Fisher Scientific). CT value ≥35 was considered negative for *F. noatunensis*. Relative bacterial DNA concentration was determined by preparing a standard curve of known DNA concentration of the pathogen.

2.8. Library preparation and RNA sequencing

Total RNA from 36 samples (T0, *N* = 4; T1, *N* = 5 per temperature group; T3, N = 5 in 12 °C, uninfected, *N* = 6 in 12 °C, infected, N = 6 in 17 °C, uninfected, N = 5 in 17 °C, infected) were sent to the Norwegian Sequencing Centre (OUS, Norway). The strand-specific TruSeq RNA Library Prep Kit (Illumina, CA, USA) was used to prepare RNA-seq libraries using the manufacturer's protocol. The libraries were pooled and sequenced on one lane of NovaSeq (Illumina, CA, USA) S4 flowcell, 150 bp paired-end reads. The raw sequencing data are available in ArrayExpress under accession number E-MTAB-12870.

2.9. Statistical analysis of nasal morphometries

GraphPad Prism for Windows® (version 8.0) was used to determine statistical differences among the treatment groups and time points for the different nasal morphometric parameters. Prior to performing an analysis of variance (ANOVA), the Spearman's test for heteroscedasticity and tests for normality of residuals were performed. To identify the effects of a gradual increase of temperature to 17 °C a two-way ANOVA followed by Tukey's Multiple Comparison Test was applied to determine statistical significance between the temperature groups and time points (T0 vs T1). A three-way ANOVA followed by Tukey's test was used to identify statistical significance between temperature groups (12 °C and 17 °C) and infection status (control versus infected) at two time points (T2 vs T3). A Chi-squared test was employed to identify significant differences in the damage score. The level of significance was set at $P < 0.05$.

2.10. Bioinformatic processing of RNA-seq data

Raw sequence data were pre-processed to remove/trim adapter sequences and low quality reads using BBDuk (part of BBMap v.38.18; parameters: ktrim = r k = 23 mink = 11 hdist = 1 tbo tpe qtrim = r trimq = 15 maq = 15 minlen = 36 forcetrimright = 149) [7]. Cleaned data were aligned against the *Gadus morhua* genome (ENSEMBL gadMor3.0) using HISAT2 v.2.2.1 (parameters: –rna-strandness RF) [24]. FeatureCounts v.1.4.6-p1 (parameters: -p -s 2) [33] was used for estimating the number of reads and aligning against the reference genes in gadMor3.0 ENSEMBL r106 GTF annotation.

Initial data analysis of raw counts was performed using SARTools (v.1.7.4) [61] and R (v.4.1.1) [43]. Normalisation of raw count and differential analysis were done according to the DESeq2 (v.1.34.0) R library [34]. The independent filtering method of DESeq2 was applied to remove features with very high dispersion or very low count. Adjusted *p*-values were calculated according to the Benjamini-Holchber (BH) method. A description of the analysis, including R session information and parameters can be found in Supplementary Material S2 'Statistical report: Pairwise comparison of conditions with DESeq2'.

Lists of significantly differentially expressed genes (DEGs, padj<0.05) between pairwise comparisons of samples were plotted in UpSet-plots (UpSetR v.1.4.0) [11]. Following this heatmaps were generated using the vst-normalised DESeq-output and the R-package pheatmap (v.1.0.12) [25]. Using the mean expressions for each condition, a distance matrix between the different conditions was used to created row- and column- clustered heatmap (default settings). Further, the DEGs between infected cod at 12 °C and 17 °C were used for subsequent heatmaps. Two row-scaled heatmaps were generated, one containing all 754 DEGs, and one limited to the top 20 variable genes (expression at the final sampling point). Both heatmaps were clustered by row (default clustering, applying Euclidian distance and complete linkage method) and show all groups/conditions throughout the timeline of the experiment.

Analysis of gene ontology was performed in R (v.4.2.1) with the package topGO (v.2.48.0) [1]. Information on gene ontology annotation for *Gadus morhua* (gadMor3.0) was downloaded from ENSEMBL Biomart (24.02.2023) and used to create the gene universe for topGO. Input lists consisted of genes showing a log2 fold change > |2| in the DESeq pairwise contrasts between infected and control fish at the two different experimental temperatures (T3). Fisher statistics and the 'elim'-algorithm were applied [1], with a significance threshold of padj<0.05 for enrichment. Visualisation of resulting GO enrichment was prepared using Goplot (v.1.0.2) [63] and ggplot2 (v.3.4.0) [64].

3. Results

3.1. Gross pathologies and qPCR detection of the pathogen in the olfactory organ

Typical pathologies associated with *F. noatuanensis* have been observed in infected fish (Fig. 2B-F) at termination, and the tendencies suggest that occurrence was higher at 17 °C. White, partly protruding nodulations of various sizes were observed in the skin, liver and spleen of infected fish (Fig. 2C-E). In addition, signs of splenomegaly and hepatomegaly were exhibited by infected fish. In some infected fish at T3, extensive distribution of nodulations was observed in the internal cavity (Fig. 2F).

The pathogen was sporadically detected in the olfactory organ by qPCR, with a CT value of around 33. Three days after experimental infection (T2), the pathogen was detected in 2/3 (sample 1 = 1.11 femtogram bacterial DNA; sample 2 = 2.47 fg bacterial DNA) olfactory organ samples from 12 °C group, while samples from fish reared at 17 °C were all negative. After 7 weeks of infection (T3), the olfactory organ of 1/3 fish (0.02 fg bacterial DNA) from the 17 °C group was positive for the pathogen. On the other hand, none of the analysed olfactory organ samples from the 12 °C group was positive. The pathogen was re-isolated from the head kidney of diseased fish used in the analysis.

3.2. Changes in the nasal histological morphometries as an acute response to elevated temperature

Nasal parameters, including the number (Fig. 3A) and size of sacciform cells (Fig. 3B), the number of mucus cells (Fig. 3C), the thickness of the epithelium of the olfactory lamella (Fig. 3D) and *lamina propria* (Fig. 3E) and tissue damage scores (Fig. 3F) were similar between T0 and T1 in both temperature groups.

Ciliated epithelial cells and sparse distribution of mucus cells on the tip (Fig. 3H) and at the base (Fig. 3H) are typical characteristics of an olfactory lamella. Occasional occurrence of an aggregate of lymphocyte-like cells was observed (Fig. 3H), as well as packets of red blood cells (Fig. 3I). Although the frequency was not striking, some fish from both temperature groups exhibited lamellar fusion with *lamina propria* degeneration. Sacciform cells were present on the outer epithelial layer leading to the nostrils (Fig. 3J).

3.3. Changes in the nasal histological morphometries in response to *F. noatunensis*

A similar suite of nasal morphometries was determined in fish infected with *F. noatunensis* at either at 12 °C or 17 °C (T2 vs T3, Fig. 4). Regardless of the temperature, most parameters did not significantly change between the control and infected fish at both time points. Only the number of mucus cells (Fig. 4C) exhibited significant inter-treatment differences at T3. In the 12 °C group, the number of mucus cells in infected fish was significantly higher than the control. In addition, the number of mucus cells in infected fish reared at 12 °C was significantly higher than the control fish at 17 °C, while the number of mucus cells of infected fish reared at 17 °C was significantly lower than the infected fish reared at 12 °C (Fig. 4C).

Sensory cells on the epithelial surface close to the nostrils frequently appeared in fish at 17 °C, though infection status presented no apparent effects (Fig. 5A). Melanocytes in the nostrils seemed to increase in infected fish at 17 °C, especially at T3 (Fig. 5B). Signs of focal inflammation in the *propria submucosa* were observed in infected fish; however, the frequency was not clear to establish a temperature-dependent response (Fig. 5C). The occurrence of packets of red blood cells increased in infected fish and were found on the nasal cavity wall and in *lamina propria* (Fig. 5 DE). Lamellar fusion and vacuolisation appeared to be common in infected fish at 17 °C (Fig. 5F).

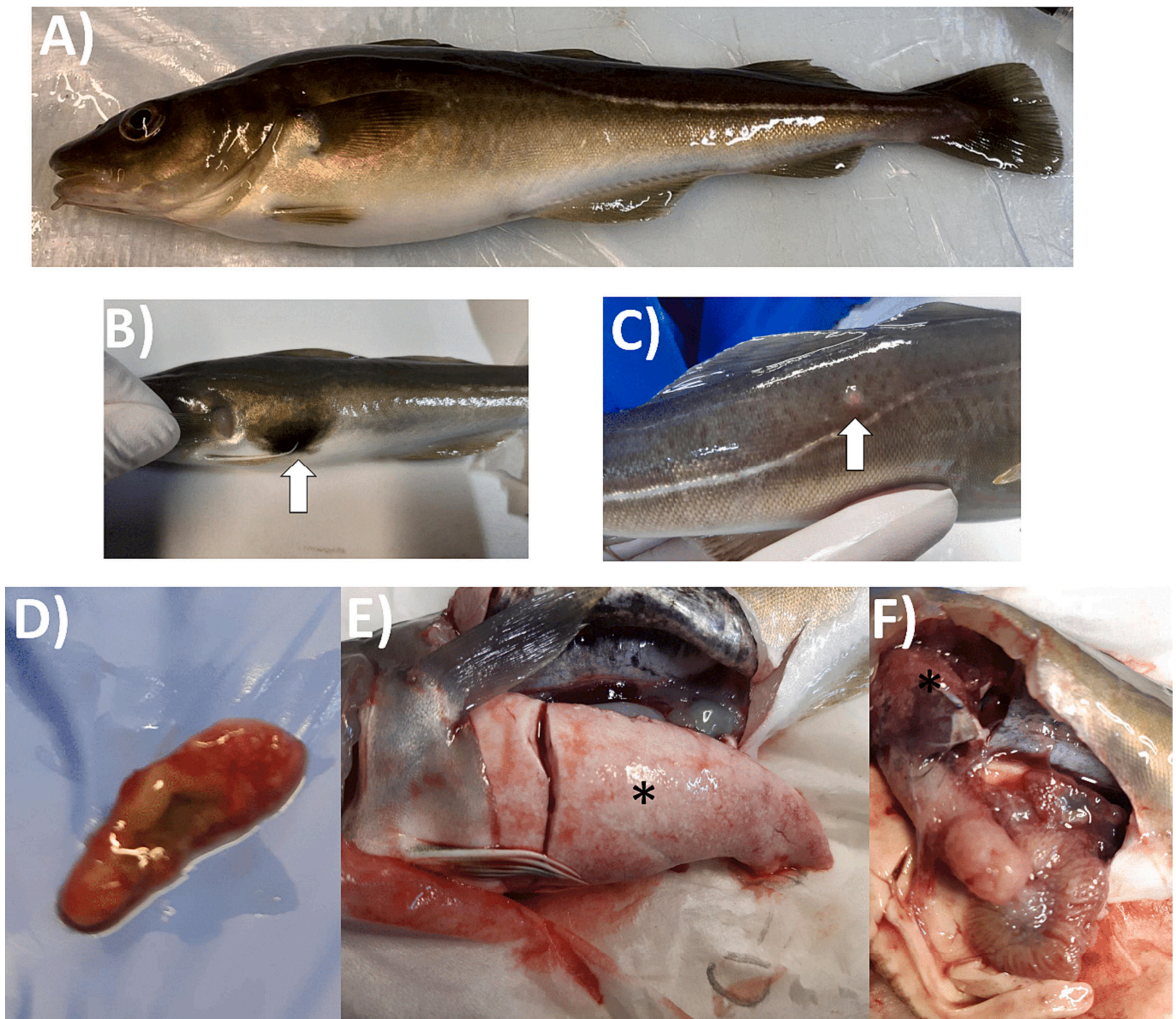

Fig. 2. Gross pathologies (A-F) of *Francisella*-infected Atlantic cod. (A) Uninfected fish reared at 12 °C. Infected fish showing darkening of the skin (B). White nodulations were observed in the (C) skin, (D) spleen and (E) liver. Hepatomegaly was exhibited by infected fish. (F) Extensive white nodulations were also observed in the internal cavity.

3.4. Differentially expressed genes in the olfactory organ

A total of 29,063 features were mapped to the Atlantic cod genome (gadMor3.0 ENSEMBL r106). Principal component analysis (PCA, Fig. 6A) shows no apparent clustering of groups, except for the 17 °C group sampled at T1.

To identify shared and unique DEGs between different comparisons (padj<0.05, a complete list of DEGs for each comparison can be found in Supplementary Material S3), three different UpSet-plots [11,32] were generated showing the association of the different experimental stages and accounting the effects of various factors (e.g., timepoint, temperature, infection status (Fig. 6B-D).

First, we identified the acute nasal response to the increase in temperature (T0 vs T1, Fig. 6B). The temperature increase to 17 °C resulted in 193 DEGs (T1_17 vs T0). During the same period, 12 DEGs were identified in the group that remained at 12 °C (T1_12 vs T0). Comparing the two temperature groups after the temperature increase revealed 31 DEGs (T1_17 vs T1_12). There were 7 shared genes between DEGs in T1_17 vs T0 and T1_12 vs T0, while 17 shared genes when T1_17 vs T1_12 and T1_17 vs T0 were compared (Fig. 6B).

Then, we compared the temporal nasal response (T1 vs T3) of the control and infected groups reared at either 12 °C or 17 °C (Fig. 6C). There were 241 DEGs found in the infected group reared at 17 °C, most of which were unique genes in that particular comparison (T3_17i vs T1_17). For its counterpart in the control group, 128 DEGs were identified (T3_17c vs T1_17) at T3. In the fish reared at 12 °C, infection resulted in 89 DEGS (T3_12i vs T1_12), while 11 DEGs were identified in the control group (T3_12c vs T1_12). There were few shared genes between these comparisons except in the 17 °C groups (Fig. 6C). There were 35 shared DEGs between the infected and control fish at 17 °C (T3_17i vs T1_17: T3_12i vs T1_12).

Lastly, we compared the responses of the control and infected groups reared at either 12 °C or 17 °C at T3, the termination of the trial (Fig. 6D). Around 754 DEGs were found between the infected groups reared at either 12 °C or 17 °C (T3_17i vs T3_12i). Only 14 DEGs were identified when the control and infected groups at 17 °C were compared

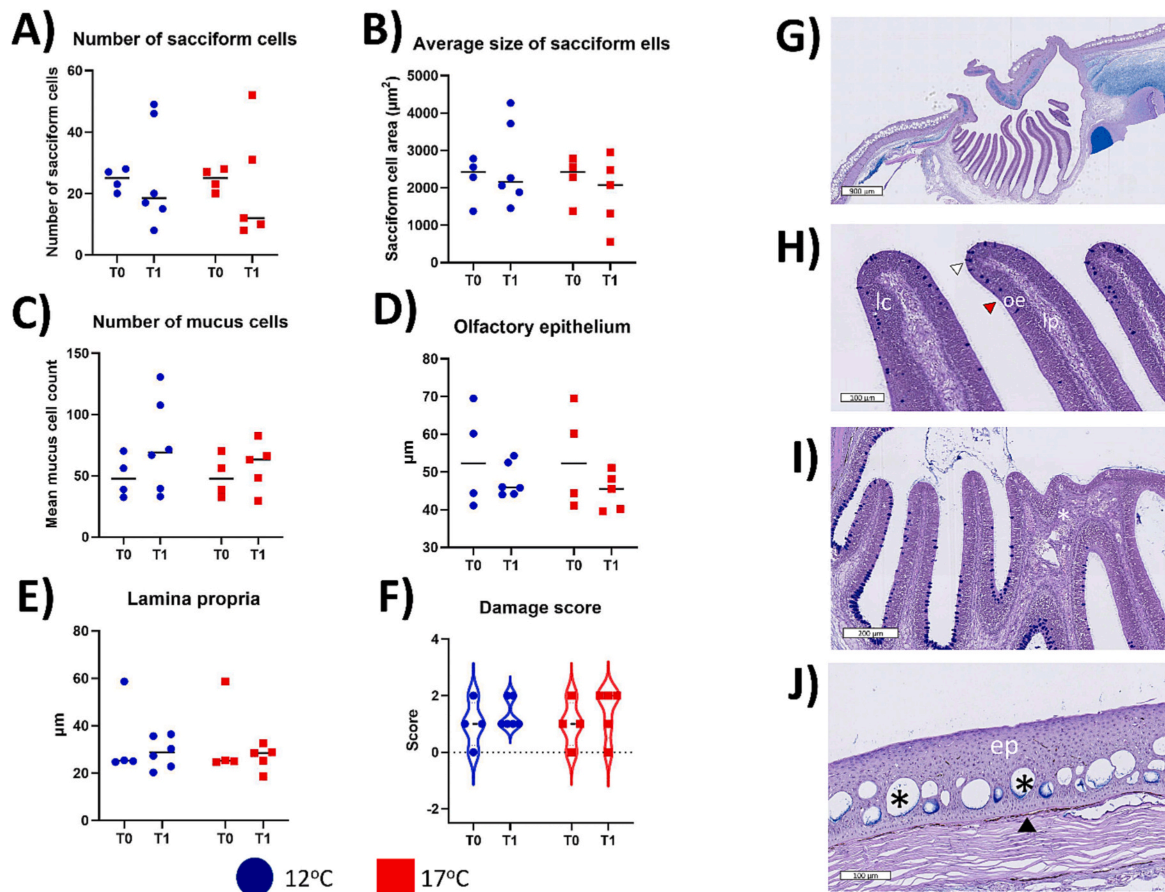

Fig. 3. Nasal parameters four days after the target temperature of 17 °C was reached (T0 versus T1). The A) number and B) size of sacciform cells close to the base of nostrils (distance of 1000 μm) were determined. In the olfactory rosette, the C) number of mucus cells in olfactory lamella and thickness of D) olfactory epithelium and E) lamina propria were measured. F) Structural damage in the olfactory lamella was scored using 0-to-3 damage score. The black horizontal line represents the mean of 4–6 individuals per group. G) Overview of the nasal section where morphometries were taken. H) Olfactory lamella, highlighting the mucus cells (white arrowhead) and ciliated epithelial cells (red arrowhead). lp = lamina propria, oe = olfactory epithelium, and lc = aggregate of lymphocyte-like cells. I) Evidence of lamellar fusion, lamina propria degeneration and packet of red blood cells (*), a representative fish from 17 °C group. J) Epithelium of the skin close to the base of the exterior area of the nostril. * = sacciform cells embedded in the epidermis (ep). Note the differences in the scale bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(T3_17c vs T3_17i). Both T3_12c vs T3_12i and T3_17c vs T3_12c yielded very few DEGs. In addition, very few shared DEGS in the different comparisons at this time point.

We selected DEGs showing a high variability among the infected fish at two different temperatures at T3; expression at T0 and T1 was also included for comparison (Fig. 7). These genes include *apoptosis-associated tyrosine kinase a* (*aatka*), *ABL proto-oncogene 2* (*abl2*), *apolipoprotein Eb* (*apoeb*), *chromobox homolog 7* (*cbx7a*), *niban apoptosis regulator 1* (*niban1a*) and *NOP2/Sun domain family, member 2* (*nsun2*). The transcript level of *aatka* and *cbx7a* was higher in the infected group than the control within the 12 °C treatment group, but such a difference was not observed within the 17 °C group; *cbx7a* expression decreased from T1 to T3 in the 17 °C group (Fig. 7A, D). *Abl2* transcript level was higher in infected fish within the 17 °C, but such treatment contrast was not observed within the 12 °C group (Fig. 7B). On the other hand, *niban1a* was lower in the infected fish than the control regardless of the temperature (Fig. 7E), while such difference was only identified in the 12 °C for the expression of *nsun2* (Fig. 7F). The *apoeb* expression was higher in the 17 °C group than in the 12 °C group regardless of the infection status (Fig. 7C).

3.5. Heatmaps for targeted DEG analysis

Expression data (all genes) from the different sampling groups (accounting for the time, temperature, and infection status) were compared for the magnitude of similarities/differences and the sample-to-sample distance heatmap generated is provided in Fig. 8A. The comparisons revealed that the nasal responses of infected fish seven weeks following infection at 12 °C grouped with the fish responding to a temperature increase to 17 °C and the uninfected fish reared at 17 °C for seven weeks (top-left quadrant). In the bottom right quadrant, the 12 °C group at T1 was more linked to the infected group at 17 °C at T3.

We further explored the 754 DEGs identified between the infected groups at either 17 °C or 12 °C at T3. The heatmap (Fig. 8B, the expression at other time points was also in the heatmap) shows an apparent effect of temperature at T3 where the magnitude of either downregulation or upregulation in gene expression was distinct between the 12 °C and 17 °C groups. However, health/infection status appeared to have a minor effect. Looking at how this group of genes behaved before the challenge (T0 and T1) indicated that the impact of temperature seemed to be minimal.

We then focused the analysis on the top 20 infection-responsive genes and identified four main clusters (Fig. 8C). The topmost cluster consists of one gene, showing a strong response to infection at 12 °C. The next cluster primarily contains genes exhibiting an increase in expression in response to infection at 12 °C, while the response at 17 °C appears minimal or in the opposite direction. The third cluster primarily contains temperature-responsive genes showing a high expression at

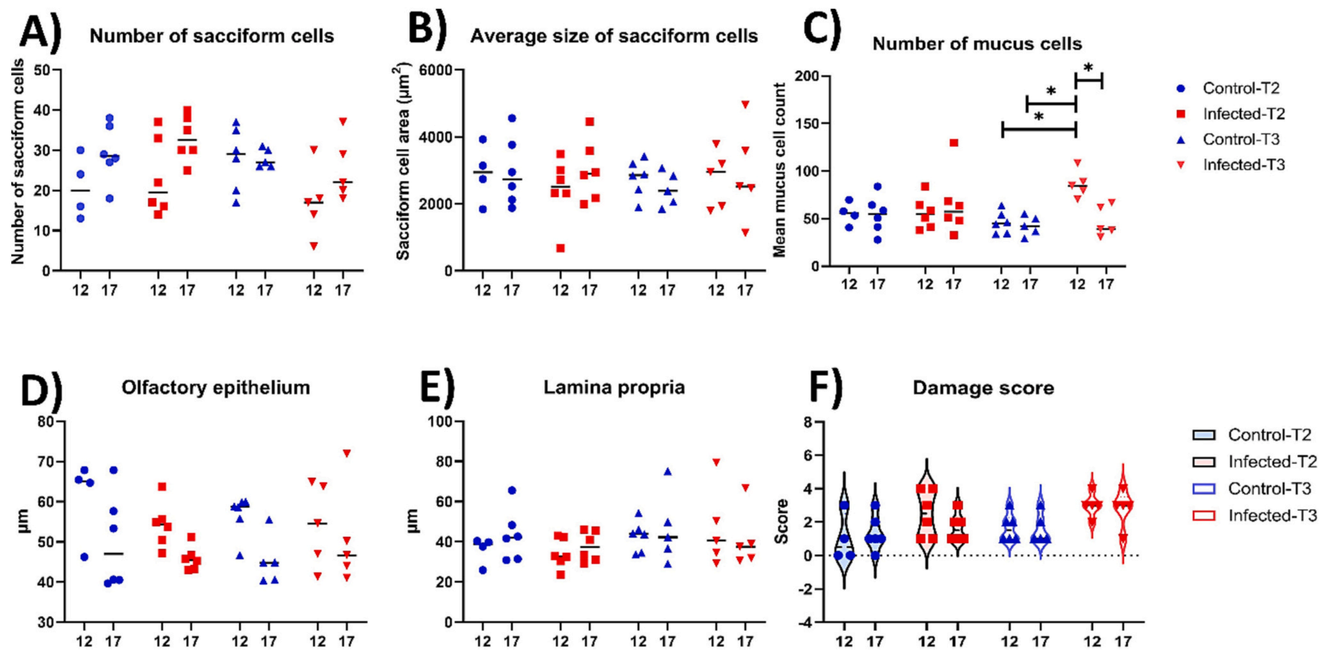

Fig. 4. Nasal parameters of Atlantic cod infected with *F. noatunensis* under two different temperatures (12 °C versus 17 °C), samples collected at T2 and T3. For additional information, please refer to Fig. 3. Asterisk (*) denotes a significant difference between the two groups.

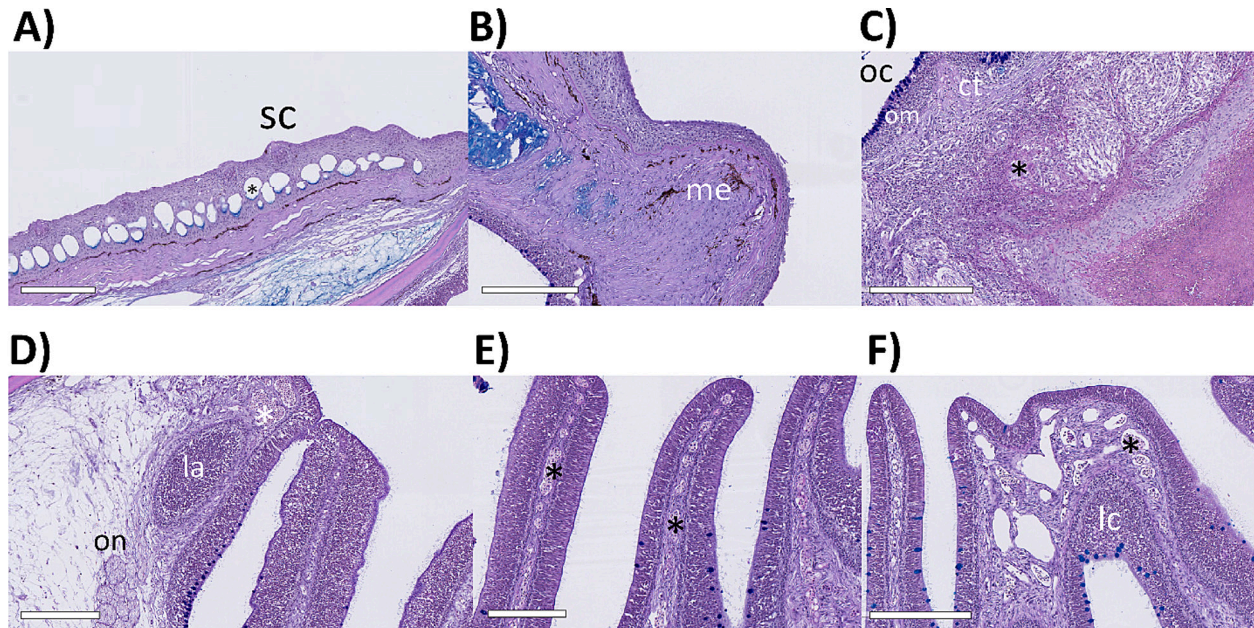

Fig. 5. Histological changes in the nasal mucosa of fish infected with *F. noatunensis*. These representative photographs were from fish infected at 17 °C. A) Sacciform cells (*) are found on the external epithelial layer near the nostrils. Sensory cells (sc) appeared to increase in frequency. B) Melanocytes (me) were found in the basal lamina of the epithelial layer (A), and their occurrence in the nostrils increased at 17 °C (B). C) Though inflammation was uncommon, cases were observed in the propria submucosa. oc = olfactory cavity; om = olfactory mucosa; ct = connective tissue. D-E) Packets of red blood cells (*) could be found in the wall of the olfactory mucosa and the lamina propria. on = olfactory nerve; la = lymphocyte aggregate. F) Lymphocyte-like cells (lc) could be found in areas where olfactory lamella fused. Vacuolisation was observed, and packets of red blood cells could be observed in the periphery. Scale bar = 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

17 °C, regardless of infection status. The last cluster contains infection-responsive genes regardless of the temperature groups, though an opposite trend was observed: at 12 °C, the genes were primarily upregulated, while at 17 °C the genes were predominantly downregulated. When exposed to the pathogen, these genes appeared to be regulated similarly in both temperature groups.

3.6. Gene Ontology

We further explored the functional characteristics of genes found to have a log2FC-change> |2| between treatments by performing Gene Ontology enrichment analysis, allowing the identification of functional changes as a response to temperature, infection, and their interaction (Figs. 9-10, Tables 1-2).

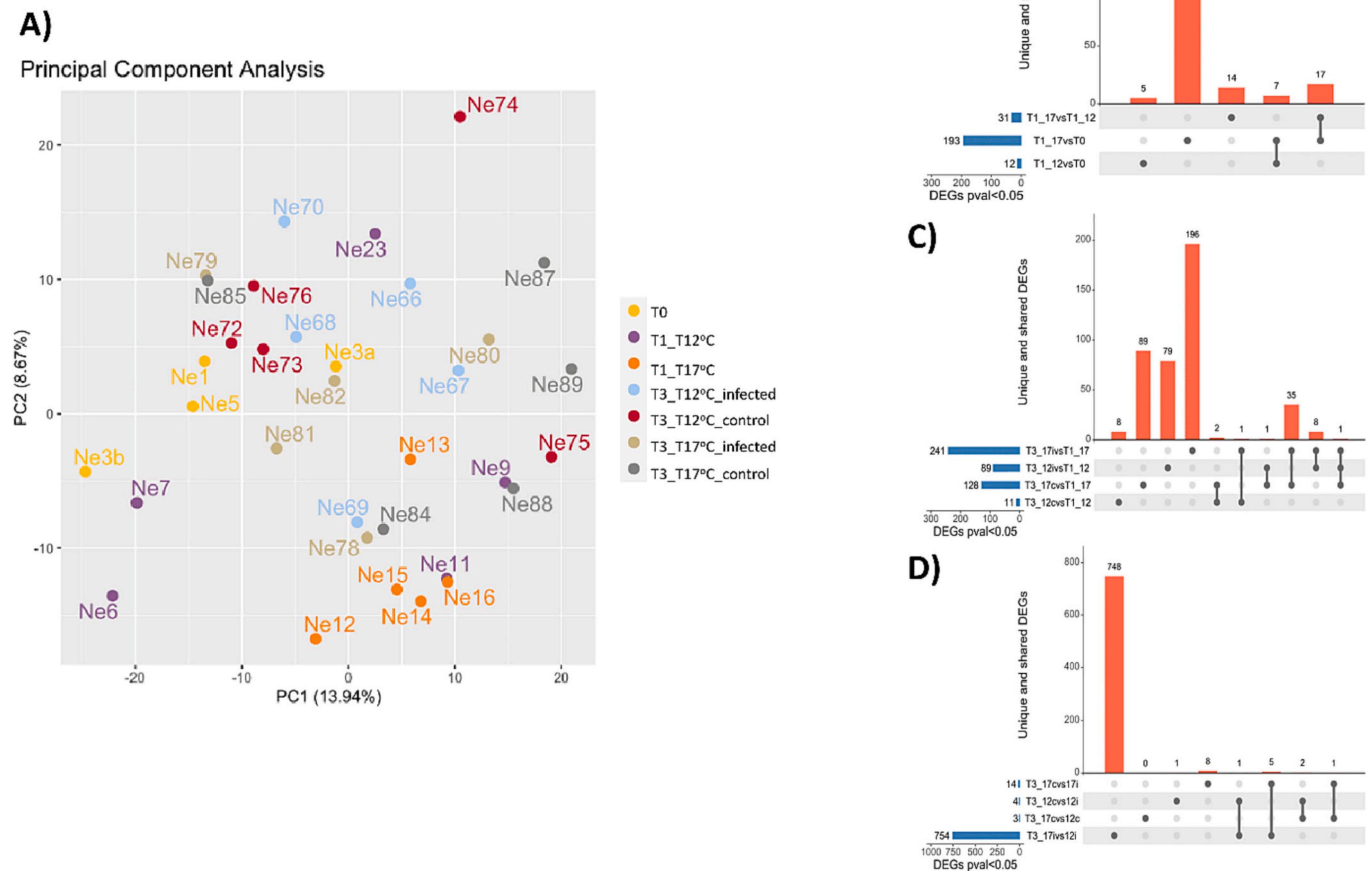

Fig. 6. General overview of the nasal transcriptome of Atlantic cod. A) Principal Component Analysis, with percentages of variance associated with each axis. Olfactory organs collected from 3 time points were subjected to RNA sequencing. T0 = before the temperature was increased; T1 = four days after the temperature reached 17 °C. The other group remained at 12 °C; T3 = 7 weeks after experimental infection at either at 12 °C or 17 °C. control = uninfected control, infected = in cohabitation with *F. noatunensis* shedder fish. T3 is also equivalent to week 9 of the whole experimental trial, including the temperature increase and disease development stages. B—D) Upset plots showing the number of DEGs for biologically relevant comparisons. There were three main comparisons aiming at B) identifying DEGS related to the short-term response to elevation of temperature to 17 °C; C) identifying DEGS related to long-term effects of elevated temperature and infection status at either 12 °C or 17 °C; and lastly, D) identifying DEGs between infected and control groups of the different temperature groups seven weeks after infection. The number of DEGs that are unique to each comparison is shown alongside those that are identified in several comparisons. For the complete list, refer to Supplementary Material S3.

The 1466 genes identified when the temperature reached 17 °C (i.e., T1_17 vs T0) generated 98 enriched GO terms, including GO:0015074 DNA integration, GO:0005576 extracellular region; GO:0016021 integral component of membrane, GO:0016706: 2-oxoglutarate-dependent dioxygenase activity (Fig. 9A). On the other hand, the 1204 genes identified after prolonged rearing at 17 °C (i.e., T3_17c vs T1_17) revealed 78 enriched GO terms (Fig. 9B). Comparing these two periods, GO:0015074 DNA integration, GO: 0005576 extracellular region, GO:0016422 RISC complex and GO: 0016706 2-oxoglutarate-dependent dioxygenase activity were the commonly enriched GO terms (Table 1).

Moreover, the functional characteristics of genes responsive to infection at two different rearing temperatures were explored. The 1164 genes identified between uninfected and infected fish reared at 12 °C revealed 26 significantly enriched GO terms (Fig. 10, Table 2). These enriched DEGs included GO:0006952 Defence response, GO:0007600 sensory perception, GO:0005922 Connexin complex and GO:0008188 Neuropeptide receptor activity. For the same treatment comparisons in fish reared at 17 °C (1 204 genes), 29 enriched GO terms were identified. Some of the enriched GO terms include GO:0034220 Monoatomic ion transmembrane transport, GO:0016442 RISC complex, and GO:0008168 Methyltransferase activity. There were 32 enriched GO terms when uninfected fish reared at either 12 °C or 17 °C were compared (1227 genes). GO:0006313 DNA transposition and GO:0008270 Zinc ion binding were some of the enriched GO terms. The comparison within infected cod reared at either 12 °C or 17 °C (1 359 genes) revealed 37 significantly enriched GO terms, including GO:0007186 G protein-coupled receptor signalling pathway, GO:0110165 Cellular anatomical entity, and GO:0004867 Serine-type endopeptidase inhibitor activity. GO:0015074 DNA integration, GO:0005576 Extracellular region and GO:0005179 Hormone activity were some of the GO terms common in all four comparisons. The complete list of GO terms per comparison is provided in Supplementary Material S4.

4. Discussion

Several pathogens evade the physical and biological barriers at mucosal surfaces to gain access and eventually cause systemic infections. The gills, gut, skin, and olfactory organs are known to have

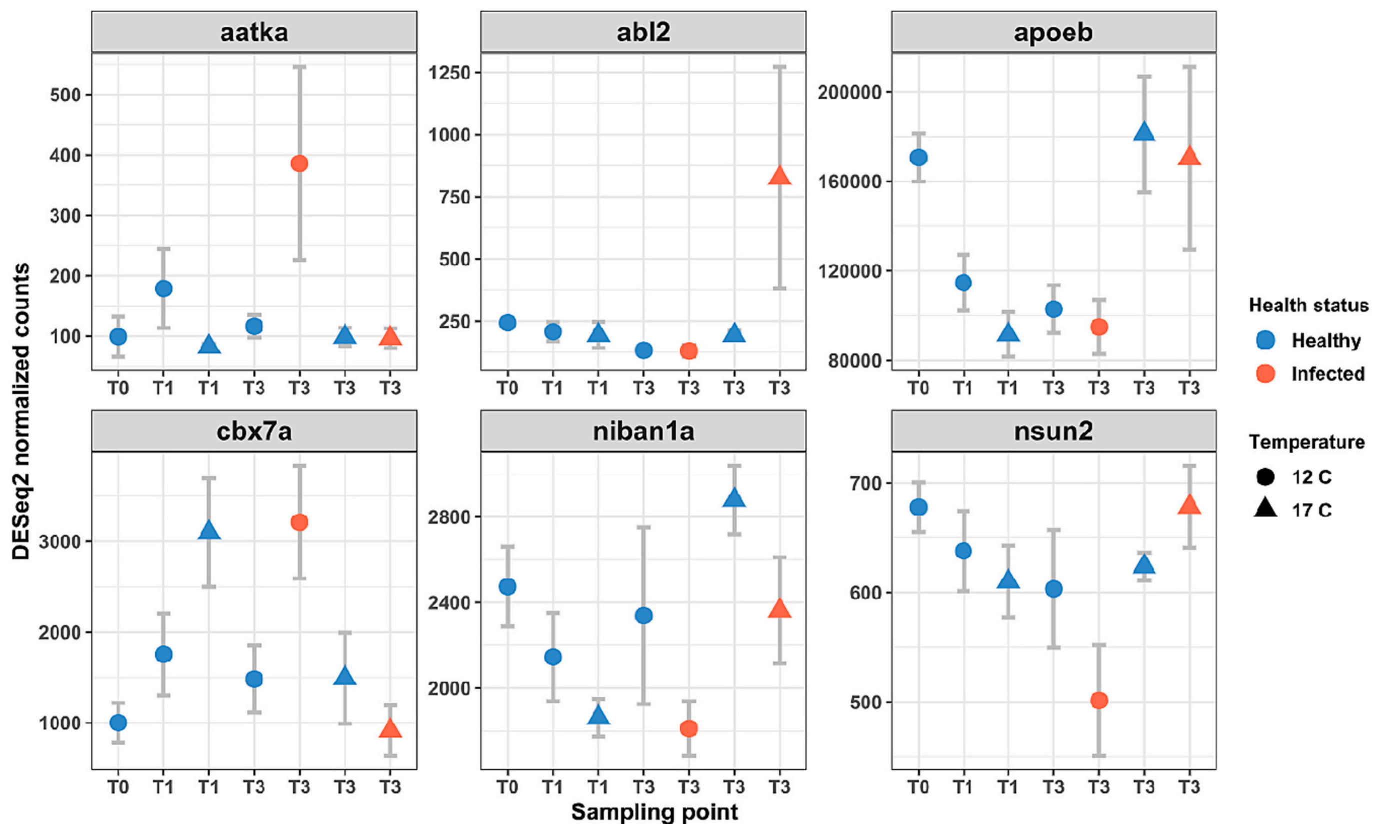

Fig. 7. Selected DEGs from the T3_17i vs T3_12i DESeq analysis, showing the temperature-influenced transcription in response to *F. noatunensis*. Transcript counts from the other time points are also given for comparison.

mucosa-associated lymphoid tissue, which plays a crucial role in mounting immune responses to pathogenic challenges. Here, we show that *F. noatunensis* infection could trigger immune responses in the olfactory organ of Atlantic cod. In addition, temperature plays an important factor in modulating the defence mechanisms against *F. noatunensis* in the olfactory organ.

4.1. Elevated temperature affects the nasal mucosa of Atlantic cod

Fish, as ectothermic animals, are heavily influenced by temperature. The mucosal surfaces of fish are susceptible to drastic changes in environmental temperature, especially those beyond the optimal range supporting their basic biological functions [8,60]. The measured optimal range for Atlantic cod is between 8 and 15 °C, and is size-dependent [47]. In the present study, there were no significant structural alterations in the nasal mucosa of fish reared at 17 °C compared with the 12 °C group. All measured morphometric parameters were unaltered at T1 and T3 after rearing at 17 °C. Low damage scores indicate that the structural integrity of the olfactory epithelium remained in good condition even after several weeks at the upper thermal threshold. Nonetheless, sporadic cases of lamellar fusions were observed at a higher temperature, especially at T3. Fusion is often associated with inflammation and epithelial proliferation and could be a form of a defence mechanism. In a sister paper, we documented structural alterations in the skin after exposure to 17 °C, including a thinner epidermis, which is more susceptible to oxidative stress, melanocyte fragmentation, and reduced keratocyte migration capacity [66]. The interaction between the olfactory organ and the rearing water might be less than for the skin, and this anatomical distinction could explain this difference. The hydrodynamics in the fish olfactory organ, especially the expansion or contraction of the accessory sac, permit the fish to differentially sample the surrounding environment, where the mechanism has been likened to sniffing [12]. Whether this mechanism played a role in the distinction observed remains to be studied. Nonetheless, this observation suggests that the olfactory epithelium was less susceptible to structural alterations at a higher temperature than the skin, at least within the period and environmental parameters of the present study.

Transcriptomics revealed acute transcriptional responses in the olfactory organ following the temperature elevation to 17 °C. Some of these molecular responses persisted after prolonged exposure to 17 °C (control group, at T3). The number of DEGs during acute and prolonged rearing at 17 °C was not very different. This indicates that the olfactory organ of Atlantic cod, though not highly affected structurally by higher temperature, could mount responses at the transcriptional level to thermal stress and the magnitude of the alterations was not heavily influenced by the duration of exposure. Some core enriched GO terms between the two periods showed similarities, revealing core thermal stress-responsive gene sets in the olfactory organ. GO term RISC complex (GO:0016442) was enriched in acute and prolonged exposure to heat stress. RNA-induced silencing complex, or RISC, is a multiprotein complex that incorporates one strand of a small interfering RNA (siRNA) or microRNA (miRNA) and uses them as a template for recognising complementary mRNA [67]. Earlier evidence suggests that miRNAs are important modulators of the molecular pathways governing responses to environmental stressors, including temperature in fish [45], and might likely be at play for the olfactory organ in cod reared at their upper thermal limit. This perhaps ensured the fidelity of post-transcriptional organisation of gene expression in response to stress. Another GO term common for both acute and prolonged response to 17 °C was 2-oxoglutarate-dependent dioxygenase activity (GO:0016706). Members of the family of 2-OG (2-oxoglutarate)-dependent dioxygenase enzymes regulate hypoxic and epigenetic responses, particularly the demethylation of histones and DNA. Their dependence on molecular oxygen provides evidence for their role as metabolic sensors [62]. Higher temperature

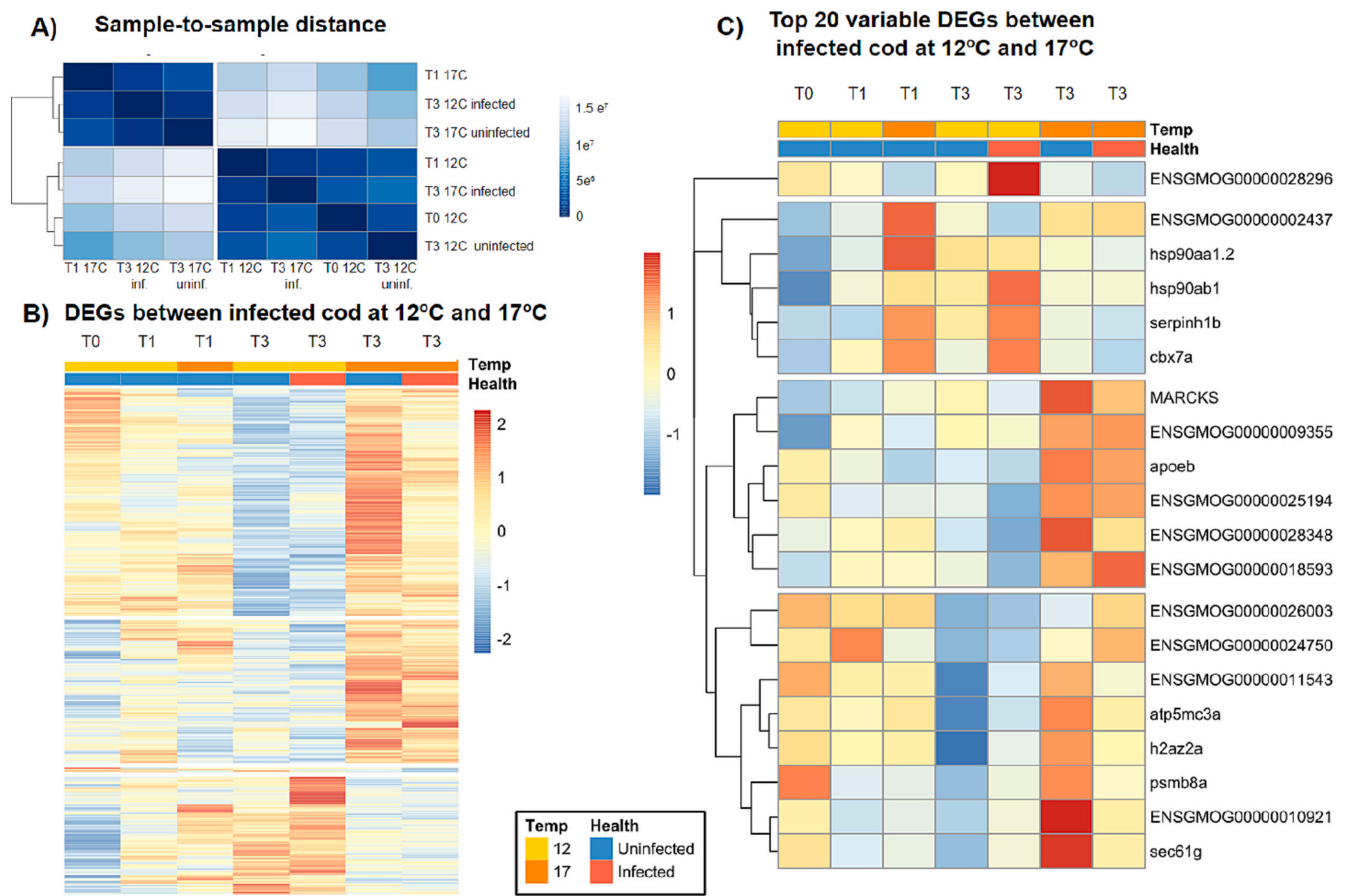

Fig. 8. The magnitude of change in the expression of genes found in the olfactory organ as a response to temperature, infection and their interaction. A) Sample-to-sample distance heatmap of all genes, accounting for temperature and infection status interactions. B) Heatmap showing expression of the 754 DEGs between infected groups at two different temperatures (T17i and T12i). C) The heatmap shows the top 20 most variable DEGs between T17i and T12i, based on expression changes at the final sampling point (T3). Expression in other sampling points was provided for comparison.

decreases gas solubility and thus can be a predisposing factor for hypoxia. It is plausible that this group of enzymes maintained the metabolic status of the cells residing in the olfactory organ during conditions that might create a hypoxic environment. Neuropeptide receptor activity (GO:0008188) was one of the GO terms identified to be affected during acute but not prolonged exposure to a higher temperature. Neuropeptides are neurotransmitters that regulate the stress-related behaviour and adaptation of the organism to environmental challenges, including temperature beyond the physiological optimum [46]. The enrichment of this GO term as an acute response to the temperature increase to 17 °C indicates that neuropeptides likely functioned as immediate regulators of the neuroendocrine response to heat stress in the olfactory organ, thus maintaining the balance at the mucosa, but such regulatory response diminished following prolonged exposure to a higher temperature. Serine proteases are ubiquitous enzymes that cleave peptide bonds in proteins, thus ensuring protein quality control. In fish, they are often associated with the immune defence mechanism, including the mucosa [16,57]. GO:0004252 serine-type endopeptidase activity was enriched only after prolonged exposure. We speculate that the alteration perhaps participated in the protection from higher temperatures after a prolonged period, which lent insights into the negligible changes in the defences at the phenotypic level.

4.2. Molecular detection of F. noatunensis in the olfactory organ - a possible olfactory route of infection?

Francisellosis in Atlantic cod is characterised by extensive chronic granulomatous inflammation with multiple granulomas in all organs but mainly observed in the spleen, kidney and liver [4]. Infected fish in the current study manifested the classic gross pathologies associated with francisellosis, and their presence was further supported by their re-isolation in the head kidney and molecular detection in the spleen [66]. However, the infection did not result in mortality. Mortalities of up to 40% have been described earlier, though levels of relatively acute mortality are rarely experienced in the field [10]. It was also possible that the isolate was pathogenic, as diseased fish displayed classic pathologies, but not highly virulent to cause mortality.

As in many other systemic pathogens, hijacking the mucosal barriers is a classic mechanism to gain entry and cause systemic infection. *F. noatunensis*, is a neurotrophic pathogen and has been identified in the brain of infected fish, including Atlantic cod [10,42], and several neurotrophic fish pathogens have been shown to target the olfactory organ as an entry point [13]. It remains to be experimentally proven in the future that *F. noatunensis* is using the olfactory organ as an entry point. The infrequent pathogen detection and limited time points made it difficult to describe the pathogenesis in the olfactory organ. However, their detection four days and seven weeks after infection in the olfactory organ posits a possibility that they might have used the nasal route as another entry point. The kinetics of infection in the olfactory organ is currently under investigation in our group, which will further shed light on this mechanism.

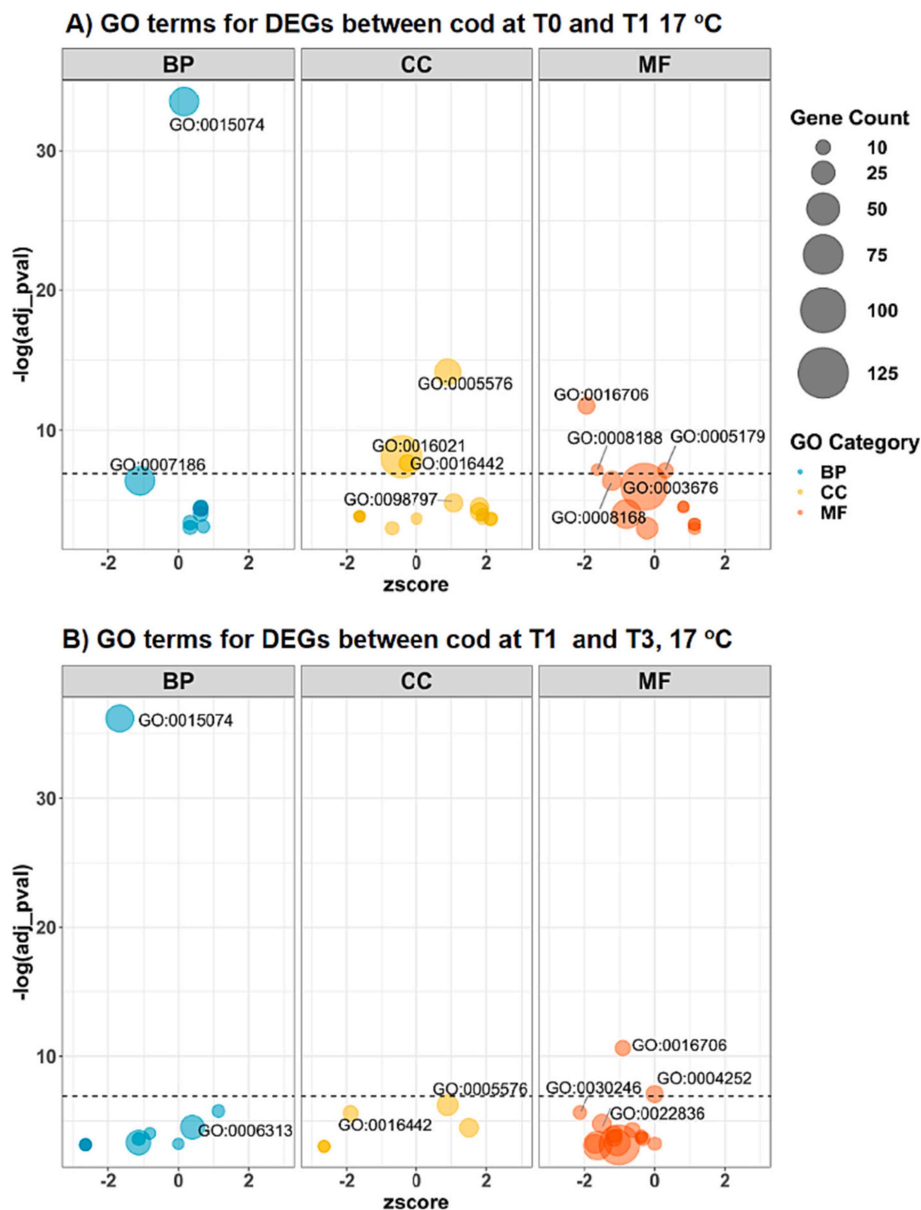

Fig. 9. The enriched GO terms, representing the genes that were affected in control/uninfected fish by short-term (T0 vs T1) and prolonged (T1 vs T3) rearing at 17 °C. BP: Biological process; CC: Cellular component; MF: Molecular function. Gene Ontology plots (labelled terms padj<0.01, count≥ 80) include genes with a log2FC. The dashed line indicates padj 0.001.

4.3. *Francisellosis has minimal effects on nasal structural morphometries*

Most nasal morphometric parameters, except the number of mucus cells, did not change following infection at either 12 °C or 17 °C. Five weeks after infection (T3), mucus cell number increased in infected fish at 12 °C compared with the uninfected group. This change was not observed in the 17 °C group. Moreover, infected fish at 12 °C had higher mucus cell numbers than infected fish at 17 °C. An increase in mucus cells is often associated with infection and stress, as this facilitates the production of mucus that provides the mucosa with the biophysical barrier [20,30]. Mucus contains several defence proteins that can prevent the adhesion of bacteria on mucosal surfaces or directly act upon them by killing or inactivation [5,8]. Decreased mucus secretion was earlier documented in sturgeon subjected to heat stress [65]. We observed that higher temperature interfered with nasal mucus production, an important defence mechanism against infection. Nonetheless, infection at two different temperatures did not cause substantial structural changes in the olfactory organ.

There were tendencies, however, that changes were more frequent at a higher temperature. For instance, we observed an increased prevalence of melanocytes, especially in the external nostrils of infected fish at 17 °C. Melanocytes are melanin-producing neural crest-derived cells, and their presence on fish skin is often associated with stress responsiveness. Melanocytes from human epidermis contribute to the phagocytosis of pathogens and the presentation of antigens to competent immune cells. Moreover, they alert macrophages, neutrophils, fibroblasts by producing cytokines such as IL-1β, IL6 and TNF-α as well as chemokines [19]. In salmon, melanocytes have been shown to be active in granulomatous inflammation [27]. Therefore, the increase in the occurrence of this cell group in infected fish is likely associated with the immune response to *F. noatunensis,* though it remained to be fully verified whether this is a typical phenotypic cellular response to francisellosis since we had a limited number of specimens. In addition, cases of lamellar fusion were quite frequent in infected fish at 17 °C. In gills, lamellar fusion likely occurred following epithelial cell hyperplasia, often associated with infection and stress [18,68]. These structural

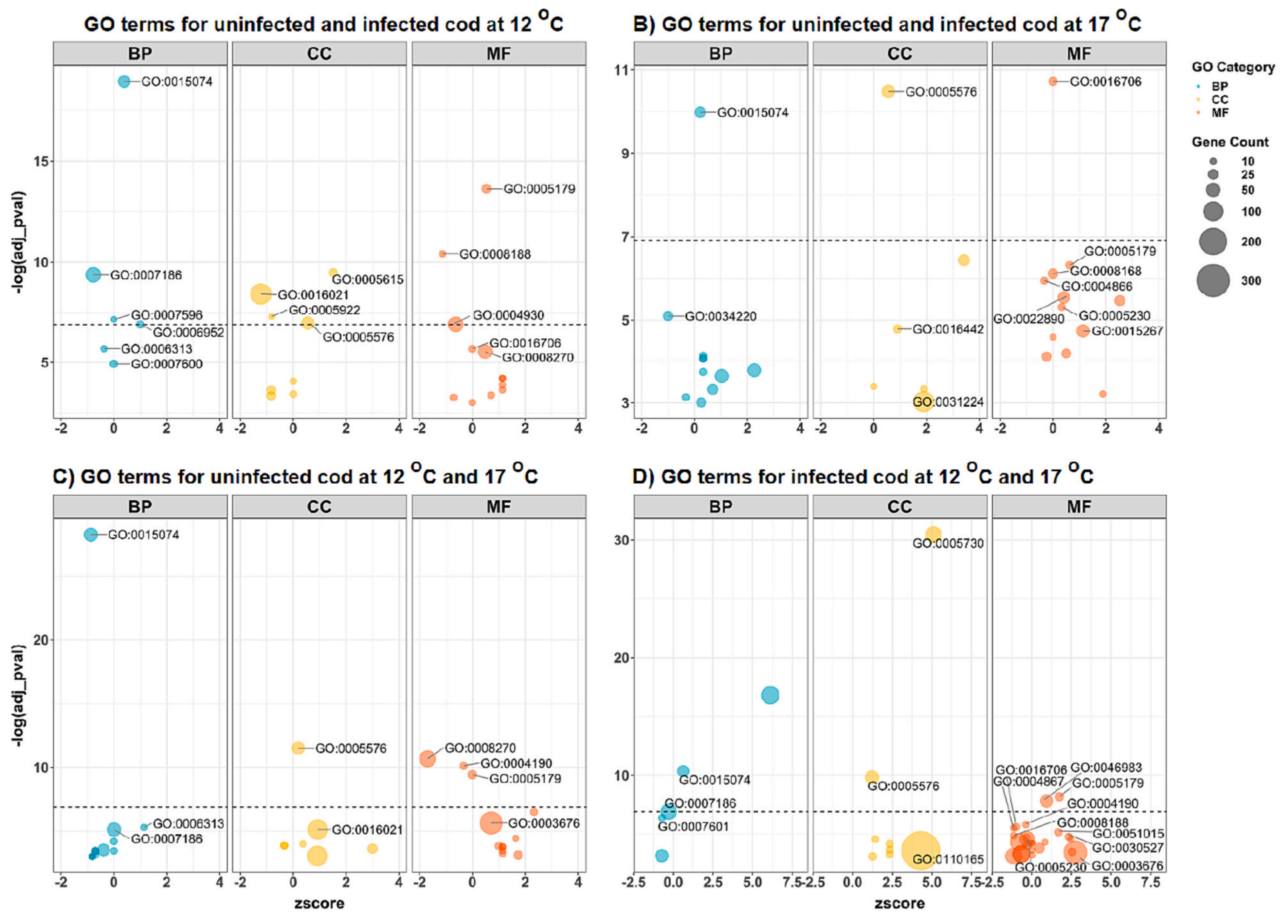

Fig. 10. The enriched GO terms represent the genes responsive to the interaction of infection and temperature (T3, uninfected and infected at either 12 °C or 17 °C). Gene Ontology plots (labelled terms padj<0.01, count≥ 80) for genes with a log2FC > |2| between the different experimental groups five weeks (T3) after infection at two different temperatures. The dashed line indicates padj 0.001.

Table 1
Summary of annotated GO terms from plots in Fig. 9. Sub-ontologies of GO : BP = biological process; CC = cellular component; MF = molecular function.

Most important GO terms for DEGs between T0 and T1 17 °C		
BP	GO:0015074	DNA integration
BP	GO:0007186	G protein-coupled receptor signalling pathway
CC	GO:0005576	Extracellular region
CC	GO:0016021	Membrane
CC	GO:0016442	RISC complex
CC	GO:0098797	Plasma membrane protein complex
MF	GO:0016706	2-oxoglutarate-dependent dioxygenase activity
MF	GO:0005179	Hormone activity
MF	GO:0008188	Neuropeptide receptor activity
MF	GO:0003676	Nucleic acid binding
MF	GO:0008168	Methyltransferase activity
Most important GO terms for DEGs between T1 and T3, 17 °C		
BP	GO:0015074	DNA integration
BP	GO:0006313	DNA transposition
CC	GO:0005576	Extracellular region
CC	GO:0016442	RISC complex
MF	GO:0016706	2-oxoglutarate-dependent dioxygenase activity
MF	GO:0004252	Serine-type endopeptidase activity
MF	GO:0030246	Carbohydrate binding
MF	GO:0022836	Gated channel activity

alterations were related to poor mucosal health, as they impaired the function of the mucosal organs, e.g., the respiratory surface of the gills was limited following lamellar fusion. We believe that severe lamellar fusion in the olfactory organ could possibly affect sensory function, leading to altered detection of odorants and pathogens. This is partly supported by the enrichment of GO term G protein-coupled receptor signalling pathway, the family where olfactory receptors belong, in uninfected and infected fish at 17 °C (Table 2). Functional studies are required to explore the biological importance of this observation, particularly in the ability to detect stimuli in the environment at sub-optimal conditions.

4.4. *Franciscella infection induces transcriptional changes in the olfactory organ and is influenced by elevated temperature*

The effects of *F. noatunensis* on the olfactory organ can be viewed in three ways. First, the molecular detection of the bacteria in the olfactory organ indicates that the nasal transcriptomic changes could be considered local responses to the pathogen. Second, internal immune organs (i.e., head kidney and spleen) were positive for the pathogen as inferred by the classic gross pathologies, and re-isolation of the pathogen, therefore, the transcriptomic changes in the olfactory organ perhaps were remote responses to systemic infection. Lastly, the nasal responses were a combination of the two possibilities, but whether there was a simultaneous coordination of responses remains to be elucidated in the future.

Looking at the effects of infection through time (T1 vs T3), it was evident that the olfactory organ from the infected group reared at 17 °C exhibited more transcriptomic alterations based on the number of unique DEGs compared with the infected group reared at 12 °C. This implies that rearing at the upper thermal limit could affect the temporal effect of infection. It has been suggested that elevated water temperature increases the risks of fish to some known diseases [23], making them

Table 2
Summary of annotated GO terms from plots in Fig. 10. Sub-ontologies of GO: BP = biological process; CC = cellular component; MF = molecular function.

Most important GO terms for DEGs between uninfected and infected cod at 12 °C		
BP	GO:0015074	DNA integration
BP	GO:0007186	G protein-coupled receptor signalling pathway
BP	GO:0007596	Blood coagulation
BP	GO:0006952	Defence response
BP	GO:0006313	DNA transposition
BP	GO:0007600	Sensory perception
CC	GO:0005615	Extracellular space
CC	GO:0016021	Membrane
CC	GO:0005922	Connexin complex
CC	GO:0005576	Extracellular region
MF	GO:0005179	Hormone activity
MF	GO:0008188	Neuropeptide receptor activity
MF	GO:0004930	G-protein-coupled receptor activity
MF	GO:0016706	2-oxoglutarate-dependent dioxygenase activity
MF	GO:0008270	Zinc ion binding
Most important GO terms for DEGs between uninfected and infected cod at 17 °C		
BP	GO:0015074	DNA integration
BP	GO:0034220	Monoatomic ion transmembrane transport
CC	GO:0005576	Extracellular region
CC	GO:0016442	RISC complex
CC	GO:0031224	Obsolete intrinsic component of membrane
MF	GO:0016706	2-oxoglutarate-dependent dioxygenase activity
MF	GO:0005179	Hormone activity
MF	GO:0008168	Methyltransferase activity
MF	GO:0004866	Endopeptidase inhibitor activity
MF	GO:0022890	Inorganic cation transmembrane transporter activity
MF	GO:0005230	Extracellular ligand-gated monoatomic ion channel activity
MF	GO:0015267	Channel activity
Most important GO terms for DEGs between uninfected cod at 12 °C and 17 °C		
BP	GO:0015074	DNA integration
BP	GO:0006313	DNA transposition
BP	GO:0007186	G protein-coupled receptor signalling pathway
CC	GO:0005576	Extracellular region
CC	GO:0016021	Membrane
MF	GO:0008270	Zinc ion binding
MF	GO:0004190	Aspartic-type endopeptidase activity
MF	GO:0005179	Hormone activity
MF	GO:0003676	Nucleic acid binding
Most important GO terms for DEGs between infected cod at 12 °C and 17 °C		
BP	GO:0015074	DNA integration
BP	GO:0007186	G protein-coupled receptor signalling pathway
BP	GO:0007601	Visual perception
CC	GO:0005730	Nucleolus
CC	GO:0005576	Extracellular region
CC	GO:0110165	Cellular anatomical entity
MF	GO:0016706	2-oxoglutarate-dependent dioxygenase activity
MF	GO:0046983	Protein dimerisation activity
MF	GO:0004867	Serine-type endopeptidase inhibitor activity
MF	GO:0005179	Hormone activity
MF	GO:0004190	Aspartic-type endopeptidase activity
MF	GO:0008188	Neuropeptide receptor activity
MF	GO:0051015	Actin filament binding
MF	GO:0030527	Structural constituent of chromatin
MF	GO:0005230	Extracellular ligand-gated monoatomic ion channel activity
MF	GO:0003676	Nucleic acid binding

particularly vulnerable when they are not in the optimal environment. Infection and higher temperatures are metabolically demanding conditions for fish that require efficient mobilisation of physiological countermeasures for survival [15,35]. Despite a mild infection as shown by lower bacterial load from qPCR, rearing infected fish at 17 °C presented a potential compound stress, which might partly explain the higher number of DEGs in infected fish reared at 17 °C. There was also a negligible number of shared genes between the DEGs of the infected fish at 12 °C and 17 °C seven weeks following infection. These profiles suggest two potentially temperature-influenced distinct responses to *F. noatunensis* in the olfactory organ. Some individual genes showed thermal-dependent regulation related to infection. It was described earlier that delay in apoptosis is a typical response for *Francisella* infecting humans and fish, including cod [22,52]. We identified several genes involved in apoptosis, such as *aatka*, *abl2* and *niban1a*, at seven weeks after infection, which were differentially affected by temperature. Apoptosis is a fundamental cellular process that eliminates damaged cells from an organism, therefore, their identification in the group of genes responsive to infection was likely related to ensuring cellular balance and turnover in the olfactory organ.

However, if we looked at the transcriptome profile at termination, it appeared that the effect of infection was diminished in the infected groups regardless of the temperature; there were insignificant transcriptional changes compared with the uninfected fish at T3. One probable explanation for this insignificant response could be that as systemic infection progressed severely, the mucosa was no longer actively fighting off the infection since the pathogen had already breached the barrier, and the fish could be relying heavily on circulating cellular and humoral factors to address the need for protection and survival. Future studies should be targeted on profiling the responses in the early days after infection, as a previous transcriptome-wide study documented that cod displayed classic innate immune response with inflammation, acute-phase proteins and cell recruitment in the head kidney days after infection with *F. noatunensis* [52]. We did not perform such an approach in the present study since we were primarily interested in the prolonged impact of infection under two thermal scenarios.

The effects of temperature were far more compelling within the infected groups where a little over 700 DEGs were identified, and the transcriptome profiles were distinct, i.e., DEGs in the infected group at 17 °C were predominantly upregulated while those in the infected group at 12 °C were downregulated. Dissecting the enriched GO terms in these two infected groups revealed some genes involved in membrane, cell signalling and motility, and enzyme activities. Some GO terms affected by temperature (Section 4.1), including neuropeptide receptor activity and 2-oxoglutarate-dependent dioxygenase activity, were enriched in infected fish, suggesting their involvement in thermal stress and infection in the olfactory organ. Neuroendocrine and immune systems have interlinked functions, where neuropeptides could act as primary mediators of innate defence [54]. Hence, neuropeptides in the olfactory organ were likely crucial for nasal response to thermal stress and infection. Another GO term enriched when the two infected groups were compared was G-protein coupled receptor (GPCR) signalling pathway. GPCRs play important roles in inflammation, particularly critical to migrating phagocytes to the site of inflammation and eventually contributing to resolution [56]. We believe such a similar mechanism might be at play in protecting the olfactory organ from thermal stress and infection. The enrichment GO term actin filament binding partially supports this implication; it is an important mechanism for cell mobility [6], including immune cells that possibly participated in orchestrating the defence mechanism in the olfactory organ.

5. Conclusions

The study provides evidence that *F. noatunensis*, elevated temperature and their combinations could trigger responses in the olfactory organ of Atlantic cod. These changes were presented at the molecular but not at the morphological level in the olfactory mucosa. The elevated temperature alone resulted in significant transcriptomic alterations in the olfactory organ. The magnitude of response to short-term and prolonged rearing at 17 °C was almost similar, which was further substantiated by several shared GO terms. *F. noatunensis* infection induced molecular alterations in the olfactory organ, which could be local responses but could also suggest remote responses to systemic infection. The infection-induced transcriptomic changes in the olfactory organ were influenced by temperature, where the temporal effects of infection were more significant at 17 °C. However, the effects of infection

appeared to be diminished after 7 weeks, as the changes in the transcriptome of infected and uninfected fish at different temperatures were almost negligible. Nonetheless, substantial transcriptomic changes were observed within the infection groups, and many of the genes affected were involved in signalling, defence, transmembrane and enzymatic processes. The results of the study provide implications of how the changing climate, such as long periods of elevated temperature, could affect the fish mucosa and its defense mechanisms against infectious diseases.

Author contributions

Carlo C. Lazado: acquired the funding, conceptualised the project and designed the experimental trial, performed the analysis, interpreted the data, wrote, edited and reviewed the manuscript.

Marianne Iversen: analysed, visualised, and interpreted the data, co-wrote the first draft of the manuscript.

Lill-Heidi Johansen: conceptualised the project and designed the experimental trial, performed the infection trial, interpreted the data, wrote, edited and reviewed the manuscript.

Hanne Brenne: performed the infection trial, collected the samples, performed lab analysis.

Arvind Y.M. Sundaram: analysed and interpreted the data, reviewed the manuscript.

Elisabeth Ytteborg: acquired the funding, conceptualised the project experimental trial, interpreted the data, wrote, edited and reviewed the manuscript.

All authors contributed to the writing and review of the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Accession number of the sequencing data is provided in the methodology

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2023.110735.

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