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# Climate change with increasing seawater temperature will challenge the health of farmed Atlantic Cod (*Gadus morhua* L.)

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Aquaculture is one of the fastest growing food production sectors in the world and further expansion is expected throughout the 21<sup>st</sup> century. However, climate change is threatening the development of the sector and action is needed to prepare the industry for the coming challenges. Using downscaled temperature projections based on the Intergovernmental Panel on Climate Change (IPCC) climate projection (Shared Socioeconomic Pathway, SSP2-4.5), we analysed potential future temperatures at a selected Atlantic cod (*Gadus morhua* L.) farm site in Northern Norway. Results showed that the farming area may experience increased temperatures the next 10–15 years, including more days with temperatures above 17°C. Based on the predicted future conditions, we designed a study with Atlantic cod (*Gadus morhua* L.) to evaluate effects from high temperature alone and in combination with *Fransicella noatunensis* infection. Fish were kept at 12°C and 17°C for eight weeks and samples of skin and spleen collected at different timepoints were analysed with transcriptomics, histology, scanning electron microscopy and immunohistochemistry. Results showed that high temperature had a stronger effect on the barrier functions of skin than the infection. Increased temperature induced gene expression changes in skin and spleen, *heat shock protein 47* and *cold inducible RNA binding protein* were identified as potential gene markers for thermal stress. The effect of bacterial challenge was small at 12°C. At high temperature, the development of severe pathology in spleen coincided with a significant decrease of immunoglobulins transcripts, which contrasted with the activation of multiple immune genes. In addition, we used an *in vitro* model of skin biopsies and scale explants exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to assess the effects of thermal and oxidative stress. High temperature and H<sub>2</sub>O<sub>2</sub> reduced proliferation and migration of keratocytes, and increased expression of stress markers, and compounding effects were observed with combined stressors. Results suggest that the projected increased seawater temperature will pose a significant threat

to Norwegian cod farming, affecting various biological processes and making fish more vulnerable to stressors and pathogens. Cod farming needs high attention to temperature changes, and special precautions should be taken if the temperature increases beyond cods' thermal optimum.

#### KEYWORDS

Atlantic cod, aquaculture, climate change, francisella, IPCC, temperature

## 1 Introduction

Aquaculture is one of the fastest growing food production sectors in the world and further expansion is expected throughout the 21<sup>st</sup> century (FAO, 2022). However, climate change is already threatening the development of the sector and action is needed to prepare the industry for the coming challenges (Falconer et al., 2022). Climate stressors relevant for aquaculture include increased air and sea temperatures, rising sea level, more frequent extreme weather events, ocean acidification, altered salinity, and changes in rainfall (IPCC, 2014; IPCC, 2019). Continued emissions will result in further atmospheric and oceanic warming, with long-term changes to the climate system, which will exacerbate existing risks and create new challenges for life on earth (IPCC, 2014; IPCC, 2023). Since aquaculture includes a diversity of production systems, species, and farming environments (Metian et al., 2020), assessment of climate change is especially complex, and a holistic approach is needed to understand the impact and severity of future changes (Falconer et al., 2022).

Atlantic cod (*Gadus morhua* L.) is one of the most important species for commercial fisheries in the North Atlantic (Welch et al., 1992; Hop and Gjøsaeter, 2013). In the last decades, spawning stock biomass and cod catches have declined, and traditional fisheries may become further reduced due to climate change (Misund et al., 2016). Reduced availability of wild cod has renewed the interest in cod farming (Henriksen et al., 2018; Puvanendran et al., 2022). The potential in Norwegian cod farming gradually declined from 2008 due to multiple factors, but new knowledge on biological performance, requirements, and production methods has in recent years initiated new investments in cod farming (Puvanendran et al., 2022). Novel methods and strategies that assess and predict cod health and welfare, as well as insight into how climate change will affect production, are important for successful further growth of the industry. For critical factors such as seawater temperature, targeted approaches are required to investigate the links between biological performance and projected future farm conditions. Seawater temperatures are increasing and there are more frequent marine heatwave events (Oliver et al., 2018; IPCC, 2019), thus farmed cod are likely to be subjected to higher temperatures in the future than currently experienced. Optimal seawater temperatures for mature cod are between 6 and 11°C (Holt and Jørgensen, 2015). Temperatures above 15°C enhance stress, impair swimming and haemoglobin

oxygen affinity, and mortality increases when temperatures reach 20°C (Pörtner, 2001; Pérez-Casanova et al., 2008a; Norin et al., 2019; Zanuzzo et al., 2019). These thermal thresholds are worth a closer look, alongside projections of future temperatures in cod farming areas, to understand the limitations and possible strategies of adaptation, when planning to increase cod farming under climate change.

Increasing seawater temperatures, beyond thermal optimum, can make Atlantic cod more vulnerable to infections. The intracellular bacterium *Furunculosis noatunensis*, the causative agent of francisellosis (Mikalsen, 2008; Ottem et al., 2009), is considered one of the most important disease problems in Norwegian cod farming and was one of the reasons why the industry collapsed around 2010. Current expectations of increased production in combination with increasing sea water temperatures, could cause the disease to re-emerge as a challenge. Diseased cod lose appetite, the skin may appear darker and small ulcers may occur together with nodules (granulomas) in the skin, but usually there are no clear external signs of the disease. Internally, large numbers of yellow granulomas in the kidney, spleen and other organs can be found (Ottem et al., 2008). Bacteria cause cell and tissue damages (Nylund et al., 2006; Olsen et al., 2006), which can be aggravated by thermal stress. Water temperature above 15°C seems to play a significant role in development of francisellosis. The number of published reports on the effect of high seawater temperature on Atlantic cod immunity is limited. Expression of immune genes and respiratory burst were measured in leukocytes of cod exposed to gradual increase of temperature from 10°C to 19°C (Pérez-Casanova et al., 2008b). Transcriptome analysis in spleen of cod acclimated to 10°C and 16°C revealed thermal stimulation of immune responses to viral mimic polyinosinic:polycytidylic acid but not to formalin killed *Aeromonas salmonicida* (Hori et al., 2013). Immune stimulation in cod may coincide with increased mortality as shown in Atlantic cod infected with *Brucella pinnipedialis* (Larsen et al., 2018). Acclimation to high temperature increased immunoglobulin and innate antibody levels in cod serum (Magnadóttir et al., 1999), and have previously been shown to compromise immunity in other teleosts (Cabillon and Lazado, 2019; Scharsack and Franke, 2022). Reduced, or changed, immune functions may result in species being more vulnerable to challenges and secondary stressors and must be taken into consideration when evaluating the effects of climate change and warmer temperatures.

Using climate projections from the Intergovernmental Panel on Climate Change (IPCC) we have evaluated potential future temperatures at a commercial cod farm in Norway. Climate change scenarios represent the consequences of anthropogenic activities at global scale (Kriegler et al., 2012; O'Neill et al., 2014), but with the inclusion of dynamical downscaling and bias correction methods they can be used to evaluate how the environment will change at local scale (Falconer et al., 2020). Based on these results we designed a temperature study with cod, and further challenged the fish with *F. noatunensis*. Thermal effects alone and in combination with the infection, were studied using transcriptomics and several microscope imaging techniques. An *in vitro* skin model was used to further study how temperature affects migration capacity and response to a secondary stressor, i.e., oxidative stress. Overall, important mechanisms involved in cod health will be severely affected by the high temperatures predicted to occur in the near future.

## 2 Materials and methods

### 2.1 Climate scenarios

Prior to designing the fish trial, temperature data from a commercial cod farm in Dønna (Northern Norway, 66.17°N) was evaluated and climate projection models used to look at possible future temperatures at that location. This farm was selected based on location and availability of farm data. Regional climate model projections of temperature were obtained from a 3D ocean circulation model NEMO-NAA10km (Hordoir et al., 2022) based on the NEMO ocean engine (Madec and The NEMO System Team, 2015) forced with atmospheric data from climate projections from the Norwegian Earth System Model (NorESM2) (Bentsen et al., 2013; Seland et al., 2020). Temperature data from the farm (daily average, 3m) was used to calibrate climate projections (IPCC, Shared Socioeconomic Pathway SSP2-4.5) according to the

published bias correction method (BC1), (Falconer et al., 2020). The SSP2-4.5 climate scenario represents a “middle-of-the-road”, medium emissions scenario (Fricko et al., 2017). Seven individual years (2016 – 2022) of temperature data (farm measurements), and 7 individual years (2030 - 2036) of modelled temperatures (BC1 SSP2-4.5; 5-day averages, bias-corrected and interpolated to daily values) were analysed to evaluate the changes in temperature in the near-future, as this timeframe is relevant to present-day decision making.

### 2.2 Ethics statement

All fish handling and manipulations in the study adhered to the guidelines and protocols of European Union Directive 2010/63/EU and the trial was approved by the Norwegian Food Authorities (FOTS ID 23048).

### 2.3 Fish trial and sampling

Atlantic cod, produced at the Center of Marine Aquaculture (Nofima, Norway), were transferred to The Aquaculture Research Station (Tromsø, Norway). Fish were kept for acclimatization for 4 weeks in flow-through tanks at 8°C, and fed Amber Neptune (Skretting, Norway). Temperature increase was performed in two stages. First the fish were acclimated to 12°C for one week. Second, 120 fish were distributed in 4 tanks, 30 fish per tank, and temperature in two tanks increased to 17°C. Temperature in the two remaining tanks were kept at 12°C. After acclimatization, fish were moved to the challenge facility at the station where they were kept in 200 L tanks with similar conditions in two replicate tanks per temperature (Figure 1). Average weight and length were 123.4 grams (SD 39,50 grams) and 23.3 cm (SD 2,08 cm), respectively.

First day after transfer (T0), three fish from each tank were killed by an overdose of anaesthetics and sampled. Skin and spleen

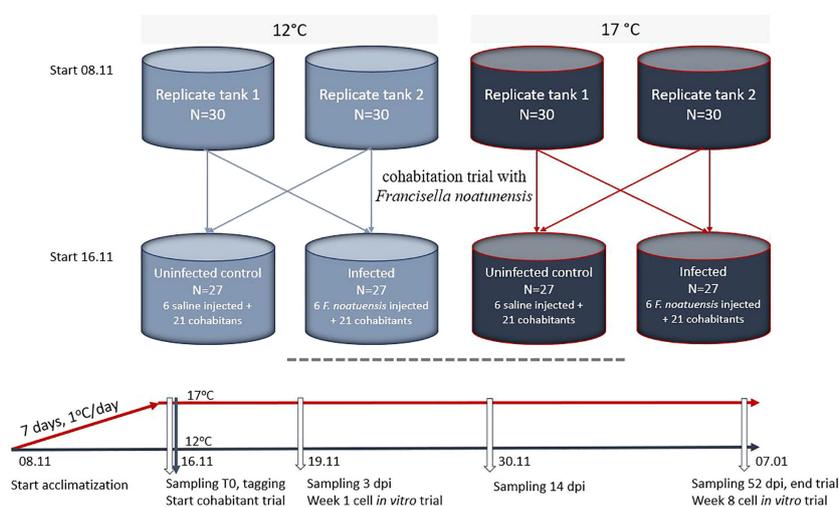


FIGURE 1  
Experimental setup and sampling. White arrows indicate sampling points.

were transferred to RNA later (Life technologies, Carlsbad, CA, USA) and formalin (Cellstore™, CellPath, Newtown, UK). RNALater samples were stored at  $-20^{\circ}\text{C}$  and formalin samples on  $4^{\circ}\text{C}$ , according to the manufacturer's protocols.

On the same day, a cohabitation trial with *Francisella noatunensis* (type strain NCIMB 14265) was started. The bacterium, recently re-isolated from infected Atlantic cod and kept frozen in glycerol, were thawed, and grown on CHAB plates for 4 days at  $20^{\circ}\text{C}$ . Multiple single colonies were resuspended in saline and  $\text{OD}_{600\text{nm}}$  measured. To calculate CFU/ml a duplicate series of ten-fold dilutions of the bacterial suspension was titrated on CHAB plates and incubated at  $20^{\circ}\text{C}$  for 4 days before counting. At challenge, 6 fish per replicate tank at both  $12^{\circ}\text{C}$  and  $17^{\circ}\text{C}$  were anesthetised and injected intraperitoneal with 0,1 ml *F. noatunensis* suspension at a challenge dose of  $10^8$  cfu/fish, or with saline (uninfected control group). For each of the two temperature groups, 3 injected and 10 or 11 naïve (cohabitants), from each of the two replicate tanks were distributed into each of 4 new tanks as shown in Figure 1, in total 6 injected and 21 naïve fish per tank and with one infected and one uninfected (saline injected) tank per temperature. To be able to know which replicate tank the fish were transferred from, fish in one tank at  $12^{\circ}\text{C}$  and one tank at  $17^{\circ}\text{C}$  were tagged by VIE (visible implant elastomer, Northwest Marine Technologies Inc). The injected fish were tagged with an additional implant. At 3, 14 and 52 days post infection (dpi), 6 cohabitant fish per tank were sampled as described for the T0 sampling (Figure 1). To confirm infection by *F. noatunensis*, the bacterium was reisolated on CHAB plates from head kidney and spleen of all sampled fish and RTqPCR was run on selected spleen samples using *F. noatunensis* specific primers (Ottem et al., 2008).

## 2.4 Histology

Skin and spleen biopsies ( $N = 6$  per treatment per timepoint) were examined in a stereo loupe (Leica Thunder) before embedding in paraffin. In brief, samples fixed in buffered 4% formalin, were carefully dissected, orientated, and placed in tissue embedding cassettes (Simport, Quebec, Canada). The samples were dehydrated through 100% alcohol and then in a clearant Xylene bath, using an automated tissue processor (TP1020, Leica Biosystems, Nussloch GmbH, Germany), before infiltrated in melted  $60^{\circ}\text{C}$  paraffin (Merck KGaA, Darmstadt, Germany). Paraffin-embedded tissue samples were cut in  $2\ \mu\text{m}$  sections using a Microtome (Leica RM 2165), mounted on polysin coated slides (VWR, Avantor, Pennsylvania, USA) and dried overnight at  $37^{\circ}\text{C}$ . The sections were deparaffinized and rehydrated and stained using automated special stainer (Autostainer XL Leica Biosystems, Nussloch GmbH, Germany). Paraffin sections of skin were stained with Alcian Blue Periodic Acid Schiff (AB/PAS, pH 2.5, Alcian Blue 8GX, Sigma Aldrich, Darmstadt, Germany), spleen was stained using Hematoxylin Eosin (Sigma Aldrich, St. Louis, MU, USA). Stained samples were scanned using a Leica light microscope slide scanner (Aperio CS2 fra Leica Biosystems, Wetzlar, Germany).

Skin was evaluated in Aperio Image Scope (Leica Microsystems, Wetzlar, Germany). Measurements were done in a region of ca.  $1000\ \mu\text{m}$  per section. In each region, epidermal mucous cells were

counted and defined as either “outer” (those touching the outer border of the epidermis) or “total” (outer mucous cells including those distributed in the epidermis). Epidermal thickness was measured in 5 different locations of the selected region. The general appearance of the epidermis and the quality of the epithelial surface were characterised using a semi-quantitative 5-point scale skin health scoring system. Spleen was evaluated in QPath (Bankhead et al., 2017). Granulomas in the spleen were evaluated based on their morphology and categorised as either “early” or “developed”. These were counted, area was measured together with total area of the section, and percentage of affected spleen calculated. Statistics were done by ANOVA, followed by Tukey *post hoc* test showing significant differences ( $P < 0.05$ ).

## 2.5 In vitro studies

Atlantic cod keratocytes were cultured from scale explants according to previously described protocol (Ytteborg et al., 2020), at weeks 1 and 8. Scales from fish reared at the two different temperatures were used for cultivation. To evaluate the impact of temperature in combination with secondary stressors other than infection, we exposed skin biopsies to oxidative stress *in vitro* according to previously described methods (Ytteborg et al., 2020). Skin biopsies were exposed to 1000 ppm hydrogen peroxide ( $\text{H}_2\text{O}_2$ , Merck, Rahway, NJ, USA) for 40 min, before scales were picked and further cultured. Control biopsies were kept 40 min in salt water. Samples from these biopsies were stored in formalin and RNALater, for histological evaluation and transcriptomics, respectively.

For keratocyte migration assays, 12 well tissue culture plates (Falcon Multiwell™ Becton Dickinson, NJ, USA) were used, 5 scales per well, 3 wells per fish, 5 fish per treatment. Each well contained 1ml L-15 supplemented with fetal bovine serum (FBS) 10%,  $25\ \mu\text{g}$  amphotericin B, 10ml/L antibiotics, antimycotics and 0.01M HEPES (Sigma Aldrich). Plates were incubated at  $12^{\circ}\text{C}$  or  $17^{\circ}\text{C}$ , corresponding to the temperatures in respective fish tanks, in a cell incubator without  $\text{CO}_2$ . After one, two, three and five days, wells were microscopically analyzed (Leica). Scales with migrating cells (defined as cells moving from the scales to the bottom of the well) were counted at every timepoint and percentage to the total number of attached scales was defined as an indicator of migratory potential. Morphology of migrating cells was documented, and wells were fixed for immunohistochemistry (IHC) using 4% formalin at the end of the experiment (day 7). Live cells were stained with EdU (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocols and visualized using a Zeiss Axio Observer Z1 equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

## 2.6 Immunohistochemistry

Fixed cells were washed twice in FBS, incubated 10 min in 50 mM  $\text{NH}_4\text{Cl}$ , washed twice in PBS, and permeabilized in 0,1% Triton X-100 for 10 min before blocking with 5% BSA for 30 min. Slides were incubated with primary rabbit anti-mouse iNOS

from Affinity Bioreagents Inc. (Golden, CO, USA) diluted 1:2000 overnight at 4° and washed with PBS-T before secondary antibody (Goat anti-rabbit Alexa 488, A11008, Invitrogen) diluted 1:400 including Hoechst 1:1000 (H1399, Invitrogen) and incubated 1 h in room temperature. All dilutions were done with 0.5% BSA. Slides were washed 3x in PBS-T followed by PBS and microscopically examined using Zeiss Axio Observer Z1 (Carl Zeiss Microimaging GmbH).

## 2.7 Scanning electron microscopy

Skin samples for scanning electron microscopy (SEM) from the two temperature regimes ( $N = 3$  per species per treatment) were dehydrated from PBS to 100% EtOH and dried using a Critical Point Dryer (CPD 030, Bal-tec AG, Schalksmühle, Germany) with liquid carbon dioxide as the transitional fluid. The samples were then mounted on stubs with carbon tape and coated with gold-palladium (Polaron Emitech SC7640 Sputter Coater, Quorum technologies, East Sussex, UK). Imaging was performed at the Imaging Centre, Faculty of Biosciences, Norwegian University of Life Sciences (Zeiss EVO-50-EP, Carl Zeiss SMT, Cambridge, UK).

## 2.8 RNA isolation

Total RNA was extracted from skin samples and spleen ( $N = 6$  per organ, per treatment, per sampling point) and from skin from the *in vitro* skin biopsies ( $N = 6$  per treatment). An automated Biomek 4000 (Beckman Coulter, Pasadena, CA, USA) including an on-column DNase treatment were used, according to the manufacturer's protocol. 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Santa Clara, CA, USA) were used to verify the integrity of the RNA samples (RIN values  $> 7.5$ ). RNA purity and concentration were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific). Total RNA samples were stored at  $-80^{\circ}\text{C}$  until prepared for multiple gene expression profiling.

## 2.9 cDNA and real time qPCR

Skin biopsies from the *in vitro* trial were analysed using real time quantitative PCR. cDNA was synthesised using Taqman Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) in a 20  $\mu\text{L}$  reaction mixture containing 9.6  $\mu\text{L}$  500 ng template RNA. Thermocycling was performed using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems), with the parameters 25 °C for 10 min, 37 °C for 30 min and 95 °C for 5 min. Transcript levels of selected genes were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) in QuantStudio™ 5 (Applied Biosystems). Each assay consisted of 5  $\mu\text{L}$  of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 0.5  $\mu\text{L}$  10  $\mu\text{M}$  of each forward/reverse primer (Invitrogen) and 4  $\mu\text{L}$  of 1:10 cDNA, with the parameters: pre-incubation at 95 °C for 20 s, 40x

amplification cycles at 95 °C for 1 s and 60 °C for 20 s, and a dissociation stage of 95 °C for 1 s, 60 °C for 20 s and 95 °C for 1 s. A five-step standard curve of 2-fold dilution series was prepared from pooled cDNA to determine the amplification efficiencies. Primers are listed in Table 1. Results were analysed using REST (Pfaffl, 2001; Pfaffl et al., 2004).

## 2.10 Microarray

Transcriptome analyses were performed using Nofima's 44k genome-wide oligonucleotide Atlantic cod microarray. Analyses included skin and spleen of intact and infected fish, two temperatures and two time-points (one week at different temperatures and seven weeks after challenge with *Francisella*), totally 59 samples were analysed. Microarrays were manufactured by Agilent Technologies, and all reagents and equipment were purchased from the same provider. RNA amplification and labelling were performed with a One-Color Quick Amp Labelling Kit, and a Gene Expression Hybridization kit was used for fragmentation of labelled RNA. After overnight hybridization in an oven (17 hours, 65°C, rotation speed 0.01 g), arrays were washed (Gene Expression Wash Buffers 1 and 2) and scanned. Subsequent data analysis was performed with Nofima's bioinformatic package STARS (Krasnov et al., 2011). Global normalization was performed by equalizing the mean intensities of all microarrays. The individual values for each feature were divided by the mean value of all samples producing expression ratios (ER). The log<sub>2</sub>-ER were calculated and normalized with the locally weighted non-linear regression (Lowess). Differentially expressed genes (DEG) were selected by criteria: 1.75-fold and  $p < 0.05$ . STARS annotations were used for comparisons of functional groups of genes, difference was assessed with t test ( $p < 0.05$ ).

# 3 Results

## 3.1 Future seawater temperatures

Looking at the temperature data from the farm at Dønna, two years from 2016 to 2022 had average daily temperatures above 15°C, with a maximum temperature of 16°C (Figure 2). At this site, the BC1 model projections suggest higher temperatures in future years (2030 – 2036); six years had days when the temperatures were above 16°C, and more than half of the years had temperatures above 17°C, with a maximum temperature of 19.5°C.

## 3.2 Fish trial

No significant differences in growth were observed between any of the groups during the trial period, but at the end of experiment the tendency was a higher growth in fish groups kept at 12°C compared to the 17°C groups (data not shown). No mortality was registered during the trial. While uninfected control fish at both temperatures had very

TABLE 1 List of primers.

Gene name	Accession no.	Forward	Reverse
<i>Elongation factor 1a, EF1a (Housekeeping)</i>	DQ402371.1	CCTTCAACGCCAGGTCAT	AACTGCAGGCGATGTGAG
<i>β-actin (Housekeeping)</i>	AJ555463	ATGGTCGGCATGGCCAGAAGGACAG	TGAGGTCTCGCCGGCCAAGTCCAG
<i>Caspase 6_ apoptosis-related</i>	XM_030352134.1	TTCCCTGGAGTTACAGAGC	GAAGCAAAGCAGGGAATCTG
<i>Tumor necrosis factor_ alpha, TNFα</i>	XM_030371627.1	CACGTGTTCAACCATTACGC	TCTCTCTCCACCATTTTG
<i>Heat shock protein 90, hsp90</i>	XM_030344648.1	CGAGGAGCACTACAACGACA	GTCCTGCTTCTCCTTCATGC
<i>Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing), SIP1</i>	XM_030361652.1	CCGATGTCCTGAAGAGGTGT	TCATGGCTAAGGGGTAGTCG
<i>G-type lysozyme</i>	AY614594	ATAAACTGGAATACGACGGTGAAG	GACTCTACCGTCTCCAGTGTGTAG
<i>Fortilin, tctp</i>	EY971322	CGATGGTCACCAGGTCCGGA	CCGTTCCCAATTTGCTTCA
<i>Interleukin-1β, Il1β</i>	EU007443	GCTTACCACGATCGAGCCGTCACA	AACAGGAAGCGACCATGTCAGTCTC
<i>Interleukin-8, Il8</i>	EU007442	TCCCCATCGGCTCCTACTGGTTCT	GACCCATGGAGCATCAGCATCCAG
<i>Cu/Zn-Superoxide Dismutase, SOD</i>	CO542220	TGAAGGAAACCGGGGACGTTACCG	TCACTCCACAGGCCAGACGACTAC
<i>Catalase</i>	DQ270487	AGCCAAGTTGTTTGAGCACGTTG	AACTTGAATAGACGGGATGGCC
<i>GSH-Px</i>	EB677081	TATCCTGGCGGTGCCCTGCAAC	AATATCAGCTTCGATTTCCGCTG
<i>Heat shock Protein 70, hsp 70</i>	ES478301	TGATTGGACGAGGTTTGATGACGC	ATCTTCAATGGTCAAGATGGACACG

few internal macroscopic changes throughout the trial, increasing macroscopic changes were observed on internal organs in infected fish, specifically granulomas in one or several of the organs; head kidney, spleen, liver and skin (fins), and swollen spleen and head kidney (results not shown). These observations were done on 2/6 sampled fish 14 dpi and on 4/5 sampled fish 52 dpi in the 17°C group, but only on 1/6 fish 52 dpi in the 12°C group. *F. noatunensis* was reisolated from head kidney and spleen in sampled fish from the infected groups at 52 dpi, while all of the sampled fish from the control groups were negative. The infection was also confirmed by qPCR in 2

out of 6 spleens at 14 dpi and in 3 out of 4 spleens 52 dpi in the 17°C group (results not shown).

### 3.3 Development of granulomas in the spleen is temperature dependent

A significantly higher number of granulomas were detected in the spleen from the 17°C group (Figure 3). This temperature-dependent profile was further reproduced in both the number of

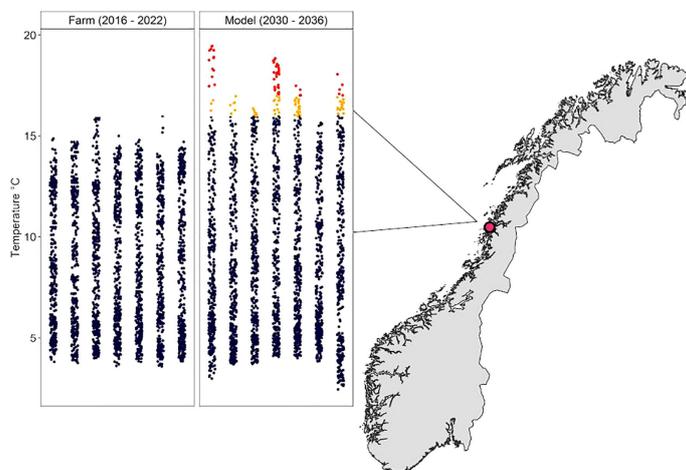


FIGURE 2

Map showing Dønna's location in Norway and the temperatures at the farm 2016 – 2022 and the model projection temperatures 2030 – 2036, temperatures below 16°C in blue, 16–17°C in orange, and above 17°C in red.

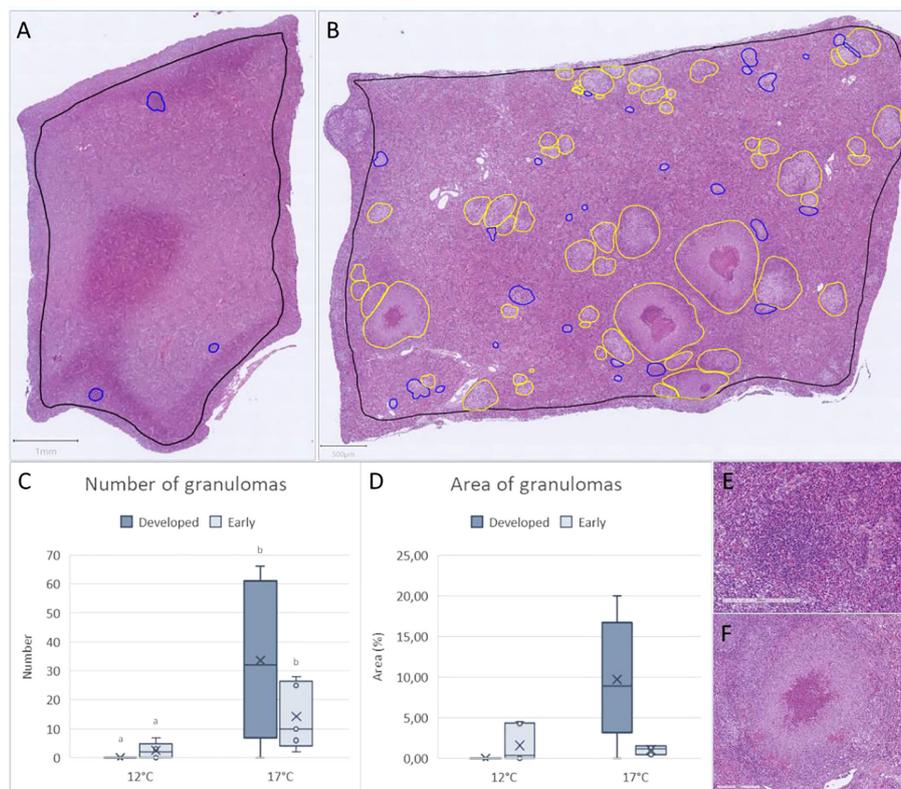


FIGURE 3

The number and area of early (blue) and developed (yellow) granulomas in spleen of (F) noatunensis-infected Atlantic cod. (A) Example from early developing granulomas (scale bar 800µm). (B) Example from a highly affected spleen with several developed and early granulomas (scale bar 500µm). Histological scoring of granulomas in spleen showing (C) Number of granulomas and (D) area of granulomas in spleen from 12°C (n=6) and 17°C (n=5). (E) Example of early and (F) developed granuloma in spleen (scale bar 200 µm).

early and developed granulomas, as well as the area of affected tissue (measured as % of spleen affected).

### 3.4 Temperature and francisellosis have compounding effects on skin

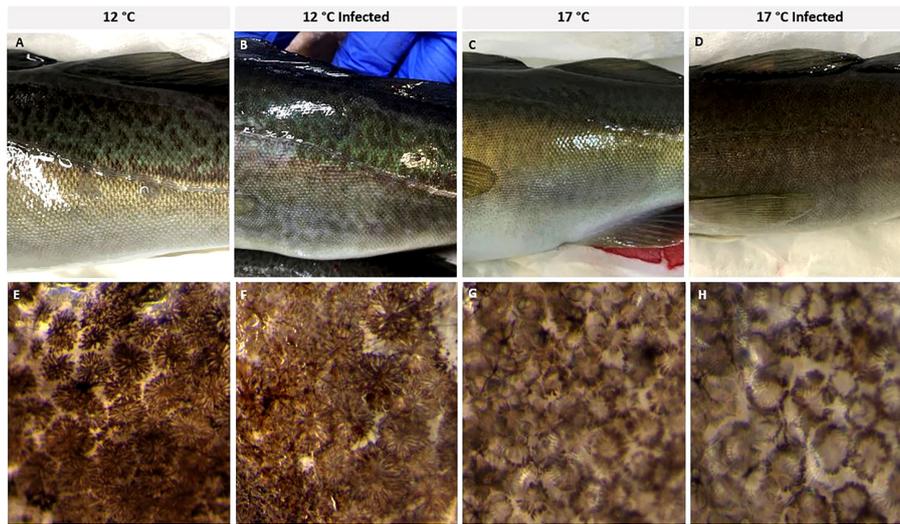
Fish from the 17°C group had a lighter skin color and weaker spots compared to fish from the 12°C group (Figure 4). This could be clearly observed in the whole fish, and the difference became more apparent when evaluating skin biopsies under the stereo loupe. Melanocytes in skin from the 12°C group were darker and denser, while melanocytes from the 17°C group had spindle shaped appearance and weaker color. There were no obvious differences between non-infected and infected fish from the 12°C group, but infected fish from 17°C had darker skin color and higher frequency of melanocytes. Melanocytes in this group were darker and denser compared to non-infected fish from the same temperature regime.

Histological measures (Figure 5) showed increased epidermal thickness in skin from fish from the 12°C uninfected group after 5 weeks compared to fish from 17°C, both in uninfected and infected fish and to infected fish from the 12°C group. Eight weeks into the trial, the 12°C group still had thicker epidermis, and infected fish from

the 17°C group had significantly thinner epidermis compared to 17°C control fish. Numbers of sensory cells were higher in the 17°C group 2 weeks into the trial, but this was changed at week 5, when the 12°C fish had higher numbers of these cells compared to the 17°C group. Sensory cells did not change in the infected fish, but these fish had higher numbers of club cells. Infected fish from the 12°C group showed this pattern already from week 3, infected fish from both temperature regimes had increased club cell number at week 5 and 8. Position of the club cells in the epidermis did not differ between the groups. Histological scoring further showed that the epidermis in fish from the high temperature regime was more damaged compared to the low temperature regime, for both non-infected and infected fish. Spacing of the epidermis was more pronounced in fish from 17°C, and more melanin was observed in infected fish from the same temperature (Figures 5H–J).

### 3.5 High temperature increases the impact of oxidative stress in skin

Skin samples from week 8 exposed to H<sub>2</sub>O<sub>2</sub> showed damages like rougher outermost border of the epidermis, increased spacing of the epidermis, and detachment of the epidermis to the basement membrane. Differences between the two temperature regimes were

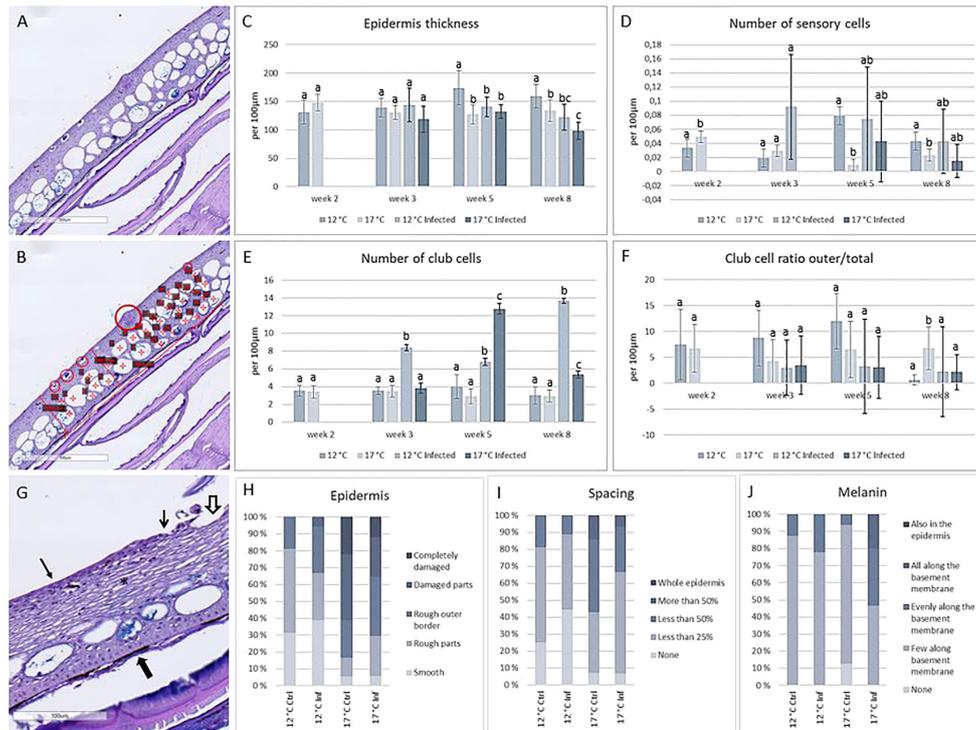


**FIGURE 4**  
Skin appearance of representative specimens from the non-infected and infected fish from 12°C (A, B) and 17°C (C, D) group. (E-H) Loupe images of skin from the same groups showing the different morphology of the melanocytes. N = 6 per treatment.

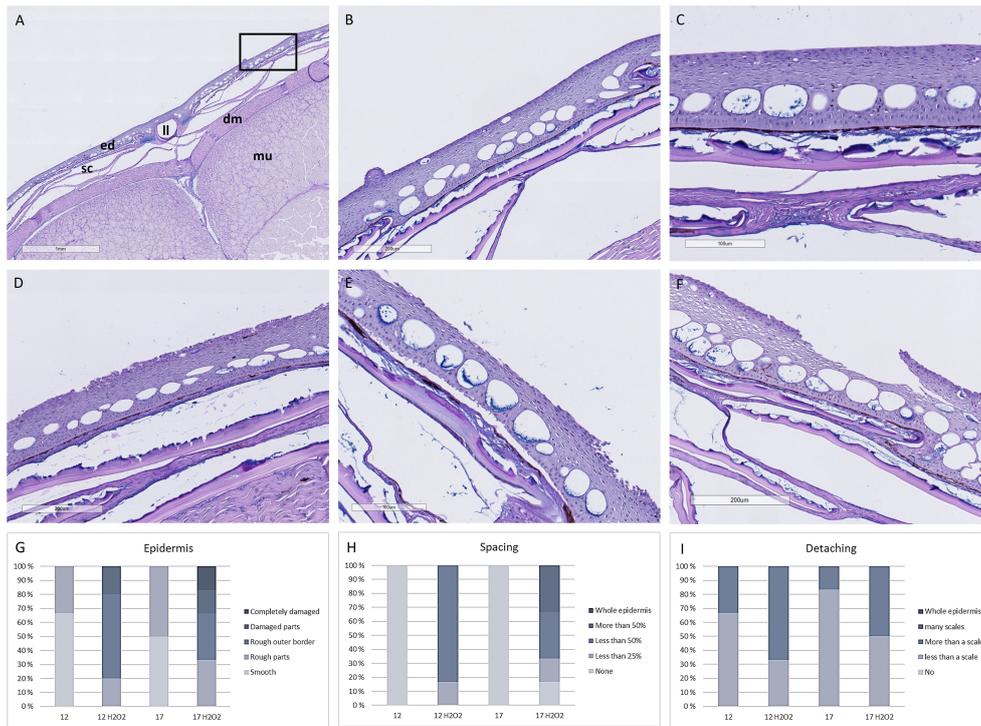
minor, but more severe cases of damage could be found in the high temperature group (Figure 6).

Analysing skin biopsies with SEM mirrored the results of histology (Figure 7). More severe damages to the outermost

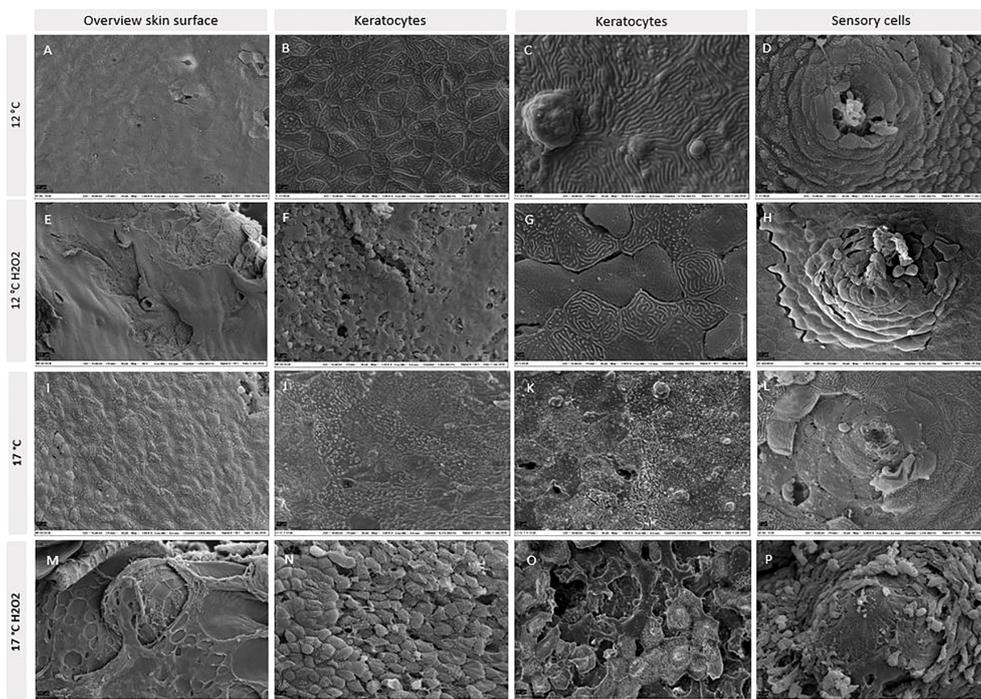
keratocytes, including breaches in the cell layer, dead cells and loss of epidermis were found in the high temperature group and in both groups exposed to H<sub>2</sub>O<sub>2</sub>, with pronounced damages in the high temperature group. In skin samples from the high temperature



**FIGURE 5**  
Histological measurements and scoring of skin. (A) Skin sample from cod, 12°C non-infected. (B) Same sample with measures; large circle: sensory cell, small circles: outer club cells, asterix: club cells, line: epidermal thickness. (C) Thickness of epidermis. (D) Number of sensory cells per 100µm of skin. (E) Number of club cells per 100µm of skin. (F) Club cell ratio outer/total. (G) Histological evaluation of smooth epidermis (arrow), rough epidermis (open arrow), damaged epidermis (hollow arrow), melanin (filled arrow) and spacing (asterix). (H) Epidermis, (I) Spacing and (J) Melanin scoring in the four treatment groups. N = 6 per treatment per timepoint, significant differences (P < 0.05) marked with different letters.



**FIGURE 6** Histological measures of skin biopsies. (A) Overview of skin, (B) Higher magnification of box in A showing smooth outer border of epidermis with a sensory cell (arrow) and (C) No spacing between keratocytes in the epidermis in skin samples from fish from the 12°C group. Representative images of skin with (D) Rough outer border (arrows), (E) Detaching epidermis from the basal membrane (arrows) and damaged epidermis and (F) Damaged epidermis with spacing (arrow). Scoring of N=6 samples from each treatment of (G) Epidermis, (H) Spacing and (I) Detachment from the basal membrane. mu, muscle; sc, scales; dm, dermis; ed, epidermis; ll, lateral line. N = 6 per treatment.



**FIGURE 7** SEM images of skin biopsies from 12°C (A–D), 12°C exposed to H<sub>2</sub>O<sub>2</sub> (E–H), 17°C (I–L) and 17°C exposed to H<sub>2</sub>O<sub>2</sub> (M–P). N = 6 per treatment.

group, areas were found with severe loss of keratocytes, exposing the deeper layers of the epidermis. Damages in the keratocytes lining the sensory cells were also found in exposed groups. With increasing temperature and H<sub>2</sub>O<sub>2</sub> exposure, epidermis got more fragmented, keratocytes lost definition of microridges and detached from the continuous outermost epidermal layer, leaving breaches and exposed areas.

### 3.6 Keratocytes are affected by temperature and oxidative stress

The migration capacity of the keratocytes from scale explants was analysed at week 1 and 8. Differences between the temperature regimes and between the exposed and non-exposed explants were clear. Keratocytes from scales picked from fish at 17°C had higher migration capacity compared to keratocytes from the 12°C group (Figure 8) at days one and three (only at week 8, Figure 8F), but decreased migration at days six and seven, when more scales from the 12°C group had migrating cells. Scales from 12°C showed 55-65%

migration compared to 25-30% at 17°C after a week in culture. Scales exposed to H<sub>2</sub>O<sub>2</sub>, had lower migration rates compared to the unexposed controls, with the 17°C group having migration from less than 10% of the scales after seven days. In comparison, scales from the 12°C group reached almost 70%. The proliferation marker EdU, used to assess the proliferation capacity, showed more positive cells identified in cell sheets from the 12°C group. Immunohistochemistry using antibodies against iNOS (stress marker) showed less activity in scale explants from the 12°C group compared to all other groups.

Observations on scales from the two different temperature regimes were in concordance with the overall skin appearance: Melanocytes in scales from the 12°C group were denser and darker compared to melanocytes in the skin from the 17°C group. After H<sub>2</sub>O<sub>2</sub> exposure, melanocytes from both temperature regimes changed. In the 12°C group, melanocytes appeared spindle-shaped with a weaker colour, while melanocytes from the 17°C group were fragmented and nearly disappeared from the scale explants (Figure 9).

The expression of selected genes in skin biopsies, which were further exposed to H<sub>2</sub>O<sub>2</sub>, was determined in the two temperature

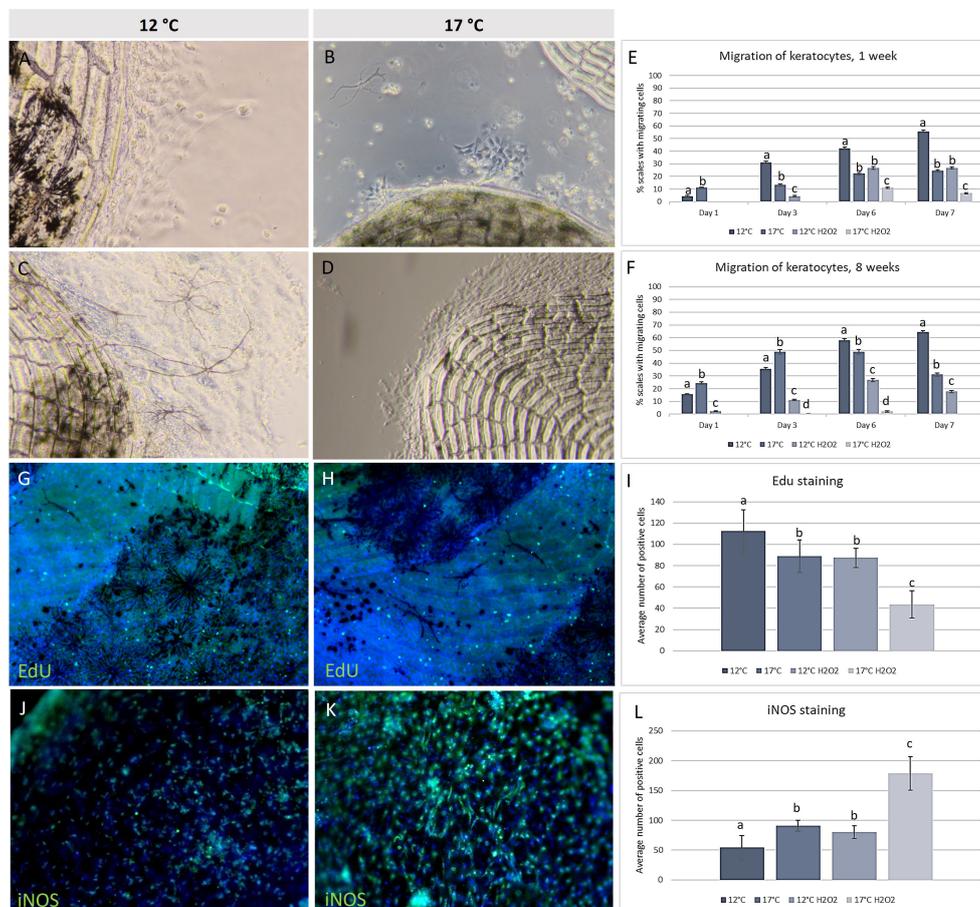
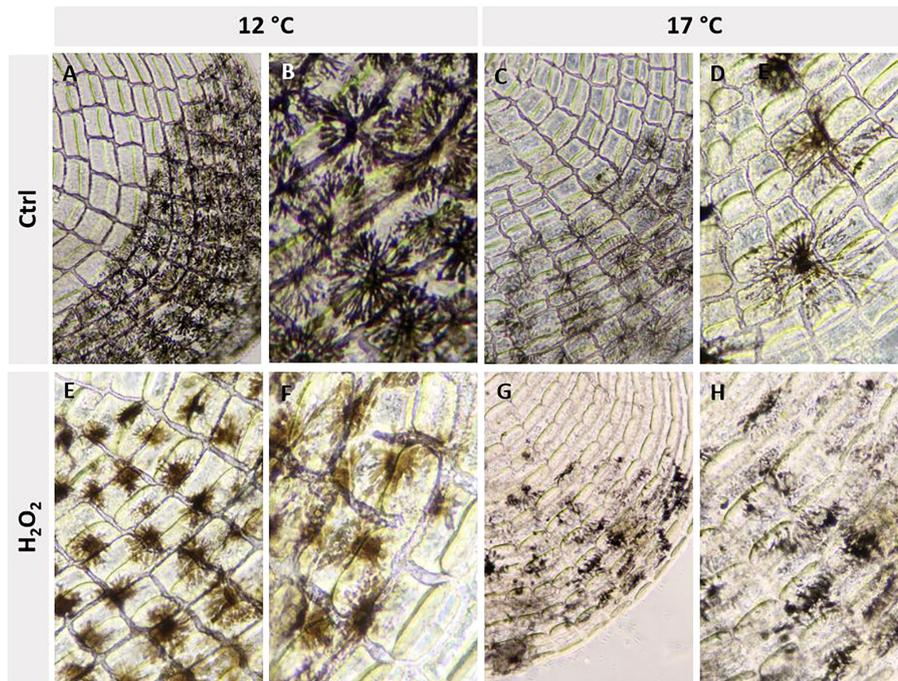


FIGURE 8

*In vitro* keratocyte cell culture. Migrating keratocytes from scales at (A) 12°C, (B) 17°C and same temperature after H<sub>2</sub>O<sub>2</sub> exposure (C, D). Migration capacity showing % of scales with migrating cells, at (E) week 1 and (F) week 8. Cells were stained with EdU and showed higher proliferations rate at (G) 12°C compared to (H) 17°C, as measured (I). IHC with iNOS showed weaker staining at (J) 12°C compared to (K) 17°C, as measured (L). Different letters indicate significance ( $P \leq 0.05$ ). Scale bar = 200  $\mu$ m. Green color = positive EdU/iNOS, blue color = Hoechst. N = 5 scales per well, 3 wells per fish, 5 fish per treatment, significant differences ( $P < 0.05$ ) marked with different letters.



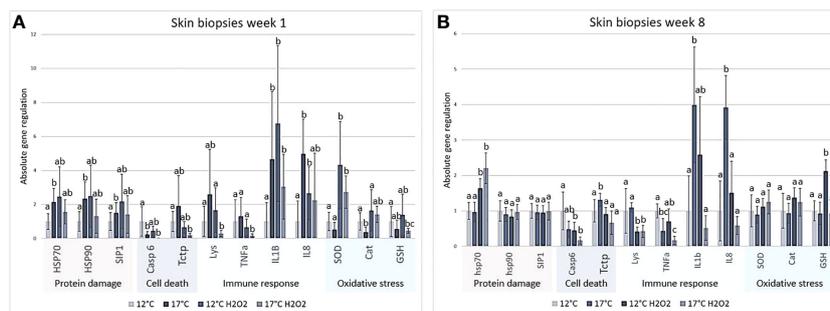
**FIGURE 9**  
The melanocytes had different morphology at 12°C (A, B) and 17°C (C, D), and after exposure to H<sub>2</sub>O<sub>2</sub> (E–H).

groups at weeks 1 and 8. RT qPCR analysis (Figure 10) included proinflammatory cytokines (*tnfα*, *il1β* and *il8*) and antibacterial agent *lysozyme*, gene markers of responses to protein damage (*hsp70*, *hsp90* and *sip1*) and oxidative stress (*sod*, *catalase* and *gsh*). *Caspase 6* and *fortilin* are involved in variety of cellular processes including apoptosis and inflammation. At week 1, *il1β* and *il8* were stimulated with the increase temperature, exposure to H<sub>2</sub>O<sub>2</sub> and their combination. *Tnfα* and *lysozyme* were suppressed by joint treatment as well as *caspase 6* and *fortilin*. The markers of protein damage showed a tendency to upregulation though significant difference from 12°C was found only in 17°C group. The expression profiles of enzymes involved in oxidative stress response were different. *Catalase* and *gsh* (not significant) were downregulated in respectively 17°C and H<sub>2</sub>O<sub>2</sub> 17°C groups, while *sod* expression was

stimulated with H<sub>2</sub>O<sub>2</sub> at both temperatures. Analysis performed at week 8 indicated reduced activation of several genes. Four genes did not show any difference between the groups (*hsp90*, *sip1*, *sod* and *catalase*). High temperature stimulated, *tctp*, *il1β* and *il8*, while *tnfα* was downregulated. H<sub>2</sub>O<sub>2</sub> exposure led to increased transcription of *hsp70* and *gsh* (only at 12°C) and reduced transcription of *caspase 6* and *lysozyme* at both temperatures, and *tnfα* and *il1β* at 17°C.

### 3.7 Microarrays

Microarray analyses (GEO Accession viewer (nih.gov)) were performed on skin and spleen samples from the non-infected and infected fish at two temperatures, at weeks one and eight



**FIGURE 10**  
Real time qPCR of skin biopsies exposed to H<sub>2</sub>O<sub>2</sub> from (A) 1 week and (B) 8 weeks. Relative gene expression is shown as mean ± std, different letters indicate significance (P ≤ 0,05), N = 6 per treatment per timepoint. Results were analysed using REST (Pfaffl, 2001; Pfaffl et al., 2004), significant differences (P < 0.05) marked with different letters.

(Figures 11A–C). Judging by the number of DEG, effect of temperature was equal at both time-points in skin and increased with time in the spleen. The gene expression differences between the temperature regimes markedly decreased for both tissues at high temperature. Responses to challenge with *Francisella* were markedly greater at 17°C in both tissues. At week 1, the effect of high temperature was apparently larger in the spleen as shown by the substantially higher number of DEGs compared with the skin. Several functional groups of genes showed co-ordinated expression changes (Figure 11D). Genes involved in cell cycle were downregulated in both tissues. Their number was twice higher in skin that also showed decreased expression of genes encoding chromosomal proteins.

Effects of temperature alone and the interaction of temperature and infection status at individual gene levels was further evaluated. Eleven genes responded to temperature in both tissues including *heat shock protein 47*, *heat shock protein 90*, *cold inducible RNA binding protein*, and *metallothionein* (Figure 12). *Heat shock proteins* and *metallothionein* coding genes were upregulated while the other genes were downregulated. Protein stress was indicated by upregulation of eight chaperones in addition to two chaperones presented in Figure 12A. Putative suppression of cell proliferation in the skin could be to some extent compensated by stimulation of genes that control the development of tissue, such as genes encoding *connective tissue growth factor*, enzymes modifying extracellular components and regulators of differentiation (Figure 12B). High temperature also affected genes associated with histogenesis of the spleen (Figure 12C). In contrast with the skin, downregulation prevailed, and most DEG encoded the components of extracellular matrix including the proteins of cartilage and bone.

Elevated water temperature could affect the full range of immune functions including pathogen detection, signalling via chemokines, cytokines and lipid mediators, antigen presentation, humoral and cellular effectors, lectins, lymphocyte-specific genes,

and regulators of various pathways; these effects were seen at both time-points (Figure 13). Of interest are *toll receptor 8* and *nilt1* that also can be involved in the detection of pathogens (Stet et al., 2005). The greatest 76.6-fold increase was seen in *il-2 receptor* in spleen at week 1. The expression of several immunoglobulins transcripts (*ig*, highlighted with bold blue in Figure 13) showed a decrease in both tissues at week 1 and accompanied by a massive drop in the spleen at week 8: 21 features with average 2.65-fold decrease.

We then evaluated the effects of *F. noatunensis* infection at two different temperatures. Six weeks following challenge with *Francisella* gene expression changes were minor at 12°C, especially in the skin (data not shown). Thereby we focused the assessment on the groups reared at 17°C. Stimulation of blood circulation indicated by the 8.1-fold increase of eight hemoglobin transcripts in the skin and chemokine signaling in the spleen was in parallel with the downregulation of cell cycle proteins and *collagens* in these tissues (Figure 14A). A small number of immune genes was downregulated including *tlr9*, a *lectin*, extracellular proteases (*chymase* and *three granzyme-like genes*), and a protease inhibitor (*tnfa-induced protein 8*) in the spleen (Figure 14B), four genes encoding *arachidonate 12-lipoxygenases*, the enzymes of eicosanoid metabolism, *protease htra1b* and two *lectins* in the skin (Figure 14C). However, immune stimulation prevailed in both tissues. In the spleen, the upregulated pathogen recognition receptors were represented with *nilt1*, *tlr8* and *tlr21*. Signalling was mediated with a suite of *chemokines* and *il1b* known as a highly active proinflammatory cytokine. Activation of antigen presentation was suggested by several *mhci* components, and the antigen transporter *tap*. The effector arm included *complement factors*, multiple *lectins* that may bind to pathogens and promote leukocyte traffic, and *b-245*, a component of oxidative burst; *arginase ii* is characterized with strong responses to bacterial infections in teleosts (Krasnov et al., 2021). *Lectins*, *complement*

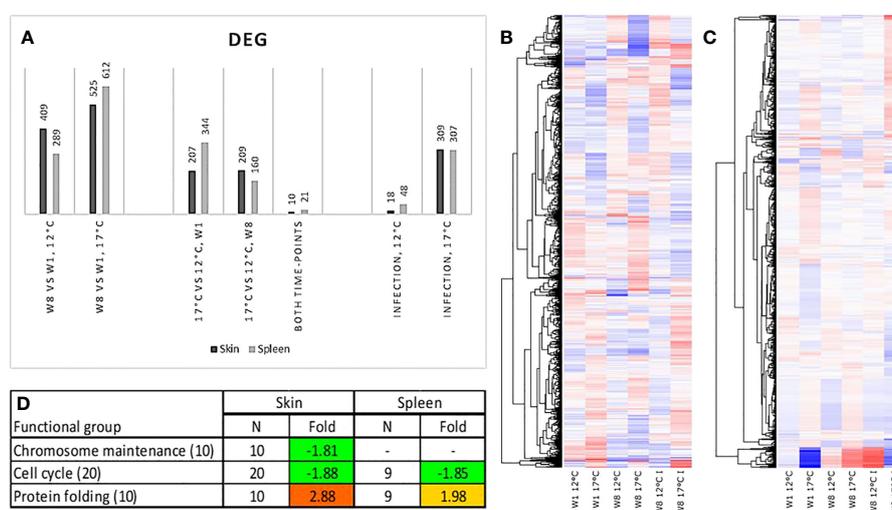


FIGURE 11

(A) Number of differentially expressed genes (DEGs). Heat maps of gene expression in (B) skin and (C) spleen. Genes with expression differences between the time-points, temperatures or infected and control fish were clustered with K means. Data are log<sub>2</sub> – expression ratios to the means of all samples (D) Functional groups with co-ordinated expression changes at week 1, differences from 12°C are significant, data are folds and differential expression is highlighted with underlined bold italics.

Gene	Skin		Spleen	
	Week 1	Week 1	Week 1	Week 8
Cold inducible RNA binding protein	<u>-2.58</u>	<u>-2.69</u>	<u>-3.10</u>	
Heat shock protein 47	<u>8.67</u>	<u>6.81</u>	<u>5.51</u>	
Heat shock protein 90	<u>2.46</u>	<u>3.44</u>	<u>2.10</u>	
Metallothionein	<u>-0.43</u>	<u>-0.49</u>	<u>-0.45</u>	
FK506 binding protein 9	<u>-2.58</u>	<u>-2.58</u>	<u>-2.31</u>	
Angiopoietin-related protein 5	<u>-1.63</u>	<u>-2.59</u>	<u>-2.55</u>	
Peregrin	<u>-2.05</u>	<u>-2.13</u>		-1.65
Roundabout homolog 2	<u>-2.19</u>	<u>-2.36</u>		-1.48

Gene	Fold
<b>Spleen</b>	
Wilms tumor protein	<u>-1.80</u>
SPARC-related modular calcium-binding	<u>-1.74</u>
Secreted frizzled-related protein 4	<u>-1.79</u>
Mucosal protein	<u>-2.90</u>
Papilin b, proteoglycan-like sulfated glycoprotein	<u>2.52</u>
Collagen_type I_alpha 3	<u>-3.29</u>
Collagen type II, alpha-1a	<u>-2.49</u>
Collagen alpha-2(I) chain	<u>-2.50</u>
Laminin, beta 2 (laminin 5)	<u>-1.75</u>
Collagen type XI alpha-2	<u>2.27</u>
Collagen, type XIV, alpha 1a	<u>-1.78</u>
Chondroadherin-like protein	<u>-1.88</u>
Osteomodulin	<u>-2.49</u>
Fibronectin 1	<u>-2.63</u>
Otoraplin	<u>-1.95</u>
Osteoglycin	<u>-2.78</u>

FIGURE 12 Genes responsive to temperature changes in the skin and spleen of Atlantic cod. (A) Genes responsive to elevated temperature in both organs, (B, C) Individual genes with tissue-specific expression changes, week 1. Data are folds and differential expression is highlighted with underlined bold italics.

factors and proteases were activated in the skin. *Francisella* also induced a panel of immune regulators including *imap gtpases*. Their role is unknown, but salmon homologs show very high immune activity. Increased expression was observed in *pu.1*, *skap2* and *samsn1a*, which may control the development and activity of B cells. The immune responses were supplemented with stimulation of apoptosis, autophagy, and detoxification. A panel of *caspases* and *dram1*, a gene marker of autophagy was upregulated in the spleen. Moreover, xenobiotic metabolism was represented with three enzymes upregulated in skin. Stimulation of *metallothionein*

points to possible sequestration of zinc that could reduce its availability for the pathogen.

### 4 Discussion

There is an urgent need to understand how climate change will impact aquaculture farming conditions so that the industry can develop appropriate responses and protective measures in advance. To study realistic scenarios and assess risks, local farm

Gene	Fold
<b>Skin</b>	
Prostaglandin D2 synthase b	<u>2.85</u>
TNF receptor superfamily member (2genes)	<u>2.17</u>
Natterin-like protein (2 genes)	<u>-3.75</u>
C-reactive protein 2	<u>1.94</u>
MHCI UDA gene	<u>-2.48</u>
Complement component 1qC	<u>-1.90</u>
Ig light chain (4 genes)	<u>-3.32</u>
Granzyme K	<u>2.13</u>
<b>Spleen</b>	
Putative NILT1 leukocyte receptor	<u>2.17</u>
Toll-like receptor 22 (3 genes)	<u>1.79</u>
15-hydroxyprostaglandin dehydrogenase (2 genes)	<u>-1.80</u>
Chemokine CCL-C17a (2genes)	<u>2.14</u>
C-C chemokine receptor type 6 )	<u>-2.26</u>
Interleukin 2 receptor beta	<u>76.41</u>
Interleukin 4 receptor alpha	<u>1.78</u>
Ifitm3	<u>3.97</u>
Interferon stimulated gene 15-3	<u>-2.05</u>
CD209	<u>1.83</u>
Complement factor D )	<u>-4.02</u>
Arginase 2	<u>2.01</u>
C-type lectin domain family 4 member A	<u>-36.47</u>
Mannan-binding lectin	<u>1.75</u>
72 kDa type IV collagenase	<u>-2.76</u>
Lymphocyte antigen 75 precursor	<u>1.87</u>
Ig light chain (4 genes)	<u>-2.36</u>
T-cell surface antigen CD2	<u>1.85</u>

Gene	Fold
<b>Skin</b>	
Toll-like_receptor_8	<u>1.76</u>
C-C motif chemokine 4	<u>-1.76</u>
Interleukin 17D	<u>-2.01</u>
TNF receptor superfamily member	<u>-2.08</u>
TNF receptor superfamily member 14	<u>1.76</u>
MHC class Ia antigen	<u>-3.43</u>
Macrophage expressed 1	<u>2.06</u>
Complement factor H-like 3	<u>-1.83</u>
Complement component 1q4	<u>2.69</u>
C-src tyrosine kinase	<u>-2.00</u>
CCAAT/enhancer-binding protein delta	<u>1.81</u>
Selectin E	<u>-1.76</u>
Granzyme A	<u>1.97</u>
<b>Skpleen</b>	
Toll-like receptor 9	<u>-2.05</u>
Prostaglandin D2 synthase b	<u>3.26</u>
Interleukin-1 beta - 2	<u>1.92</u>
Interleukin_1_beta - 1	<u>-1.88</u>
TNF receptor superfamily member	<u>-2.29</u>
MHC class I (2 genes)	<u>-2.00</u>
C-type lectin - Ident 63	<u>12.72</u>
C-type lectin domain family 4 member A	<u>7.33</u>
C-type lectin domain family 9 member A	<u>-2.26</u>
Mannose receptor C1-like protein	<u>1.86</u>
Novel immune-type receptor 3.9	<u>-2.05</u>
Ig light chain (21 genes)	<u>-2.63</u>
T-cell leukemia homeobox protein	<u>-1.82</u>
T cell receptor	<u>3.14</u>

FIGURE 13 Selected immune-related genes affected by elevated temperature at week 1 (A) and 8 (B), folds to control. If more than one paralog was affected (blue), the number of genes is indicated in parentheses. All differences from control are significant.

A				B	
Functional group	N	Fold	Tissue	Gene	Fold
Chemokines	5	4.80	Spleen	Putative NILT1 leukocyte receptor	2.39
Erythrocyte, globin	8	8.09	Skin	Toll-like receptor 21	1.88
Cell cycle	6	-1.99	Skin	Toll-like_receptor 8	1.89
Tissue ECM mucus	5	-3.17	Skin	Toll-like receptor 9	-1.77
Tissue ECM collagen	7	-1.99	Spleen	Chemokine receptor	2.23
C				MHC class I antigen (2 genes)	1.96
Gene				Fold	
Chemokine CXCL-C1c				2.66	
Cytokine-like protein 1				2.90	
Interleukin_17D				2.00	
Arachidonate 12-lipoxygenase (4 genes)				-1.94	
Properdin				4.80	
Immune-related lectin-like receptor 4				1.83	
C-type lectin domain family 4 member A				-2.47	
C-type lectin				-2.22	
Tetranectin				1.84	
Chymotrypsin (2 genes)				1.88	
Serine protease HTRA1B				-1.82	
Proprotein convertase subtilisin/kexin type 6				2.73	
GTPase IMAP family member 2 (2genes)				2.06	
Mitogen-activated protein kinase kinase kinase 8				2.06	
TNF receptor superfamily member				1.76	
T-cell-originated protein kinase				-1.76	
DNA damage-inducible transcript 4				3.66	
Sulfotransferase family 5A, member 1				1.94	
Alcohol dehydrogenase 1				2.70	
Alcohol dehydrogenase 8b				2.19	
Metallothionein				2.01	
Connective tissue growth factor				3.57	
				MHCI UDA gene	1.89
				Antigen transporting protein	1.88
				Complement C1qC	2.07
				Complement factor H-like 3 (2 genes)	2.35
				Properdin	3.29
				Arginase 2	2.39
				Granzyme (3 genes)	-3.62
				Chymase	-3.38
				Trypsin domain	13.20
				C-type lectin domain family 4 member A (2 genes)	2.50
				C-type lectin domain family 17, member A	-2.88
				Cytochrome b-245 light chain	1.98
				TNF alpha-induced protein 8	-1.94
				Transcription factor PU.1	1.93
				Igvl5 protein	1.84
				Src kinase-associated phosphoprotein 2, skap2	1.81
				SAM SH3 domain, nuclear loc-n signals, 1b, samsn1a	1.83
				CD276 antigen	1.91
				Myeloid-associated differentiation marker	-2.29
				DNA damage-regulated autophagy modulator 1, dram1	2.71
				Caspase 3 (3 genes)	2.00
				Caspase 6	2.25

FIGURE 14 Responses in the spleen and gills to challenge with *F. noatunensis* at 17°C. Samples were collected 6 weeks after experimental infection. (A) Functional groups of genes with co-ordinated expression changes. (B, C) Genes with differential expression in spleen (B) and skin (C), folds from uninfected control. All differences from control are significant.

conditions should be considered, especially when high temperatures will act in combination with secondary stressors and pathogens. Given the complexity of the interactions, multifaceted studies are required. We report a step in this direction that included profiling of climate projections at a Norwegian cod farm site, *in vivo* and *in vitro* trials, and powerful analytic methods to understand how high environmental temperatures and secondary stress affect the performance of Atlantic cod. We included *in vivo* *F. noatunensis* infection to evaluate the impact of temperature stress in combination with a pathogen, and *in vitro* exposure using H<sub>2</sub>O<sub>2</sub> as an oxidative stressor, to simulate production related stress. The analytical approach focused at finding biological significance of increasing temperatures combined with secondary stressors, including general performance, skin barrier responses, pathological changes in the spleen and transcriptomics, to understand risk factors associated with future temperatures.

### 4.1 Near future temperatures will increase beyond cods preferred temperatures

The future scenario from the IPCC (SSP2-4.5, intermediate emission scenario) downscaled to local conditions, showed that temperatures potentially will increase from maximum temperatures around 15°C in 2016 – 2022, to above 17°C in 2030- 2036. As 15°C are considered high for cod, reducing the health and welfare of the fish (Hori et al., 2013; Larsen et al., 2018), these temperatures will

challenge productive farming. The site we included in this work was located in North Norway. Most of the commercial cod farming takes place further North, but new locations further South (in the Bergen area) were recently suggested by the Norwegian Directorate of Fisheries. According to The Special Report on the Ocean and Cryosphere in a Changing Climate (IPCC, 2019), there is high confidence in the IPCC ocean climate projections and that climate-induced stressors on aquatic ecosystems present risks for commercial fisheries and aquaculture, with implications for regional economies, cultures, and the global supply of fish. From the modelling of future temperatures at the cod farm site, 17°C is far from an unrealistic scenario, although we are aware that applying a multiple of climate change scenarios with a global multi-model ensemble and several dynamical and statistical down-scaling to farm site level would increase the confidence of the projected temperature ranges. Also, this study used climate model data that had been averaged to 5-days intervals, which may mask more extreme temperatures and days with high temperatures. Challenges with temperature have also been reported in other countries that have tried to farm cod, such as Canada. In Newfoundland waters, cod can be exposed to temperatures of up to 20°C (6 m) and short-term (daily/weekly) temperature fluctuations of as much as 10°C during the summer months (Gollock et al., 2006). These large temperature fluctuations and high summer temperatures, as well as health challenges due to sudden temperature changes and long periods of temperature outwith optimal conditions, were some of the technical challenges that ended Canadian attempts at commercial farming of cod

(Nardiet al., 2021). From our study it is obvious that temperatures at 17°C are too high for cod, as also previously suggested by other studies (Pérez-Casanova et al., 2008a; Larsen et al., 2018). This study focused on conditions in the near-future, and were from an intermediate emission scenario, but even higher temperatures may be expected in the longer term, or with higher emissions scenarios such as SSP5-8.5 (IPCC, 2019/ IPCC, 2023). Other cod aquaculture locations will also be affected by increasing temperatures, thus, precautions and adaptation measures are needed to sustain current farming practice in the future, as well as increased efforts to reduce the sectors own emissions. Mean ocean temperature increase may lead to further decline, and in worst case to a collapse, of several coastal cod stocks (Drinkwater, 2005), thus adaptation of aquaculture to climate change may be of even greater importance.

## 4.2 Cod farmed at high temperature exhibit distinct stress response profiles

Atlantic cod has a low tolerance to temperature change (Lannig et al., 2004), and like other ectotherms, its physiology is directly influenced by the seawater temperature (McCormick, 2011). During our 8 weeks *in vivo* infection trial, significant differences in growth were not detected between groups. However, the individual variance in weight was relatively large at start of the trial and the weight and length data were only registered from a small number of fish (N=6) in each group at each of the sampling points. Nevertheless, there was a tendency that fish in both of the 17°C groups had a lower weight gain from start till end of the trial compared to the 15°C groups. Growth rate of Atlantic cod was not affected by disease nor temperature after infection with *B. pinnipedialis* at 15°C either, and findings were explained by multiple energy demanding processes taking place at the same time (Larsen et al., 2018). Studies including multiple stressors, e.g., high temperature and infection, may highlight cumulative stress responses and are especially important when studying biological impact of climate related stressors. Simultaneous stressors may lower the cod's thermal tolerance, as previously shown for other species, and several studies have linked increasing seawater temperatures to increased outbreaks of marine diseases (reviewed in Burge et al., 2014). We did not observe any mortality in any of the groups, but our results clearly showed that fish are more vulnerable to both oxidative stress and francisellosis at high temperature. The latter is in line with observations from the field and other experimental studies of francisellosis in cod, showing faster and more comprehensive disease development with increasing temperatures (reviewed in Puvanendran et al., 2022).

Obvious differences in skin coloration were observed between fish from the two temperature regimes, with the characteristic spotted cod skin pattern more or less lost at 17°C. In both Atlantic salmon (*Salmo salar*) and Atlantic mackerel (*Scomber scombrus*), changed skin color has been observed after exposure to stress (Thorsen, 2019; Tveit et al., 2022). In this trial, melanocytes in high temperature exposed skin had spindle shaped appearance and appeared weaker, probably causing the overall weaker impression of color in these fish. Further evaluating skin from the different temperature regimes showed reduced thickness of the

epidermis, reduced number of sensory cells and increased number of club cells in the outer border of the epidermis of fish exposed to high temperature. Similar alterations in skin have previously been connected with increased stress response in Atlantic cod (Ytteborg et al., 2020), as well as in lumpfish (*Cyclopterus lumpus*) and salmon (Karlsen et al., 2021; Ytteborg et al., 2023). Interestingly, severe morphological changes in skin were not observed until the end of the trial, at week 8, indicating long term effects of 17°C and highlighting the importance of running temperature exposure trials for longer periods. Based on these findings, a sudden reduction in the coloration of the skin and weaker spots may be considered as an indicator of temperature related stress in farmed cod, and farmers should pay special attention to these changes when entering warmer periods.

There were clear signs that the fish were under stress at elevated temperatures as shown by key stress-related genes in the core gene set responsive to temperature in both skin and spleen. Heat shock proteins (hsps), also known as stress proteins and extrinsic chaperones, are a family of highly conserved proteins of varying molecular weight (ca. 16-100 kDa) produced in all cellular organisms when exposed to a stress condition, thus often considered classic markers of heat stress in fish (Roberts et al., 2010). They function as key regulatory molecules for refolding of misfolded proteins, thereby contributing to the maintenance of cellular homeostasis (Shan et al., 2020). Here we identified *hsp47* and *hsp90* as highly responsive to changes in temperature in both organs. The results corroborated an earlier study in Atlantic cod exposed to a heat challenge where the expression of these genes was shown to be upregulated in liver, head kidney and skeletal muscle (Hori et al., 2010). Hsp47 (serpin h1), which is essential for collagen processing in endoplasmic reticulum under normal condition, prevents secretion of damaged procollagens with abnormal conformation from stressed cells and its induction by heat stress is regulated by a heat shock element in its promoter region (Nagata, 1996; Ishida and Nagata, 2011). On the other hand, Hsp90 and its co-chaperones orchestrate crucial physiological processes such as cell survival, cell cycle control, hormone signaling, and apoptosis, and utilizes its ATPase activity to drive conformational changes to facilitate client protein binding, stabilization, and activation (Hoter et al., 2018; Chen et al., 2020). Regulations of these hsps and several other chaperone protein coding genes identified allow Atlantic cod to master adaptive responses to the physiological demands of the heat stress. Moreover, responsiveness of these genes to temperature in several tissues indicates their ubiquitous role during heat stress, and perhaps, they can be considered as key biomarkers for climate-related heat stress response in cod. This function has been reported in different fish species and thus can be considered a cross-species marker for climate-related stress (Lele et al., 1997; Stefanovic et al., 2016; Wang et al., 2016). We also identified another potential biomarker, *cold-inducible RNA-binding protein (cirp)*, an intracellular stress-response protein that affects expression of many genes by regulating mRNA stability in response to various stressors (reviewed in (Zhong et al., 2021)). CIRBP encodes an 18-kD protein consisting of an N-terminal RNA recognition motif (RRM) and a C-terminal arginine-rich region and initially associated with cold-shock, but recent evidence demonstrates

hypoxia, UV radiation, glucose deprivation, and heat stress (Zhong and Huang, 2017). Though the functional role of this has not yet been elucidated in cod, upregulation of CIRP protein was observed in skin mucus of Atlantic cod infected with *Vibrio anguillarum* indicating its role mucosal immunity (Rajan et al., 2013). The identification of molecular biomarkers will facilitate a better understanding of how Atlantic cod responds and behaves to heat stress and the current study have identified potential candidates that can be further verified, especially their reproducibility under different thermal scenarios.

### 4.3 High temperature alters the immune response to francisellosis

The interaction of immune system and temperature has long been identified, but discussion in fish has recently received significant attention in light of the threats from elevated temperatures associated with climate change. It is known that when temperatures increase and reach the upper permissive range, teleost immunity is compromised thereby making them vulnerable to challenges and stressors (Cabillon and Lazado, 2019; Scharsack and Franke, 2022). The spleen is regarded as the primordial secondary lymphoid organ while the presence of mucosa-associated lymphoid tissue in the skin highlights its immunological features in fish (Björge and Koppang, 2021). Both of these organs mount immune responses to various environmental stressors, including temperature (Hori et al., 2013; Scharsack and Franke, 2022). Here we showed that elevated temperature affected the immunological functions of both organs as shown by the changes in an array of immune-related genes and the responses were both acute (week 1) and persistent (week 8), though the magnitude of responses were more pronounced in the spleen. Because immune genes showed both up and downregulation, it is difficult to judge the character and consequences of high temperature for Atlantic cod immunity. Nonetheless, some of the genes provide insights into the immunological impacts of elevated temperature in cod. Lectins mediate pathogen recognition via the carbohydrate recognition domain that specifically and reversibly binds to a carbohydrate which is widely distributed in bacteria, fungi and viruses, thereby activating the innate immune system (Elumalai et al., 2019). The present study identified that splenic lectins were heavily influenced by elevated temperature, particularly, *c-type lectin domain family 4 member A*. There was a dramatic downregulation at week 1 and upregulation was observed at week 8. In trout, *c-type lectin domain family 4* is expressed in monocytes/macrophages (Johansson et al., 2016). It remains to be functionally verified the relevance of this striking trend, but downregulation at week 1 might indicate suppressed macrophage-mediated activity but likely compensated by other immune molecules that have been activated. Immunoglobulins (Igs) are highly specialized glycoproteins that recognize a multitude of antigens from bacteria, viruses, and other disease-causing organisms and recruit other cells and molecules to destroy these pathogens (Salinas et al., 2011). Decreased abundance of *immunoglobulins* transcripts at 17°C was observed already after

one week and turned into a massive drop at the eighth week. Serum antibodies were not analyzed in this study, however, Magnadóttir and coauthors (Magnadóttir et al., 1999) did not observe decrease of antibodies in cod acclimated to high water temperatures. Apparent downregulation of immunoglobulins can be due to massive evasion of B cells from the lymphatic organs, especially in an infected fish. It is likely that recruitment of lymphocyte left spleen unprotected. The duration of the trial was sufficient for the formation of acquired immunity. Consequently, specific antibodies can participate in protection along with innate heterologous antibodies, which are always abundant in fish, including Atlantic cod.

As in previously published studies, high temperature caused complex changes in expression of immune genes without a trend towards overall stimulation or suppression. *Francisella* infection induced significant changes in gene expression at 17°C with a predominance of upregulation, while responses were minor at lower temperature. This result would be difficult, if possible, to interpret without knowledge of the pathology. A stronger immune response can be either evidence of active defense or a consequence of a more severe course of disease. Given the greater number and area of splenic granulomas at high temperature, it is evident that transcriptome changes, which were likely linked to the immune responses, did not protect cod. In challenge trials, there is usually stronger stimulation of immune genes in fish with higher pathogen levels. More serious damage could develop as a consequence of active proliferation of bacteria at a higher temperature. Increased mortality despite stronger immune response was observed in Atlantic cod infected with *B. pinnipedialis* (Larsen et al., 2018). The authors attributed this finding to the trade-off between rapid growth at high temperature which in theory could divert resources from immune defense. Some of the striking transcriptomic changes in the skin and spleen, despite of lesser magnitude as expected for an infection challenge, mirrored previous observations in *F. noatunensis* infected cod where the overall classic innate immune response was demonstrated such as upregulation of genes related to inflammation, acute-phase proteins and cell recruitment (Solbakken et al., 2019). Inflammation was activated through an array of pro-inflammatory chemokines and cytokines.

### 4.4 The skins barrier function is reduced at high temperature

Unlike the salmon industry, which uses H<sub>2</sub>O<sub>2</sub> to treat against salmon lice, this agent is not common in cod farming. However, H<sub>2</sub>O<sub>2</sub> was applied in this trial as a chemical inducer of oxidative stress in the same way as in similar *in vitro* exposure studies (Karlsen et al., 2021; Ytteborg et al., 2023). *In vitro* migration potential of keratocytes offers insight into the skins wound healing capacity, as migration of keratocytes is the first step in the healing process of skin damages (Sveen et al., 2020). From scale explants, a reduced migration capacity was observed at 17°C, with lowest migration found in scales exposed to H<sub>2</sub>O<sub>2</sub> from this temperature regime. It is interesting that changes were induced already after 1 week at high temperature, and that the changes seemed to worsen throughout the fish trial. Skin samples from the 17°C temperature regime further had thinner epidermis and

fewer sensory cells compared to samples from 12°C. SEM analyses showed superficial alterations like detaching keratocytes and breaches in the continuous keratocytes layer at increased temperature, a morphology that expanded after exposure to H<sub>2</sub>O<sub>2</sub>. With increasing temperature and H<sub>2</sub>O<sub>2</sub> exposure, epidermis got more fragmented, keratocytes lost their defined structures of microridges, and detached from the continuous outermost epidermal layer, leaving breaches and exposed areas. Similar changes have already been shown after exposure to oxidative stress at low temperatures (Ytteborg et al., 2020), but it is of interest that elevated temperature worsened the effect of oxidative stress. The microridges have several functions in fish skin, i.e. enlarge the cell surface for gas exchange, serve as support to hold mucous coating, provide mechanical strength and support sensory cells (Henrikson and Matolsky, 1968; Olson and Fromm, 1973; Fishelson, 1984), thus damaged microridges may negatively affect important skin functions. In addition, elevated temperature and H<sub>2</sub>O<sub>2</sub> exposure further altered the morphology and colour of the melanocytes in skin. Fragmented and spindle shaped melanocytes were observed at both treatments, and more severe when temperature and H<sub>2</sub>O<sub>2</sub> exposure were combined. Similar observations were seen in lumpfish after exposure to stress (Ytteborg et al., 2023) and in salmon after the operational procedures crowding and netting (Thorsen, 2019). Melanocytes in skin may function as antioxidants (Hardeland, 2005; Galano et al., 2013), and reduced function caused by stress may thus reduce the robustness of skin as a protective barrier in fish. Analysing gene transcription from non-exposed and H<sub>2</sub>O<sub>2</sub> exposed biopsies from both temperatures showed different transcription of proinflammatory cytokines (*tnfα*, *il1β* and *il8*), antibacterial agent *lysozyme*, gene markers of stress and responses to protein damage (*hsp70*, *hsp90* and *sip1*) and oxidative stress (*sod*, *catalase* and *gsh*). The stress markers were markedly increased at week one in the high temperature biopsies, but only significant for the infected groups after eight weeks. The use of several markers for chaperone genes, oxidative stress, and protein folding have been suggested and already used to detect thermal stress in fish (Akbarzadeh et al., 2018). Increased expression of *il1β* and *il8* were also found in skin biopsies from the high temperature regime at both timepoints. Elevated transcription of both *il1β* and *il8* has previously been reported in blood plasma from cod (Pérez-Casanova et al., 2008a) and in salmon liver (Beemelmans et al., 2021) after exposure to high temperature. Combined, the approach of using *in vitro* systems with skin and scale explants, provided valuable insight into how different temperatures affect skin. Overall, high temperatures seem to reduce the skins potential to heal quickly and alter its response to secondary stressors, two important skin functions vital for the fish health and welfare.

## 5 Conclusion

Overall, our results imply biological alterations caused by high temperature that may reduce the cod's robustness to diseases and production stress. Understanding how multiple stressors affect the

biological performance of farmed species is essential when planning sustainable production. Fish in production systems are exposed to changing environmental conditions, pathogens, and operational procedures, that may expose the animals to stress either individually or in combination. High temperature was shown to reduce skin integrity and barrier functions, leaving it more vulnerable to wounds and secondary infections. Although transcriptome analysis did not reveal global immune suppression, challenge with *Francicella* caused severe damage in spleen at 17°C, which could be due to impaired B cell responses. A weakened skin barrier combined with reduced resistance to pathogens at high temperatures poses a threat to the fish health and welfare. Taken together, our results provide valuable information on how secondary stressors combined with increased temperature may affect farmed cod, and the industry should pay high attention to environmental changes. As shown here, climate change projections show markedly increased temperature at an existing farm site close to the Arctic Circle, and future sites for cod production should be selected taking into account temperature projections and assessment of local environmental conditions.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## Ethics statement

The animal study was approved by Norwegian Food Safety Authority. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

EY, LF, L-HJ and CL conceptualized and designed the study. EY, LF, CL, GJ, SH and ØH collected and sampled the data. EY, LF, L-HJ, CL, AK, SA, VH, GJ and GT analysed the data. EY, CL, AK and GT performed the statistical analysis. EY, LF, L-HJ, CL and AK drafted the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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