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Evaluating a crowding intensity scale and welfare indicators for Atlantic salmon in sea cages

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ABSTRACT

A 5-level crowding intensity scale for directing and auditing the crowding of Atlantic salmon in sea cages based on surface observations is currently included in standards, manuals, and guidelines for fish farmers. Here we test the feasibility of using this scale to create distinct crowding levels, the effects of these different levels upon fish welfare, and the suitability of a set of possible operational welfare indicators (OWIs) and laboratory-based welfare indicators (LABWIs) to be included in toolboxes for monitoring and assessing fish welfare in relation to the crowding of salmon in sea cages. Crowding level 1 was not included in this study since this is a very light level of crowding, and also not level 5 as this level clearly would harm the fish and lead to mortalities. We were able to use the scale to create three distinct crowding levels in two of three separate crowding events in $12 \times 12 m^2$ sea cages. Although the farm personnel were experienced, it soon became evident that underwater monitoring of fish behaviour and how the net was tightened around the fish was important to make sure that no pockets or irregularities that could harm the fish were formed during the crowding. Despite evidence of increased stress and epidermal damage with increased crowding intensity, there were no clear indications that this led to any long-term detrimental effects on fish welfare. In conclusion an OWI-toolbox for crowding should include both surface and underwater observations, monitoring of oxygen conditions, and morphological injury data to steer decisions to prevent welfare problems and mortalities. In addition, qualitative assessment of fish behaviour, plasma cortisol, and skin histology can be included in a LABWI-toolbox if more in-depth information on the effects from the crowding is wanted.

1. Introduction

Crowding is a key component of nearly all handling operations in commercial fin fish aquaculture. When Atlantic salmon (*Salmo salar* L.) are farmed in traditional open sea cages, crowding operations can often involve using a seine net to crowd the fish or utilise an approach that raises part or all the cage net, to reduce the amount of available cage volume (Noble et al., 2018). The operations that utilise crowding include, but are not limited to, manual lice counting, manual grading, mechanical and thermal delousing, medicinal and non-medicinal bathing treatment, transport, and slaughter procedures (e.g., Noble et al., 2018).

During crowding, salmon are at risk of stress, immunity suppression, hypoxic conditions, the loss of mucous cells, physical damage from colliding with each other and contact with the net (Noble et al., 2018 and references therein). Crowding is therefore generally believed to be one of the factors that leads to poor welfare and increased mortality from delousing operations (Overton et al., 2019). Damage to the epidermis and mucosal barrier can also lead to secondary infections such as winter ulcers and long-term suffering (Ingerslev et al., 2010). However, given that crowding has been identified as a major welfare risk, and Atlantic salmon being a major aquaculture species, there are surprisingly few studies specifically investigating the welfare effects of crowding on salmon in sea cages.

Føre et al. (2018) investigated the feasibility of using acoustic telemetry to monitor Atlantic salmon behaviour during crowding in sea cages. The crowding was done in three steps. First, the net was raised to 7 m, kept there for 1 h, and then raised to 1 m followed by another 1 h pause, before seine nets were used to crowd the fish towards a pump into a thermal delousing system. The acoustic transmitter tags registered

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significantly higher swimming activity during crowding, and this was interpreted as being indicative of stress. However, although the study introduces acoustic telemetry as a new method for monitoring crowding before delousing, it did not focus on the effects of crowding upon fish welfare *per se*.

Most studies on the crowding of salmonids in sea cages focus on traits related to slaughter and flesh quality (Erikson et al., 2016; Lerfall et al., 2015; Merkin et al., 2010; Roth et al., 2012). From these studies it is known that crowding leads to decreased muscle and blood pH, decreased blood O_2, and increased blood Na^+, Ca, CO_2, glucose, haematocrit and lactate, and earlier onset of *rigor mortis*. All these factors are indicative of stress, but as these studies are conducted in relation to slaughter, they do not contain any information on the long-term effects of crowding on fish welfare. In the trial by Erikson et al. (2016) oxygen conditions were always above 78 % during the crowding operation and the fish were generally observed to swim calmly amongst each other. This behavioural observation was supported by data on muscle pH, where levels were approximately the same after 0–40 min and after 115–155 min of crowding. However, cortisol levels were elevated, and the authors therefore emphasized that monitoring fish behaviour as an indicator of stress must be done with caution. The difference in behavioural outcomes between Føre et al., (2018) and Erikson et al., (2016) in the form of increased activity versus calm behaviour, probably indicates differences in the experimental setups and crowding procedures between the two trials. There is also a small-scale study on the use of sedation to calm salmon during crowding (Speilberg et al., 2018), where salmon of around 4 kg in small $5\times5m^2$ cages were crowded, resulting in an increase in plasma cortisol and plasma lactate for the controls that were not sedated. They also observed some scale loss.

Although the utility of using behaviour alone to assess welfare during crowding is questioned by Erikson et al. (2016), the rational for using behaviour to describe fish welfare during crowding is that it is an instantaneous operational welfare indicator (OWI) that can be used by the farmers to modulate and direct the procedure (Noble et al., 2018). Behaviour is the central component of a 5-levels crowding intensity scale for Atlantic salmon (Mejdell et al., 2009). The levels in this scale are based upon what the farmers can see from surface observations of behaviour as outlined below (non-italic text reproduced with kind permission from Alistair Smart, Smart Aqua, Hazelwood Park, South Australia and the RSPCA, 2021 and additional italic text reproduced with permission from Mejdell et al., 2009):

1. **Goal:** Fish in the sides of the crowd swimming slowly, normal swimming behaviour (but not all in the same direction), no dorsal fins above surface, no white sides on surface.
2. **Acceptable:** Normal swimming behaviour at suction point, low stress, few dorsal fins above surface, no white sides on surface.
3. **Undesirable:** Over-excited swimming (different directions), more than 20 dorsal fins on surface, some white sides constantly on surface.
4. **Unacceptable:** Overcrowding, over-excited swimming behaviour (different directions), some fish decreasing activity, pumping rate: not possible to keep a constant rate, many fish stuck up against the crowd net, many dorsal fins on surface and numerous white sides on surface, a very few lethargic fish.
5. **Unacceptable:** Extreme overcrowding, whole crowd boiling, potential for large fish kill without rapid release, *panic in the population, the fish are exhausted, many fish floating on their side.*

The scale is also supported by a set of example images (see Mejdell et al., 2009, Noble et al., 2018, RSPCA, 2021).

However, the Mejdell et al. (2009)-report does not include any specific evidence or validation trials supporting the intensity scale. Irrespective of this, the scale is viewed to have utility, is intuitive and has been adopted in the welfare standards from The Royal Society for the Prevention of Cruelty to Animals (RSPCA, 2021), in the guidelines of the Norwegian Food Safety Authorities (NFSA, 2014) and is also repeated in the FISHWELL-handbook of welfare indicators for Atlantic salmon (Noble et al., 2018). The NFSA emphasised in their guidelines that crowding the fish to level 4 and 5 are not in line with Norwegian animal welfare regulations. As a result, Norwegian farmers are required to use the scale, but some farmers we have spoken to (pers. comm.) express concerns regarding how to adhere to it in all situations and have also questioned its validity across a range of rearing systems and operations, especially as it was primarily designed to audit crowding during a very specific operation; when fish are held in waiting cages prior to slaughter at slaughter facilities. Various retailers also employ the scale in their guidelines for welfare auditing of salmon but allow for some exceptions in its interpretation and application e.g., reducing a reliance on a specific number of fins being visible at the surface in crowding level 3 (pers. comm.).

The main goals of the present experiment were threefold. Firstly, to test and evaluate the crowding scale in a controlled experimental scale sea cage setting, and secondly to audit how the different crowding levels affect fish welfare. The third goal was to validate a set of operational welfare indicators (OWIs) and lab-based welfare indicators (LABWIs) for inclusion in toolboxes for monitoring and assessing fish welfare in relation to crowding in sea cages. The OWI-toolbox should include welfare indicators that are easy to use (operational) for the farmer, while the LABWI-toolbox can also include welfare indicators that must be sent to a laboratory for analysis or require expert skills (e.g., Noble et al., 2018). The tested OWIs include underwater observations and inspection of the fish after crowding for external injuries. The tested LABWIs include analysis of plasma parameters, muscle pH, manual and automatic analysis of skin histology and keratocyte migration analysis.

In order to describe the surface and underwater observations, we defined a set of behaviours that we then graded from 0 (not present) to 10 (totally dominating the visual impression of the recording) using a visual analogue scale (VAS). This was motivated by the need to have some way to quantify what we saw on the films and partly inspired by Jarvis et al. (2021) who have proposed a qualitative behaviour assessment for Atlantic salmon. In the Jarvis et al. (2021) article, the assessors were asked to score the presence of behavioural expressions as inquisitive, unsure etc. on a 125 mm horizontal line corresponding with how intensely they felt a particular expressive quality was seen in the salmon, and if different expressions were seen in different individual fish, to score according to the proportion of animals showing them. In our study, we use visual analogue scales to audit specific observations or behaviours during each crowd.

When testing the crowding intensity scale, one must be aware that Atlantic salmon behaviour in sea cages can vary with fish size, season and the water environment inside the cage, their clinical health status, and many other parameters (Noble et al., 2018; Oppedal et al., 2011). The original design of the present experiment was therefore to follow a commercial-like production of salmon as they grow and continuously monitor fish welfare, on triplicate cages that would be crowded to either level 2, 3 or 4 at four separate events during different seasons. Crowding level 1 was not included in this study since this is a very light level of crowding, and level 5 was also not included as this level clearly would harm the fish and lead to mortalities. Note that the premise of this study is that the crowding intensity scale is used as the guide to how much the fish are crowded. Another approach would have been to crowd the fish up to pre-set intensities based on water volume and fish biomass. This would in many ways have been easier, but here we wanted to see if we could use the scale as a tool to decide when to stop crowding the fish further. As we wanted to solely isolate the effects of crowding upon fish welfare, the fish were not pumped from the crowd into a well boat or treatment system, as would normally have been the case in an industry situation but kept at the assigned crowding intensity for two hours, and then released.

2. Material and methods

2.1. Farm and fish

This trial follows a commercial like production of Atlantic salmon at the Institute of Marine Research (IMR) research farm at Solheim (60°N), Masfjorden, Norway. The farm has ten 12×12m^2 sea cages (each 1728 m^3) fully equipped with underwater winch-cameras (Imenco, Norway), an echosounder (MK.IV, Bluegrove AS, Norway) and an environmental profiler (APB5, Saiv AS, Norway) (for more information see Stien et al., 2008). The echosounder was connected to upward facing transducers (42° acoustic beam, 50 khz) positioned ~5 m below each cage.

Atlantic salmon (Aquagen, Norway) were delivered to the farm between 12 and 15th May 2020 and were distributed into 9 cages, with approximately 6000 fish in each, at an estimated average weight of 140 g. This constitutes a start stocking density of approximately 0.12 kg m^{-3}, but with an estimated end stocking density before harvest of above 17 kg m^{-3} (if the fish were to be kept until 5 kg average weight). The upper limit for stocking density according to the Norwegian aquaculture regulations is 25 kg m^{-3} (§25, FOR-2008–06–17–822). The fish were reared with standard salmon feed (Supreme Plus 75 3 mm, Supreme Plus 150 4,5 mm, Supreme Plus 300 4,5 mm, Supreme Plus 600 7 mm, Premium 1200 9 mm, Premium 2500 9 mm, Skretting, Norway) with increasing pellet size as the fish grew. Amounts of feed distributed each day were regulated according to biomass, fish size and sea temperature as specified in the feeding tables provided by the feed manufacturer. Dead fish were collected daily by a dedicated pump system (Lift Up Akva AS, Norway) and their number recorded for each cage according to standard practices in the industry. There were no recorded disease outbreaks at the farm during the experiment.

It is important to limit the number of experimental animals used in research. This research scale farm mimics a full-scale facility and ensures that experiments can be done in sea cages without involving millions of animals. The farm is run the same way as a commercial farm, using a commercial breed of salmon and commercial feed.

2.2. Experiment plan

The experimental plan was to follow the fish in the nine cages (triplicate treatments) from transfer until slaughter, carrying out crowding at different seasons and addressing differing fish sizes to cover normal management procedures. The cages in each group were selected by block design, so that there should be minimum bias in groups, northeast and southwest on the farm, and close and far from the shore (Fig. 1).

The crowding operations were carried out in three separate events, between i) 27–29th of October 2020, ii) 26–28th January 2021, and iii) 26–28th of May 2021. At the first crowding event the fish had an estimated size of around 1.5 kg, 3 kg at the second, and 5 kg at the last crowding event. Each cage was fasted for 24 hours before each crowding episode. Fasting the fish before handling operations is standard practice, and is done to empty their gastrointestinal tract, and thereby lower their oxygen demand, and to avoid faeces in the water during crowding (reviewed in Hvas et al., 2024). The third (May 2021) crowding event (spring crowding) was originally planned for April to coincide with the coordinated spring delousing in the area but had to be delayed to May due to unusual high salmon lice levels at the farm. The fish were deloused on the 13th of March and again on the 27th of April 2021 using an industrial thermal delouser system. After the delousing event in April 2021, the site veterinarian judged it unsafe to perform the crowding trial before the fish had a few weeks to recover. Consequently, the crowding event was delayed to the end of May 2021. The planned fourth crowding event (August 2021) had to be abandoned and the fish slaughtered early due to persistent high lice infestation levels.

2.3. Water environment

The farm is located in a typical fjord environment with generally brackish, colder water near the surface and salinity approximately 33 for the remaining cage depth (Fig. 2AB). Oxygen conditions were generally close to 100 % throughout the cage depth for the entire experimental period, except for a period with slightly sub-optimal oxygen levels below ca. 10 m in April (Fig. 2C).

The water temperature was about 10 °C near the surface and about 13 °C below the pycnocline during the October crowding (Fig. 3AB). The oxygen levels were around 88 % in the deep and close to 100 % saturation near the surface (Fig. 3C). The January crowding was characterised by both very cold water and relatively saline water near the surface (Fig. 3AB). This is typical winter conditions for the site as the cold weather means that precipitation is locked in the mountains as snow. In contrast, spring delousings in a Western Norway fjord will often be characterised by very cold and brackish water in the upper meters from melting water. As the spring crowding had to be postponed till May we instead got a relatively similar water profile as the October crowding, with the notable differences that spring algae bloom resulted in high dissolved oxygen values (>100 %, Fig. 3C), and that the temperature difference between the deep and the surface waters was much less, with 8–9 °C in the surface waters and 9–11 °C in the deep (Fig. 3B).

Oxygen conditions were also monitored in the middle of the fish crowd during the crowding operations using a handheld optical oxygen meter probe (ProSolo; YSI. USA) equipped with a 10 m long cable. If the measured levels fell below the optimal range depending on temperature (Remen et al., 2016) we planned to oxygenate the crowd.

2.4. Crowding procedure

At each crowding, the farm personnel crowded the fish according to

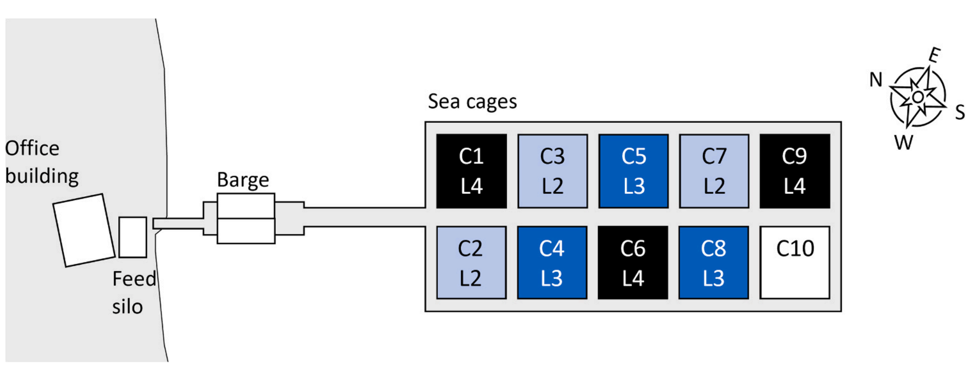

Fig. 1. Schematic overview of the fish farm and cages that were selected to be crowded to level 2, 3 or 4. C1–10: Cage identification numbering, L2 = crowding intensity scale 2, L3 = crowding intensity scale 3 and L4 = crowding intensity scale 4. C10 was not used in the experiment.

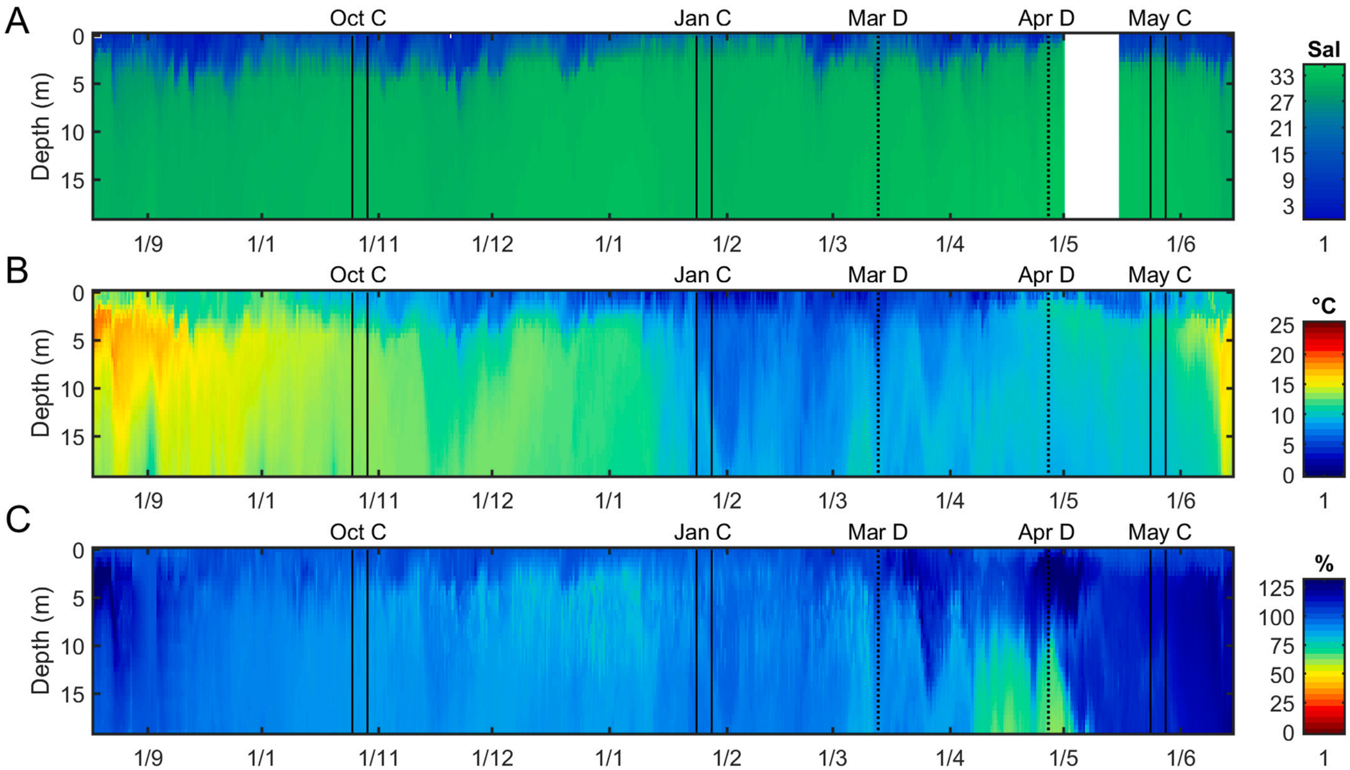

Fig. 2. Water environment conditions at the experimental site as measured by the APB5 environmental profiler, also called Welfaremeter. A) Salinity, B) Temperature (°C) and C) Oxygen saturation (%). The white area in panel A indicates a period where there was a technical problem with the salinity sensor. Oct C, Jan C and May C marks the three crowding events, while Mar D and Apr D marks the two delousing operations.

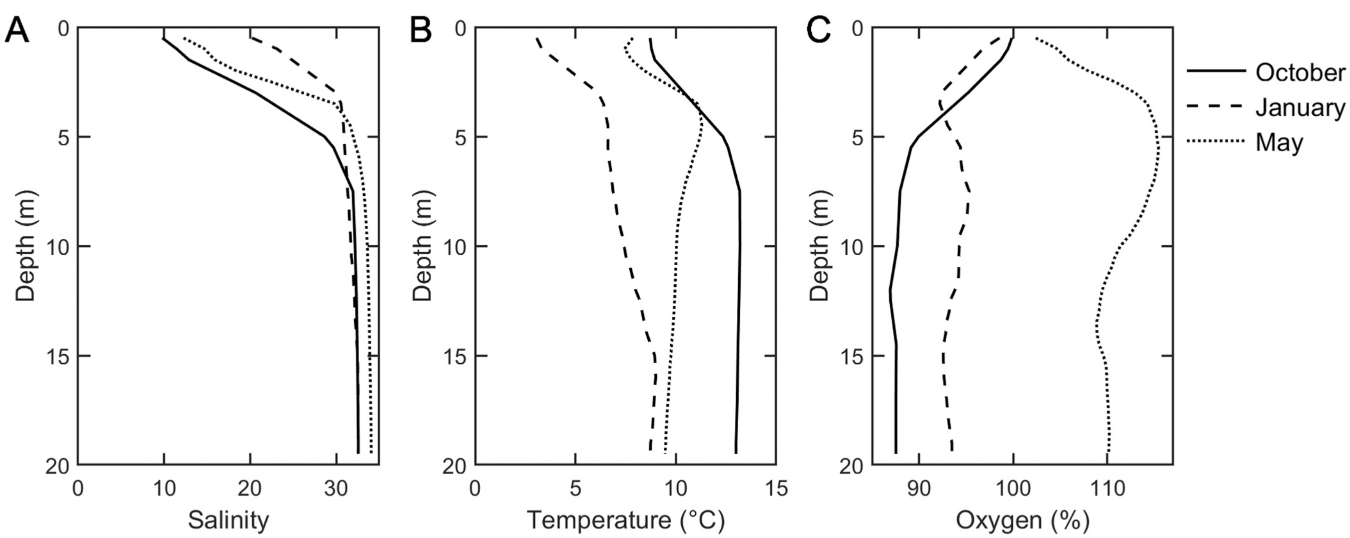

Fig. 3. Water environment during each crowding event. A) Salinity, B) Temperature (°C), and C) Oxygen (%) as measured from the surface and down to 20 m. October profiles are indicated by a solid line, January by a dashed line and May by a dotted line.

the standard practises at the farm. In short, this consisted of firstly gradually pulling up the net evenly from all sides, and then from one of the sides to crowd the fish towards the central gangway for ease of sampling. The day before each crowding episode, farm staff prepared the cages by removing the bird barrier net, the dead fish collection system and any other hardware that would obstruct the net lift procedure. With the number of people we had available to do the crowding and sampling of the fish, there was only time to crowd three cages per day. We therefore crowded three cages per day, on three consecutive days. Each day we crowded one cage to level 2, one to level 3, and one to level 4. The order of the three crowding levels each day were block-randomised to avoid that for example crowding level 2 always was performed first. At each crowding, the farm personnel, together with one of the researchers, used the description of the five crowding levels together with the illustrated reference photos (see Mejdell et al., 2009; Noble et al., 2018; RSPCA, 2021) to agree on when they thought the respective cage had reached its planned crowding intensity level from surface observations only. Each crowding was also monitored and recorded with an underwater winch camera so that the pulling of the net could be stopped and corrected if fish became trapped in pockets in the net or were otherwise at clear risk of becoming injured. For each cage the fish were kept crowded for 2 h, and thereafter 10 fish were sampled (see Section 2.7), and the net released to end the crowding event. Two hours is the maximum time salmon can be crowded according to the RSPCA-guidelines (RSPCA, 2021).

2.5. Camera observations and qualitative description of behaviours

At each crowding event the camera operator moved the winch camera (Imenco AS, Norway) to the centre of the crowd and recorded sample films from the top to the bottom of the crowd. Videos above the water were recorded via surveillance cameras mounted over the central gangway directed towards the cage in question. In addition, a ROV (Blueye, Norway) was used to monitor and record fish behaviour as seen from outside the net. Varying weather conditions, varying water parameters (and thereby visibility), fish of different size between crowding episodes and constraints of the farm and the crowding operations made it difficult to get standardised measurements from these recordings. We therefore developed a pilot approach for describing the behaviours that could be audited from the video footage. In short, we constructed a list of behaviours (Table 1), that we then scored from 0 (not present) to 10 (totally dominating the visual impression of the recording) using a visual analogue scale. Unfortunately, it was possible to deduce the crowding level in each film based on how much the net was pulled in. The scoring was therefore not blinded. The scoring was, however, carried out independently by 5 different researchers. Beforehand, these had discussed and gone through the different behaviours at a meeting to ensure that they all had the same understanding of how the scoring should be done.

2.6. Scoring of external injuries

Obligatory weekly lice counts, where the Norwegian salmon farmers are required to sample fish and count lice by law, were used as an opportunity to also score the sampled fish for external injuries and deviations, specifically those associated with emaciation status, spinal deformities, opercula deformities, scale loss, snout wounds, skin haemorrhaging, wounds/ulcers, fin damage, eye opacity and eye wounds. Pictures and detailed descriptions of each of these OWIs and scoring levels are available in the LAKSVEL-protocol (Nilsson et al., 2022). In short, the LAKSVEL-scoring system consists of evaluating each indicator according to the following primary rules (text reproduced with permission):

1. Injury and deviation free.
2. Minor injury or deviation, which is normally assumed to have little effect on fish welfare, but which nevertheless indicates that something is not entirely optimal.
3. A clear injury or deviation, typically damage of moderate importance to the fish, or that will heal under normal circumstances.
4. A serious injury or deviation that is believed to have major consequences for the fish's health and welfare.

The lice counting procedure involved the fish being firstly confined using a small seine net, and then dip netted into a louse counting tank for sedation (Tricaine methanesulfonate, 100 mg L^{-1}, Scan Aqua AS, Norway). After the fish were sedated, they were individually graded (length and weight), counted for lice, and scored for external injuries and deviations. Fish were then transferred into a recovery tank, and after awakening allowed to swim freely into a tube containing flushing water that transferred them back to the sea cage (Tellekar Lakselus, MarinHelse AS, Norway). At each weekly lice count the staff at the farm sampled 60 fish from three cages (20 from each cage), one cage from each crowding level. The lice counts were cycled, so that each of the nine cages were sampled every third week.

Table 1
Description of behaviours to be assessed qualitatively from 0 (not present) to 10 (totally dominating the visual impression of the film) based on the recordings from the surface camera, winch or ROV cameras of the different crowding events.

Observation	Description	Camera (s)
Surface activity	Degree of visible activity from fish in the surface. Including ripples in the water and various surface breaks by the fish (rolling, jumping etc.)	Surface
Fins out of water	Degree of fins sticking out of the water across the crowding surface	Surface
White sides	Degree of white sides (belly side) observed at the surface	Surface
Space to surface	Degree of space between the surface and the fish group	Winch, ROV
Queuing	Degree of fish being hindered and having to slow down and swim slowly due to other fish	Winch, ROV
Structured	Degree of the fish managing to maintain a structured school with fish swimming in the same general direction.	Winch, ROV
Swimming speed	Relative swimming speed	Winch, ROV
Space to net	Degree of closeness between the fish group and the net	Winch, ROV
Touching the net	Degree of fish touching the net	Winch, ROV
Pressed into net	Degree of fish being pressed into the net	Winch, ROV

2.7. Fish sampling procedure

Baseline sampling of fish for blood plasma, muscle pH and skin histology were carried out on the day prior to the first October (N=24), January (N=35) and May (N=28) crowding events. Fish were caught by a seine net pulled towards the central gangway for easy sampling of fish using a fine meshed dip net (55 cm × 40 cm × 43 cm, Fish Tech, Inc. Norway) to minimize injury on the fish (Moltumyr et al., 2022). To audit the effect of crowding upon fish welfare, fish (N=10 per cage, N=30 per crowding level, i.e. N=90 per crowding event) were netted from the crowds at the end of each crowding procedure using the same dip nets. No sampling to audit fish health, physiology and injury status was carried out during the crowding procedure as this would be detrimental to behavioural monitoring of the crowd at this scale of production. The sampled fish were put directly in a seawater bath and killed by an overdose of anaesthesia (300 mg L^{-1} Finquel MS 222, Tricaine methansulfonate, Scan Aqua AS, Norway). For each fish, body weight and fork length were recorded, and 1.5 ml blood samples were then drawn from the caudal vein with a heparinized syringe to assess plasma stress physiology markers. In addition, we also collected skin biopsies from the area just above the lateral line, in line with the rear edge of the dorsal fin. About 1×1.5 cm^2 of skin at a thickness of ca. 0.5 cm (which included red and white muscle) was carefully collected to preserve scales and the epithelial layer in each sample. Samples were fixated for histological analyses in 10 % formalin (CellstoreTM, CellPath, Newtown, UK) overnight at room temperature and afterwards stored at 4 ℃. At the May crowding event, scales were picked and cultured for migration assays, as described in the section below.

The blood samples were centrifugated for 5 min at 4 ℃ at 3000 g in Eppendorf tubes. The blood plasma was isolated and then stored first in dry ice and then at −80 °C for later analyses. Thereafter, in order to measure muscle pH, fish were pierced approximately 1 cm deep with a scalpel on the left dorsal muscle, centrally between the head and dorsal fin. The probe of a portable pH-meter (Seven2Go S2 Food kit, Mettler Toled) was inserted in the incision and pH value recorded. Plasma pH, ions (Na^+, Ca^{2+}, K^+, Cl^-) and metabolites (glucose, lactate) levels were measured with an ABL90 FLEX PLUS blood gas analyser (Radiometer Medical ApS, Åkandevej 21, DK-2700, Brønshøj, Denmark). Plasma cortisol concentration was quantified with an ELISA immune assay kit

(IBL International GmbH) and a Sunrise™ microplate reader (Tecan, Switzerland).

2.8. Skin histology

Skin biopsies, fixed in buffered 10 % formalin (N=10 per cage, i.e. N=90 per crowding event), were carefully dissected, orientated, and placed in tissue embedding cassettes (Simport, Quebec, Canada). The samples were dehydrated through 100 % alcohol and then in a clearent Xylene bath, using an automated tissue processor (TP1020, Leica Biosystems, Nussloch GmbH, Germany), before infiltrated in melted 60°C paraffin (Merck KgaA, Darmstadt, Germany). Paraffin-embedded tissue samples were cut in 5 µm sections using a Microtome (Leica RM 2165), mounted on polysin coated slides (VWR, Avantor, Pennsylvania, USA) and dried overnight at 37 °C. The sections were deparaffinized and rehydrated, and staining was performed using an automated special stainer (Autostainer XL Leica Biosystems, Nussloch GmbH, Germany). Paraffin sections were stained with Alcian Blue Periodic Acid Schiff (AB/PAS, pH 2.5, Alcian Blue 8GX, Sigma Aldrich, Darmstadt, Germany). Stained samples were examined by a light microscope slide scanner. Manual measurements for skin were done in a region of ca 1000 µm per section using Aperio Image Scope (Leica Microsystems, Wetzlar, Germany). Deep neural network analysis on the Aiforia® platform was then used according to an in house developed algorithm for salmon skin digital histopathological evaluation (see Sveen et al., 2021).

Many of the samples were missing parts of the epidermis. Since this is not detected by the digital algorithm, a manually scoring of the integrity of the epidermis was instead carried out. The general appearance of the epidermis and the quality of the epithelial surface were scored using a semi-quantitative 5-point scale skin health scoring system similar to Karlsen et al., (2021). Score 0: Smooth surface- Score 1: Signs of rough cells at the surface. Score 2: Clear signs of rough cells, <50 % of the surface affected. Score 3: All cells lining the outer part of the epidermis appears rough. Score 4: Rough outer part and thorn epidermis. The blinded scoring was carried out by the same histology expert for all samples.

2.9. Scanning electron microscopy (SEM)

Skin samples for scanning electron microscopy (SEM) from the different crowding levels (N = 3 per level from the first day of crowding, and for the January and May crowding events also the baseline) 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, United Kingdom). Imaging was performed at the Imaging Centre, Faculty of Biosciences, Norwegian University of Life Sciences (Zeiss EVO-50–EP, Carl Zeiss SMT Ltd, 511 Coldhams Lane, Cambridge CB1 3JS, UK).

2.10. Keratocyte migration assays

Salmon keratocytes were cultured from scale explants according to a previously described protocol (Karlsen et al., 2021), 1 h post crowding for all crowding levels. 6 scales per well in 12 well tissue culture plates (Falcon Multiwell™ Becton Dickinson, NJ, U.S.A.) were used, 3 wells per fish and 5 fish per crowding level (N = 5 per crowding level), from the first day of crowding at the May crowding event. Each well contained 1 ml L-15 supplemented with 10 % fetal bovine serum (FBS), 25 µg amphotericin B, 10 ml/L antibiotics, antimycotics and 0.01 M HEPES (Sigma). Plates were incubated at 12 °C in a cell incubator without CO_2. After one, two and three days, wells were microscopically analysed (Leica S9i, LED3000 RL). Scales with migrating cells (defined as cells moving from the scales to the bottom of the well) were measured at every timepoint and calculated as migration potential to the total number of attached scales. Scales that were not attached to the wells were not counted.

To further test the biological impacts of the crowding events in relation to potential further secondary stressors e.g., delousing by hydrogen peroxide (H_2O_2), skin biopsies were exposed to 1000 ppm H_2O_2 (Merck) for 20 min to induce oxidative stress, before scales were picked from the biopsies and further cultured as above. Control biopsies were kept 20 min in salt water. Samples from these biopsies were also stored in formalin for histological evaluation and SEM.

2.11. Mortality

Mortality is the ultimate outcome of poor welfare, and both short term (daily) and long term (daily over time or accumulated) mortalities were monitored throughout the study.

2.12. Final sampling

In addition to final accumulated mortality, we had intended to do an extended sampling of the fish at the end of production. However, due to high lice levels and the urgency of getting the fish slaughtered as fast as possible to avoid breaching the legislative lice limit, we only had time to sample 90 fish from cage 3, 5, 6, i.e. 30 fish from each level, for the final registration of weights and scoring of external injuries (Nilsson et al., 2022)

2.13. Statistical analysis

All data analyses were carried out in R (version 4.2.0, R Core Team, 2022), while the figures were produced in MATLAB (version R2022b, The MathWorks, Inc). The collected data from the various OWIs and LABWIs described above vary in type (scores, counts, percentages, real numbers). The data are tested with Fisher's exact tests in the case of count data (e.g., number of dead fish, number of fish with external injury score 2 and 3), t-tests in case of continuous data (plasma and muscle parameters), and otherwise Wilcoxon rank-sum test. The key assumption of this study is that the measured indicators will either increase or decrease with crowding level, and that a good operational welfare indicator for crowding should be able to distinguish between baseline (i.e. control), crowding intensity levels 2, 3 and 4, or at least crowding level 2 and 4. Welfare indicators that systematically increase or systematically decrease with a significant difference ($p \leq 0.5$) between each crowding intensity are marked as blue circles in the figures. Welfare indicators that satisfy the blue criteria, but where baseline either is not different from level 2 or not present are marked as green. Welfare indicators that do not satisfy the criteria of being marked as green or blue but are still significantly different between crowding level 2 and 4 are marked as yellow. Indicators that do not satisfy any of these criteria are marked as white (not meeting the minimum requirement).

2.14. Ethics statement

This experiment was conducted at the Institute of Marine Research's facilities at Solheim, part of Matre Research Station, which is authorised for animal experimentation by the Norwegian Food Safety Authority (facility ID 110), and in accordance with regulations for the use of animals in experimentation (application ID: 24315).

3. Results

3.1. Practical experiences from the crowding events

During the first crowding event, in October 2020, the fish had reached an estimated average size of around 1.5 kg. The goal was to raise the nets slowly while the farm team looked for the criteria of the level 2, 3 or 4 crowding intensities. On day one of crowding, the team started to crowd cage 1, which was to be crowded to level 4. In order to reach this level, we envisaged incrementally and briefly passing through levels 2 and 3 before arriving at the desired crowding intensity level 4. To achieve this objective, it was necessary to pull the net up to within 1–2 m of the surface and then crowd the fish further by gradually lifting one side of the net up above the water, crowding the fish towards the central gangway. This resulted in a sudden change in surface activity, from zero surface activity in one moment to dorsal fins appearing across the entire surface area in the next (Fig. A.1). The underwater camera showed that the fish were crowded from the top to the bottom of the available water volume, and that they swam very close to the net (Fig. A.2–4). It was therefore deemed unsafe to crowd the fish further. Since the fish essentially did not show any surface activity before suddenly showing an intensity that we judged corresponding with level 4, we had to support the surface observations with observations from the underwater camera to create the desired intensity level. This was a responsive decision and was not an original objective of the trial. We also adapted the crowding technique to reduce the risk of inadvertently exposing the fish to level 4, by stopping the lifting of the net towards the central gangway earlier in order to achieve a crowding level 3. This timepoint was decided based on underwater camera images showing that fish were approaching the same behaviour and proximity to the net as seen for level 4, but still with little or no surface activity. For level 2 the net was only pulled up to 4 m from the surface and not crowded towards the central gangway. Dissolved oxygen saturation levels, recorded at 1 m depth, in centre of the crowd were always above 97 % in the level 2 and 3 crowding operations, and for level 4 the minimum recorded dissolved oxygen saturation at 1 m was 82 %. It was therefore not considered necessary to oxygenate any of the crowding operations.

During the second and third crowding events in January and May 2021, the fish had reached an estimated average size of around 3 kg and 5 kg, respectively. These crowding procedures were carried out in a similar manner to the October 2020 crowding, but there was no absence of surface activity as the crowding commenced, unlike the October crowding (Fig. A.1). In these latter two crowding events, the fish exhibited noticeable surface activity from the start of the crowd and the farm team could therefore use the surface crowding intensity scale to manage the crowding procedures and judge when to stop pulling the net based on surface observations as planned. In the January 2021 crowding event, dissolved oxygen saturation at 1 m depth inside the crowd was always above 70 % at level 4 and generally above 78 % at level 3, except for the cage 4 replicate within level 3 which had a dip down to 67 % oxygen saturation but returned to 74 % saturation within 10 minutes. It was therefore judged unnecessary to oxygenate. For the May crowding events, dissolved oxygen saturation levels were always higher than 90 % for all crowding levels. During all the crowding operations a designated operator monitored the fish and the net using the underwater cameras and occasionally gave warning when a rope, or part of the net, had been pulled in such a way as to create a pocket or other potential risks to the fish.

3.2. Echosounder observations

Echo sounder observations should be interpreted with care. The echo strength is predominantly the echo from the swim bladders of the salmon inside the beam of the transducer (Folkedal et al., 2022). This means that when the fish are crowded towards the gangway, the fish are effectively pulled out of the beam weakening the echo. Salmon have an elongated swim bladder, and as a result the amount of echo reflected is also a function of the salmons' tilt angle towards the transducer (Folkedal et., 2022). Furthermore, salmon is also known for emptying their swim bladder when stressed (Bui et al., 2013). The only thing we can read for certain from the echo sounder images during the crowding operations (see Fig. A.5) is therefore the approximate maximum depth of the crowds. This showed that the farm team stopped pulling the net up earlier, i.e., deeper, at level 2 than at level 3 and 4. Level 3 and 4 were pulled up to ca. 2.5–2 m in all the three crowding events, while at Level 2 the farmers stopped at about 4 m (see Fig. A.5). Monitoring mean swimming depth in the days after the crowding events showed overlapping and very similar patterns for the fish crowded to the different levels (Fig. 4).

3.3. Mortality and final weight

The accumulated mortality curves for the nine cages at the farm were levelling out before the first crowding at the end of October 2020 (Fig. 5). The October crowding did not lead to any marked rise in mortality for any of the cages; there were either no significant changes or a continued decline in mortality rate (Fig. 5, Table 2). The crowding at the end of January did not lead to any significant changes in mortality in the cages crowded to level 2 and 3, except for cage 4 which had no mortality during the week after crowding (Fig. 5, Table 2). However, for level 4, while two of the cages had no significant increase, cage 9 had an increase from 3 dead fish the week before to 36 dead fish the week after crowding (Table 2, $p<0.001$). In May 2021, when the fish were recovering from two delousing events, the trend of a decreasing mortality rate continued after the crowding event at the end of May (Fig. 5, Table 2). Total accumulated mortality from the start to the end of the experiment at slaughter was 4.7±0.1 %, 4.4±0.4 %, 4.8±0.1 % (mean±SE) in the level 2, 3 and 4 cages respectively, with no significant increase with crowding level (Fig. 5, $p\geq0.200$, Wilcoxon rank sum test). The sampling before the expedited slaughter revealed no significant difference in weight between the different crowding levels (4472±243 g, 4438 ±222 g, and 4448±221 g, $p\geq0.751$, Wilcoxon rank sum test).

3.4. Qualitative description of behaviours from camera recordings

Note, that as it was not possible to derive objective measurements of fish behaviour from the video footage, the description of the videos had to be done as a subjective assessment how much, or to what degree, a set of behaviour categories (Table 1) were present in the different crowding levels. The purpose was to detect if there were differences in fish behaviour between levels. The measurements cannot be used as objective measurements of e.g., swimming speed; only that swimming speed was perceived as greater or smaller between the different crowding levels.

The behavioural assessment from the overwater camera recordings unveiled an overall positive correlation between surface interactions (i.e., surface activity, fins out of the water or white sides) and increasing crowding intensities for all sampling events (Fig. 6, also see Fig. A.1). However, recordings from the October crowding confirmed that there was little to see in behavioural terms from the surface for crowding levels 2 and 3, whilst at the level 4 crowding intensity there was clear surface activity, fins out of water and visible white sides (Fig. 6A-C, also see Fig. A.1). This lack of surface activity was also confirmed by underwater footage, showing that the space between the fish and the surface was judged to be much greater during the October crowding events than during both the January and the May crowding events (Fig. 6D, also see Fig. A2 and A4). For the January and May crowding events, where we were able to guide the crowding events using surface observations and the crowding scale, the qualitative analysis of the camera recordings showed an increase in white sides as a function of crowding level (Fig. 6C), while the amount of surface activity and fins out of water were similar between crowding levels 3 and 4 (Fig. 6AB). The observed queuing of fish near the surface was least for the October crowding, and the increase with crowding level was not large enough to distinguish between all three levels (Fig. 6E). The quantification of school structure near the net bottom was similar for all time points, but with little difference between crowding intensity levels 2 and 3 and a marked decrease in structured swimming for crowding intensity level 4 (Fig. 6F). As expected, the degree of space between the fish school and

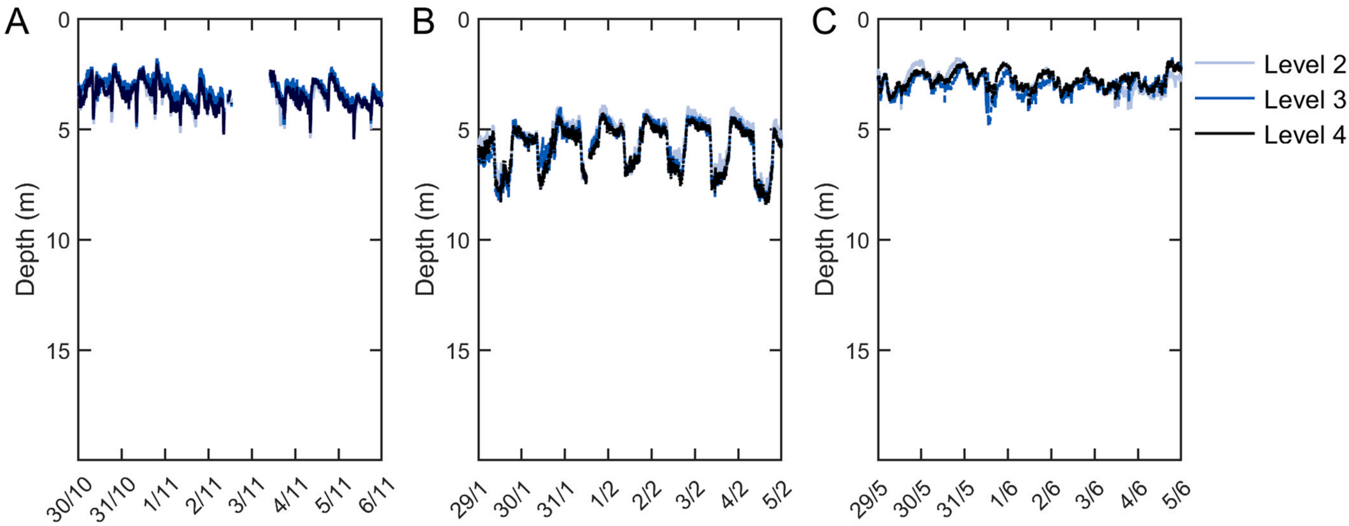

Fig. 4. Echosounder data showing the average swimming depth of the fish crowded to level 2, 3 and 4 in the week after each crowding event. A) After the October crowding, B) After the January crowding and C) After the May crowding event. The average depth behaviour of the fish crowded to level 2 are indicated by a solid light blue line, the fish crowded to level 3 by a solid blue line, and the fish crowded to level 4 by a solid black line.

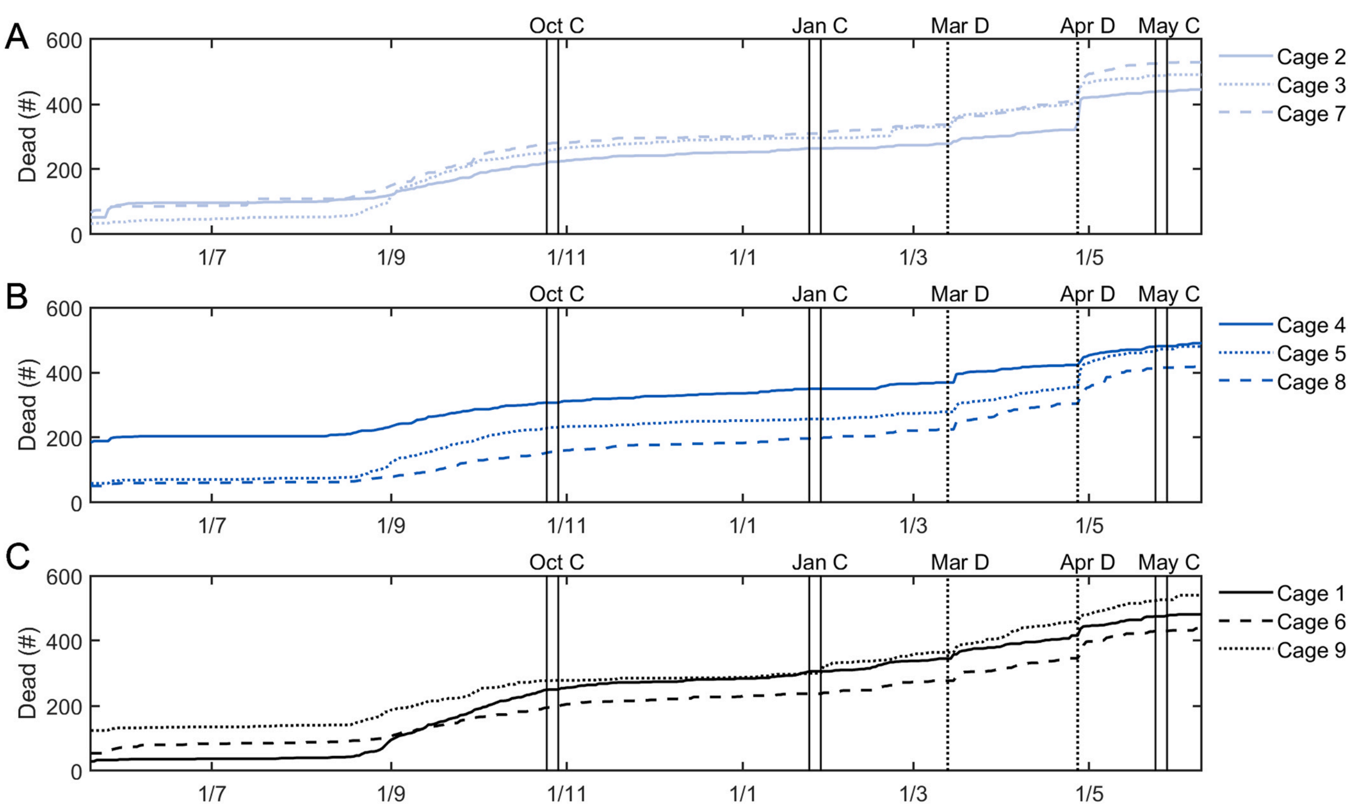

Fig. 5. Accumulated mortality starting May 2020. A) Accumulated mortality for cage 2, 3 and 7 crowded to level 2. B) Accumulated mortality for cage 4, 5 and 8 crowded to level 3. C) Accumulated mortality for cage 1, 6 and 9 crowded to level 4. 'Oct C', 'Jan C' and 'May C' marks the weeks when the October, January and May crowding operations were performed, while 'Mar D' and 'Apr D' marks the days of the March and April delousing events.

net bottom decreased between level 2 and 4, but with varying values for level 3 (Fig. 6G). Similar results were observed for the degree of fish touching the net (Fig. 6H). It was a stated goal to pull and adjust the crowd net so that fish becoming pressed into the net were avoided as much as possible. It is therefore not surprising that this indicator showed the least correspondence with crowding level in our experimental set up (Fig. 6A-H vs. Fig. 6I).

The ROV-recordings (Fig. A.4) offered an underwater view of the crowding events from outside the cage and a general overview of the fish group when crowded. This made it easier to audit the general swimming speed of the fish and this was therefore included as a behavioural parameter in the sub surface behavioural auditing toolbox. However, poorer visibility near the surface meant that the parameter 'space to surface' had to be excluded. In general, despite a diverse range of behavioural parameters being audited from the ROV-recordings, there were little or no differences in a range of sub surface behaviours across

Table 2
Weekly mortality (‰) in the week before and week after crowding operations. P: Fisher's exact test, comparing mortality before and after crowding for each cage. Note that there were ca 6000 fish in each cage, and that a difference in 1 ‰ before vs. after therefore only constitutes about 6 fish.

		October crowding			January crowding			May crowding		
Level	Cage	Before	After	P	Before	After	P	Before	After	P
2	C2	1.9	0.8	0.029	0.7	0.1	0.039	1.0	0.5	0.210
2	C3	1.8	0.9	0.070	0.1	0.1	1.000	0.8	0.3	0.146
2	C7	1.5	0.6	0.064	0.4	0.9	0.210	0.9	0.4	0.179
3	C4	0.8	0.8	1.000	0.6	0.0	0.031	1.4	0.8	0.307
3	C5	1.1	0.3	0.049	0.3	0.4	0.508	1.4	0.7	0.151
3	C8	1.5	0.8	0.168	0.3	0.6	0.226	1.3	0.3	0.007
4	C1	2.7	1.2	0.011	1.5	0.3	0.007	1.7	0.5	0.015
4	C6	1.3	1.4	0.860	0.4	0.9	0.143	1.2	0.7	0.286
4	C9	1.1	0.1	0.003	0.3	3.2	<0.001	1.5	1.3	0.855

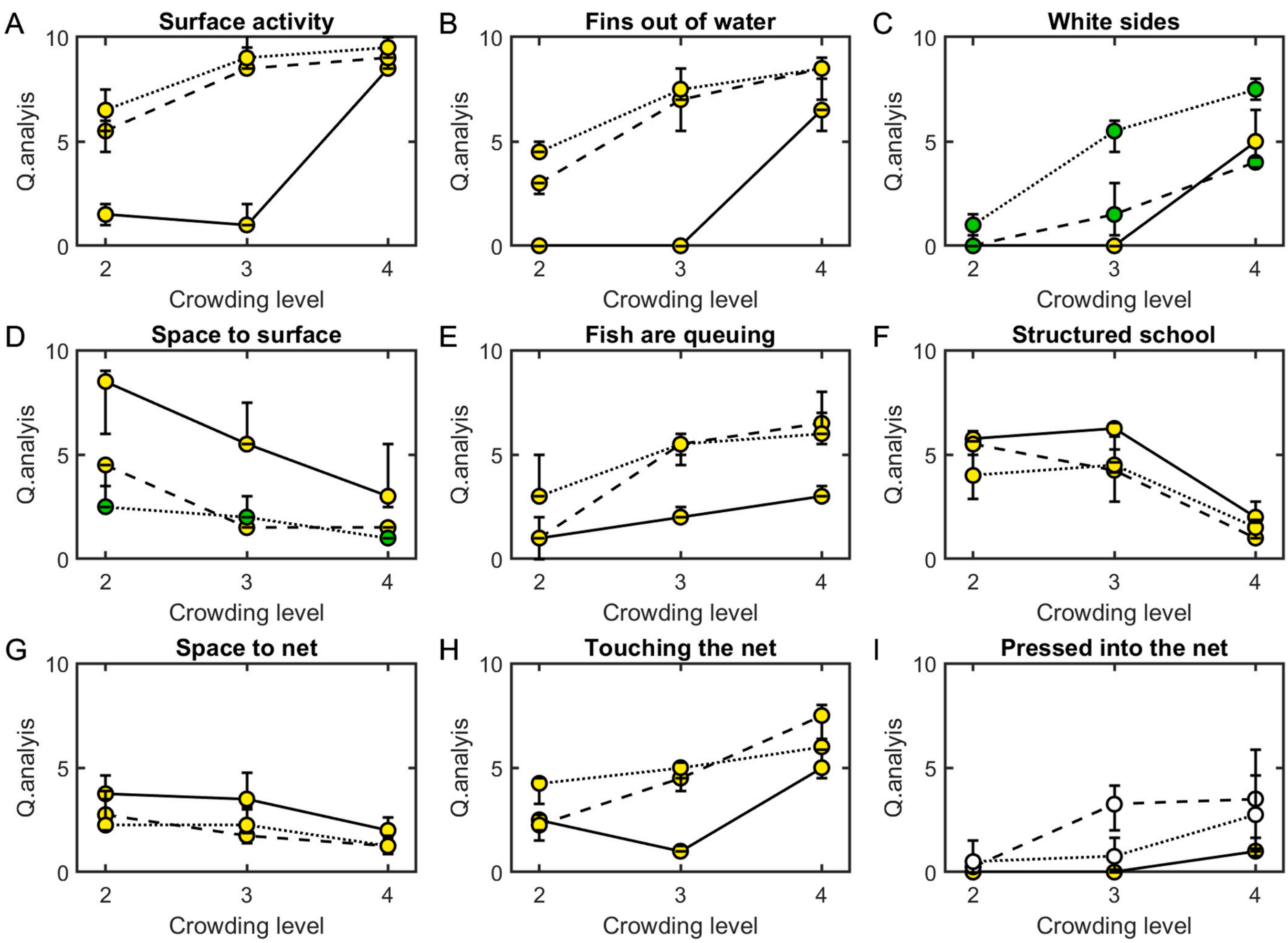

Fig. 6. Qualitative analysis (median, error-bars show 25- and 75-percentiles) of fish behaviour during the October (solid line), January (dashed line) and May (dotted line) crowding events based on the cameras positioned above the surface and the winch cameras inside the nets. A) Degree of fish activity at the surface (0 = no activity, 10 = surface totally dominated by fish activity. B) Degree of fins out of the water (0 = no fins, 10 = surface totally dominated by fins visible above water). C) White sides visible in surface (0 = no visible white sides, 10 = surface totally dominated by fish showing their white sides. D) Space between the fish and surface. E) Fish queuing near surface. F) Degree of swimming structure to school. G) Space between fish school and net bottom. H) Degree of fish touching the net. I) Degree of fish being pushed into the net. The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, white if L2 and L4 are not significantly different from each other, otherwise yellow (Wilcoxon rank sum test).

the crowding events in our set up (Fig. 7), There was generally a significant increase or decrease between crowding level 2 and 4 for all the parameters, but not significant differences for level 3 (Fig. 7, also see Fig A.4). Regarding the underwater recordings inside the cage, the degree of fish being pressed into the net again showed the least correspondence with crowding intensity (Fig. 7) due to the previously stated objective of limiting scenarios where the fish could be harmed.

3.5. *Scoring of external injuries*

The scoring of external injuries at the weekly lice counts after the October, January and May crowding events revealed no significant increases or decreases with crowding level (Fig. 8). There were more injuries on the fish after the May crowding than after the two other crowding events, but at this timepoint the fish had also recently gone

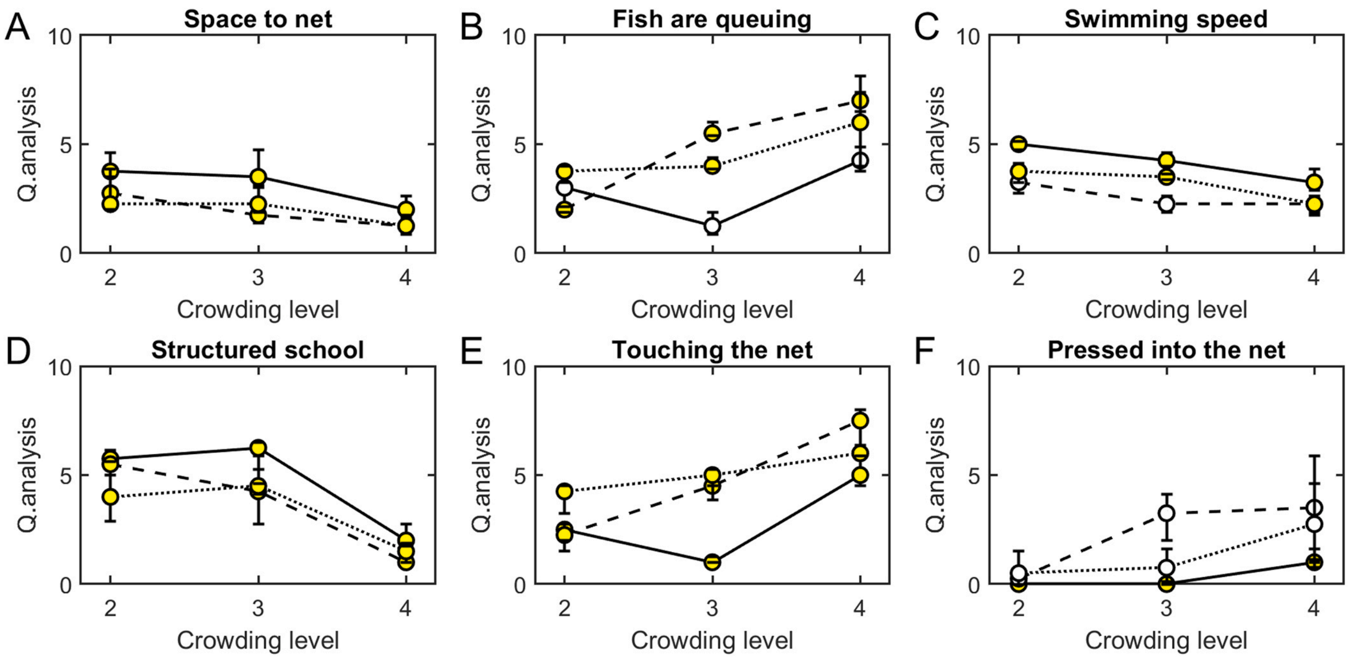

Fig. 7. Qualitative analysis (median, error-bars show 25- and 75-percentiles) on a scale from 0 (minimum) to 10 (maximum) of fish behaviour during the October (solid line), January (dashed line) and May (dotted line) crowding events based on the ROV-recordings. A) Degree of space between fish school and net, B) Degree of fish queuing, C) Swimming speed, D) Schooling structure, E) Degree of fish sometimes touching the net and F) Degree of fish being pressed onto the net. The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, white if L2 and L4 are not significantly different from each other, otherwise yellow (Wilcoxon rank sum test).

through two thermal delousings as indicated in Fig. 5.

3.6. Plasma parameters and muscle pH

The plasma analyses of cortisol showed a rise in stress response as a function of baseline and crowding level for the October crowding (Fig. 9A). The cortisol values were generally higher for the January and May crowding compared to the October crowding, and for these crowding events the cortisol values plateaued at level 3 with no further rise at level 4 (Fig. 9A). One should further note the high cortisol values at baseline for the May crowding, and also deviating values for many of the other parameters (Fig. 8). The imperfect baseline for the May crowding was probably because we had some trouble catching the baseline fish at this sampling.

Plasma sodium, plasma chloride, plasma calcium and plasma potassium all showed inconsistent results revealing no clear relationships with crowding level (Fig. 9B, F, G and H). The plasma lactate values were oddly higher for baseline fish than for the crowded at all crowding events and starting on a lower level at the January crowding compared to the October and May crowding events (Fig. 9C). For the January crowding there was a consistent increase in plasma lactate with crowding level, while this was only significant between level 2 and 4 for the May crowding, while there was no such relationship for the October crowding (Fig. 9C). Despite relatively little change in absolute values, there were always significant higher plasma pH values at level 4 compared to level 2 (Fig. 9D), while this was only the case for muscle pH at the October crowding (Fig. 9E). Note also that the muscle pH-values in January showed a wider standard error range (Fig. 9E), possibly caused by imprecise measurements due to the low air temperature. Plasma chloride (Fig. 9F), plasma calcium (Fig. 9G) and plasma potassium (Fig. 9H) also did not show a clear systematic relationship to crowding level. There was, however, a clear increase in plasma glucose from baseline and with increasing crowding level for the January crowding, but this was not the case for the October and May crowding (Fig. 9I).

3.7. Histology

No differences were detected between crowding levels using the digital algorithm in Aiforia® for the October and January crowding events (Fig. 10). However, at the May crowding event, many of the parameters showed significant differences between level 2 and 4 (Fig. 10). Epidermis was in general thinner at the May sampling, with a lower number of mucus cells and less loose connective tissue (LCT) compared to October and January. Dermis and dense connective tissue (DCT) were also increased in this group, resulting in the dermis to epidermis ratio being highest in May. The manual scoring of skin samples (Fig. 11) showed increased damage with increasing crowding level, with differences between level 2 and 4 in all sample sets for score 3–4 (Fig. 11B).

To further study the biological significance of the different crowding levels, we cultured scales to study the migration capacity of the keratocytes from the May crowding. Results after three days showed a clear pattern with a significantly reduced migration rate from level 2 to level 3 and further to level 4 (Fig. 12A). Exposing skin to H_2O_2 reduced the migration capacity even further, with a significant reduction at the highest crowding levels (Fig. 12).

4. Discussion

4.1. Did the crowding intensity scale work?

Although the farm staff at our research site expressed initial scepticism regarding the usability and validity of the crowding intensity scale, we were able to use the scale to create what we perceived as level 2, 3 and 4 crowding intensities in two of the three crowding events. The word 'perceived' is key here. Although level 2 and 3 can be distinguished by an objective observation that more than 20 dorsal fins are out of the water. Most of the descriptions of the levels are quite subjective and diffuse, leaving it up to the observer to decide where the threshold is between for instance 'some white sides' (level 3) and 'numerous white sides' (level 4) on surface. This is an inherent problem with all such

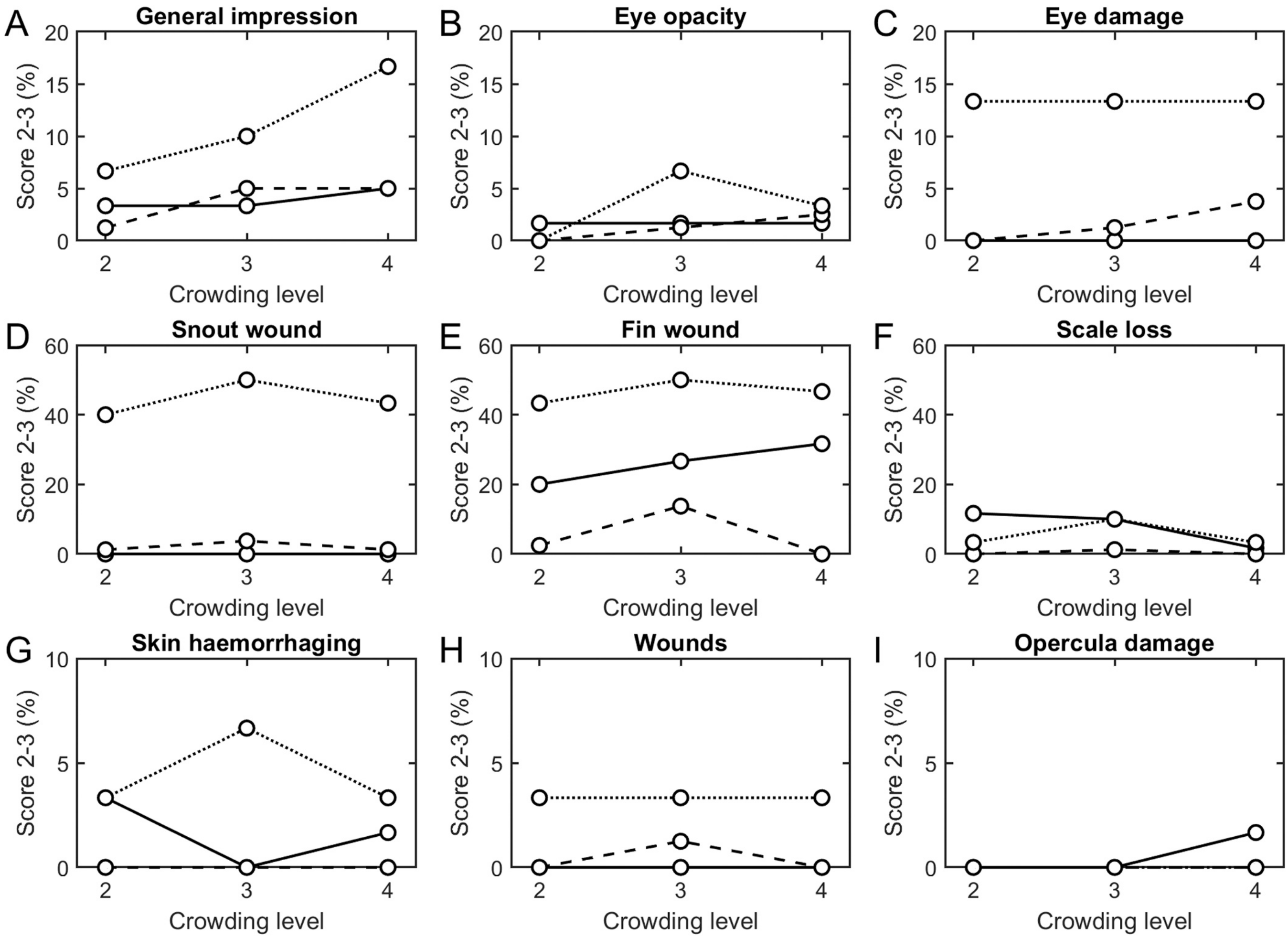

Fig. 8. Prevalence of fish scored with moderate to severe scores (2–3) at the weekly lice counts in the month after the October crowding (solid line), in the month after the January crowding (dashed line) and after the May crowding (dotted line). A) General impression of the fish, B) Eye opacity, C) Eye damage, D) Snout wound, E) Fin wound, F) Scale loss, G) Skin haemorrhaging, H) Wounds on the skin and I) Opercula damage. The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, white if L2 and L4 are not significantly different from each other, otherwise yellow (Fisher's exact test).

classification schemes, which is somewhat circumvented by the use of illustrative pictures that correspond to each level (see Mejdell et al., 2009, RSPCA, 2021 or Noble et al., 2018). These pictures were in many ways more helpful than the descriptions themselves. In addition, the crowding intensity scale does not state in the text descriptions whether it is enough that one or two of the criteria for each level are met, or that most or all must be met for the crowding to be scored as e.g. level 4. This uncertainty can lead to disagreements between different observers on how they would classify a crowding level. It is also clear that each crowding level has a range from weak to strong. This is unavoidable when dividing the real world into discrete levels but will necessarily mean that two different crowdings, although assessed to the same crowding intensity level, in reality can be quite different and have different effects upon fish welfare.

In the October crowding event, we were not able to use the scale and had to rely on the underwater cameras as the fish refused to approach the water surface until the lack of available water volume forced them to do so. This led to an acute and intense expression of surface activity that we judged consistent with level 4, bypassing level 2 and 3. At the time we believed that this was explained by cold and brackish surface water, however, brackish surface water was also present in the May and January crowding events, although to a lesser extent. Close scrutiny of the echo sounder data (Fig A.1) shows a downward movement of the school at the onset of crowding during the October event. A second hypothesis may therefore be that the fish at the October crowding event were more skittish and wary of possible dangers from above. At this time, they were smaller (around 1.5 kg) than at the January (3 kg) and May (5 kg) crowding events. Smaller salmon are more vulnerable for attacks from avian predators (e.g., cormorants). Another possible explanation is that the October crowding habituated the salmon to being crowded in cages, an operation that the fish groups had not experienced earlier. However, habituation of salmon to a potentially dangerous event does normally require more than one exposure (Bratland et al., 2010; Fernö et al., 2020; Folkedal et al., 2010), making this explanation unlikely. In any case, our study shows that surface observations alone are not always suited to estimate crowding intensity when fish avoid the surface, and that the causal drivers for this avoidance, such as at the October crowding event, can be difficult to determine.

However, the crowding intensity scale proved suitable for producing three different crowding levels during both the January and May crowding events. This is supported by data from the echosounder observations, the plasma cortisol measurements, and with data from some of the underwater parameters for fish behaviour that correlated with crowding level. The qualitative analysis of the camera recordings of the crowded fish at the January and May crowding events were also to a large extent overlapping. This suggests that the descriptions of each crowding intensity were relatively easy to understand, and robust, even when the crowding operations were separated by up to 4 months. However, in hindsight it is clear that, even though the farm staff were experienced and knew the sequence of which parts of the net and which

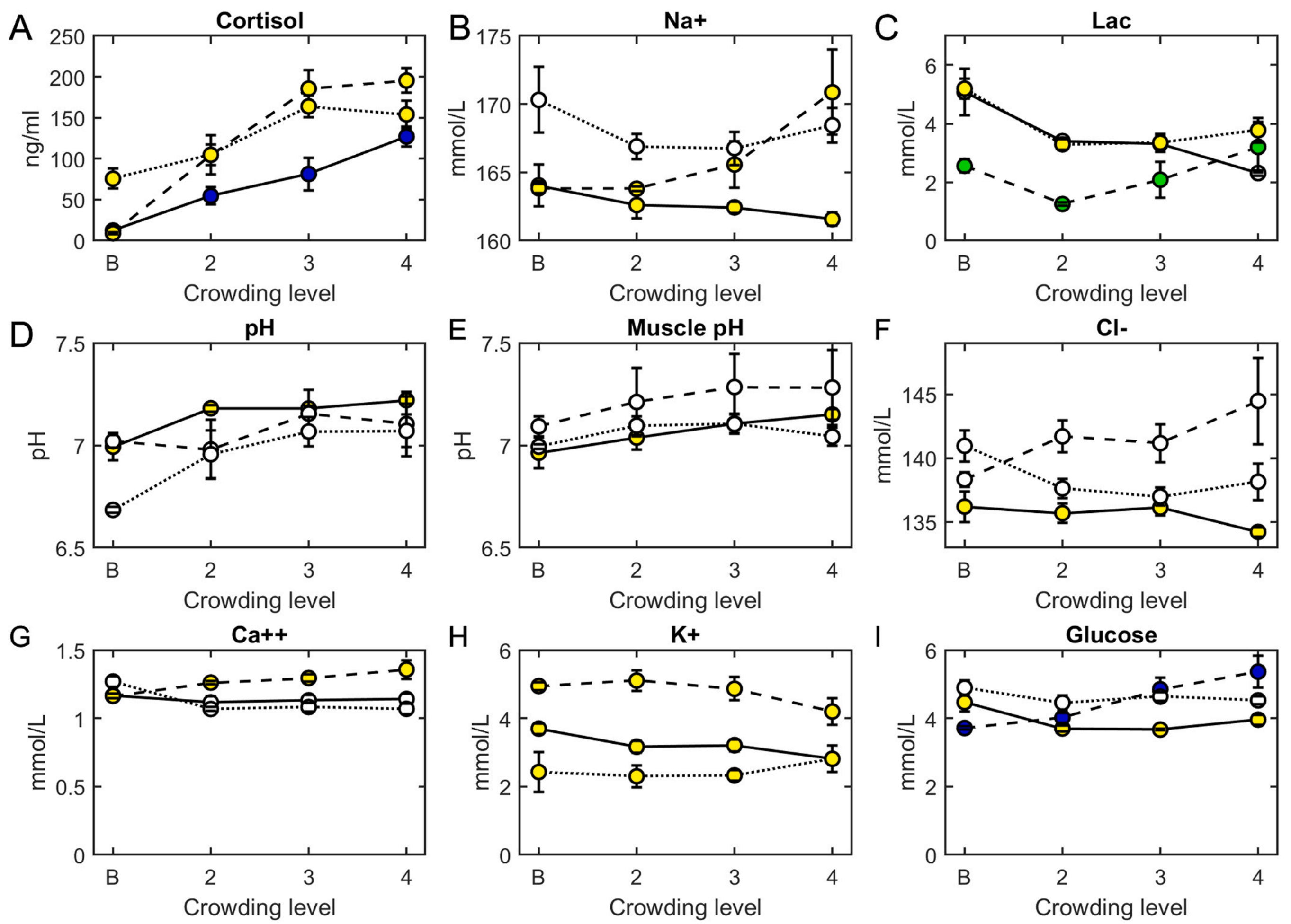

Fig. 9. Plasma parameters and muscle pH (mean ± SE) sampled at the October (solid line), the January (dashed line) and the May (dotted line) crowding events. A) Plasma cortisol, B) plasma sodium (Na^+), C) plasma lactate, D) plasma pH, E) muscle pH, F) plasma chloride (Cl^-), G) plasma calcium (Ca^{++}), H) plasma potassium (K^+) and I) plasma glucose. The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, blue if the baseline also is following this pattern, white if L2 and L4 are not significantly different from each other, and otherwise yellow (Welch two sample t-test).

ropes to pull, the operator monitoring the underwater cameras still sometimes had to signal that corrections were needed to avoid the creation of pockets, irregular net walls or situations that could otherwise trap the fish or create places where fish could collide and harm themselves against the net. It is therefore clear that although the crowding intensity scale often works as intended, crowding should not only be monitored from the surface, but also from below in order to reduce sub surface risks to the fish. Commercial sea cages in Norway are often equipped with winch cameras, and farmers usually also have access to an ROV to inspect their nets. Farmers also sometimes use these ROVs to monitor crowding operations they perceive as especially risky (pers. obs.).

4.2. Was an increased crowding intensity detrimental for long term animal welfare?

The plasma cortisol data indicated that stress increased with increased crowding intensity, and the histology of the skin sampled immediately after crowding indicated increased damage to the skin. However, the absence of increased mortality in our study, coupled with no differences in the amount of external injuries documented in the month after the crowding events, suggests that these negative effects were not long term or overtly detrimental for the fish in our settings. As documented by the ROV-recordings, the fish were usually swimming calmly, and although fish occasionally touched or became pressed into the net, there was generally a little distance between the school and the net, also at what we perceived as level 4. Skin damage from crowding alone was therefore likely not severe enough to have long term effects. There was no evidence of rapid swimming and panic in the crowded fish. The situation can perhaps be likened with that of a rock concert where one is forced to stand closely together, but where there is still room for moving through the crowd to get to the lavatory. It is likely that panic and danger first arise if one feels at risk of, or becomes stuck in the crowd, and that this level of immobility and lack of behavioural control was not present in the crowding events undertaken in this study. Calm behaviour during crowding is consistent with some previous controlled trials (Erikson et al., 2016; Speilberg et al., 2018), but not others (Føre et al., 2018). This indicates that each crowding situation is a unique combination of multiple behavioural drivers, and that for the most part salmon behaviour during crowding should be calm, and the steering of the crowding event should achieve this desired effect.

In this study, the fish were released and allowed to recover immediately with no further stress. The increased stress and the reduced epidermal integrity with increasing crowding intensities can however be more detrimental in applied settings as crowding is normally the first step in a wider handling procedure. The changes in skin status observed via histology were not detected when using skin based OWIs. Skin breaches and lost epidermis, as we observed at increasing crowding intensities, may weaken the skin's function as a barrier tissue. The integrity and continuity of the epidermal cells, the keratocytes, are

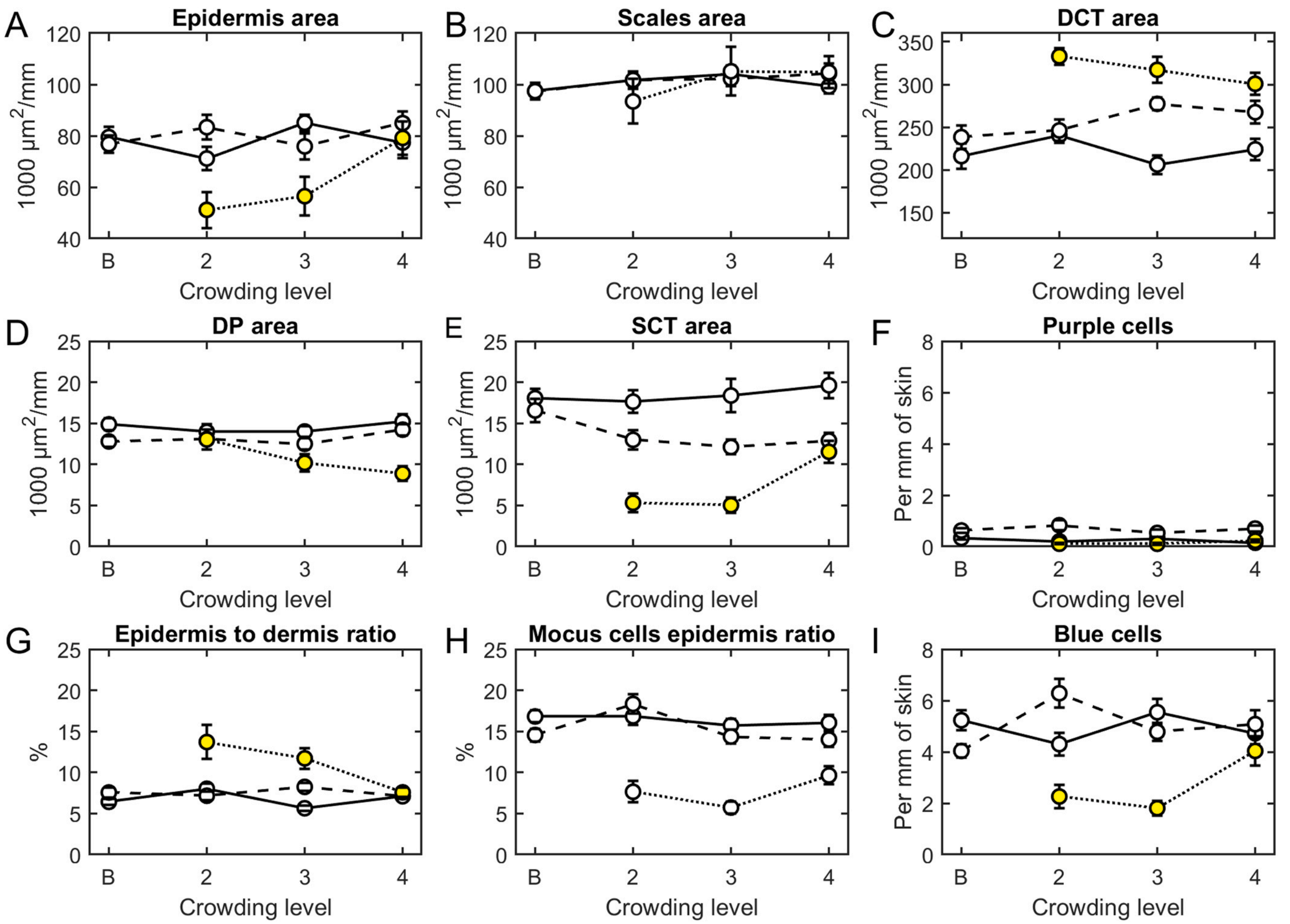

Fig. 10. Skin characteristics (mean ± SE) analysed using neural network analysis of skin samples taken at the October (solid line), the January (dashed line) and the May (dotted line) crowding events. A) Epidermal area, B) Areas of scales, C) dense connective tissue (DCT) area, D) area of dark pigment (DP), E) area of scales and scale connective tissue (SCT), F) number of purple mucous cells, G) ratio of epidermis to dermis area, H) ratio of mucous cell area to epidermal area and I) number of blue mucous cells. The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, blue if the baseline also is following this pattern, white if L2 and L4 are not significantly different from each other, and otherwise yellow (Wilcoxon rank sum test).

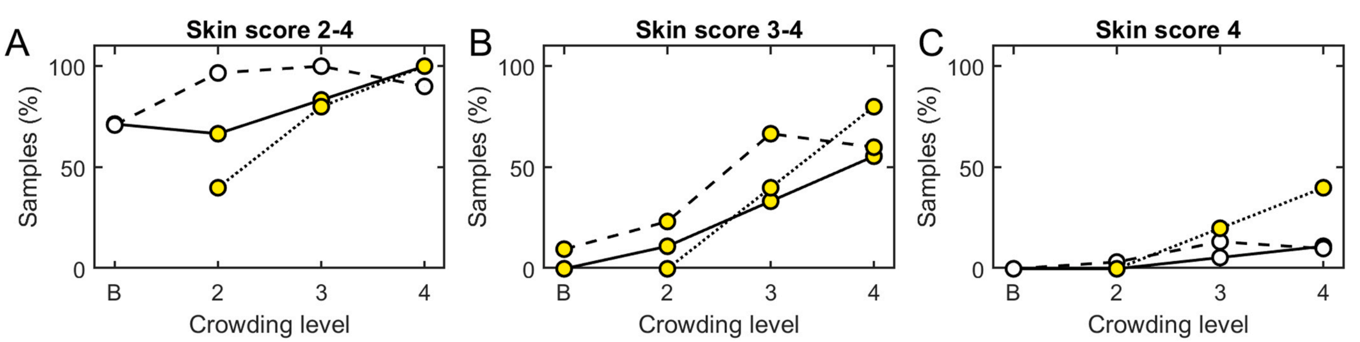

Fig. 11. Manual scoring of the skin sample sets taken at the October (solid line), the January (dashed line) and the May (dotted line) crowding. A) percentage of samples with score 2–4, b) score 3–4 and c) score 4 (see Fig. A.6 for example of scoring levels). The circles are green if L2, L3 and L4 are consistently greater or smaller than each other, yellow if only L2 and L4 are significantly different, and otherwise white (Fisher's exact test).

central for maintaining barrier functions of the skin (Chang and Hwang, 2011). Thus, the damages observed here may result in fish being increasingly predisposed to secondary infections and further wound developments. The reduced keratocyte migration rate that we documented with increased crowding intensity may further indicate a reduced healing capacity in the skin. Upon wounding, the keratocytes move rapidly as a continuous sheet to cover the wounded and exposed area (reviewed in Sveen et al., 2020). In an industry scenario, the crowding of cage held salmon would typically be followed by some kind of delousing treatment or transportation process. An increased stress load coupled with reduced epidermal integrity and loss of the outermost keratocytes seemed to affect keratocyte migration capacity. Whether the reduced migration is caused by stress from the crowding *per se* or was due to the epidermis being lost and damaged, is difficult to say with the

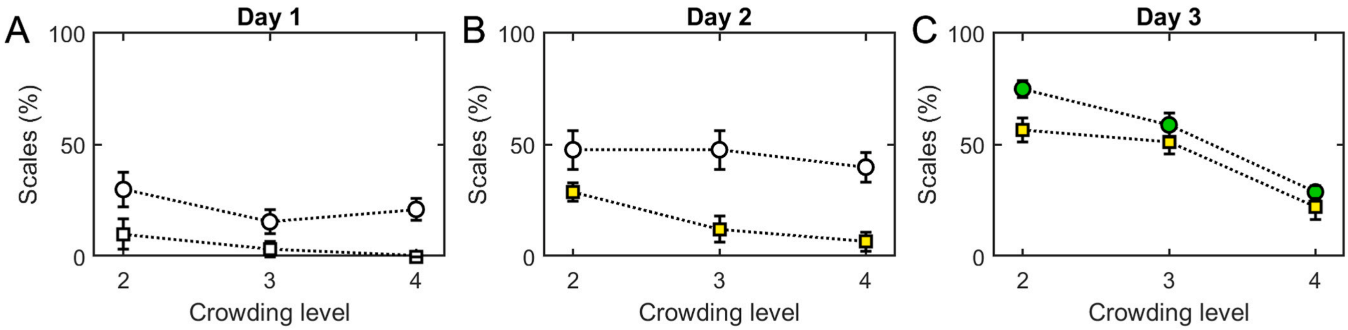

Fig. 12. In vitro cell culture model using salmon skin keratocytes, from skin sampled at the May crowding. A –C) and the migration capacity as measured as percentage of scales with migrating cells after 1–3 days. Circles are non-exposed while squares are after exposure to H_2O_2. The symbols are green if L2, L3 and L4 are consistently greater or smaller than each other, yellow if only L2 and L4 are significantly different, and otherwise white (Wilcoxon rank sum test).

applied methods. However, the *in vitro* trial showed that the migration may be further reduced when fish are exposed to an additional stressor, such as exposure to anti-parasitic oxidative chemicals like H_2O_2. Similar effects are also observed on lumpfish (Ytteborg et al., 2023) and cod (Ytteborg et al., 2020) when subjected to the same chemical treatment. Thus, precautions should be taken after crowding procedures to ensure that micro damages in the skin are allowed sufficient time to recover and the full barrier functions of skin restored. The duration of this recovery period, and factors driving this were not the subject of this experiment but could be considered in future studies.

Although not part of the planned experimental setup, the high risk associated with industrial thermal delousing was clearly demonstrated in the current study. These delousings had to be done in accordance with the standard routines of the delousing boat and each cage was therefore crowded to level 4 before the fish were pumped into the thermal delousing system. At the first delousing, average weekly mortality across all cages went from 0.6±0.1 ‰ the week before delousing to 4.7±0.3 ‰ the week after, and for the second delousing weekly mortality went from 1.3±0.2 ‰ the week before delousing to 10.8±1.6 ‰ the week after. In comparison, there was only one such isolated case of elevated post-crowding mortality for the planned crowding events conducted in this experiment (cage 9, level 4 in the January crowding event). The fact that the mortality was so high after these delousings, illustrates that although there is a risk associated with crowding, the risk for poor welfare can be far higher when it is followed by an industrial thermal delousing.

A lack of a statistically significant increase in external injuries and mortality with crowding intensity level does not mean that there was no such increasing risk in all situations, even without a subsequent treatment adding to the risk. It merely demonstrates that the crowding events in our experiment did not markedly impact upon fish injury levels. The crowding events in the current experiment were undertaken using relatively few fish compared to a commercial situation where each cage can contain up to 200,000 fish, and in relatively small, easy to handle cages (12x12x12m^3) compared to commercial cages that typically are 50 m in diameter and that can have 20–50 m deep nets. Considerations related to scaling up of both fish cages and the number of fish probably increases the risk of fish experiencing mechanical trauma related injuries by becoming trapped amongst each other, towards or against the net or becoming stuck in net pockets in an industry situation. There are also some logistical considerations to pay attention to as it is difficult to have full control when pulling ropes and adjusting nets when everything is happening under water. Even with underwater cameras (Fig. A.2–3) and ROVs (Fig. A.4) one still only has a limited overview of the situation as the crowding progresses.

Although water quality parameters in our study were not detrimental to fish welfare at the locations that we documented them, it is clear that stressed fish crowded together has the potential to create hypoxic conditions and a poor water environment. It is therefore not unreasonable to assume that level 4 is riskier to the fish than level 2 and 3. Increased risk can increase the likelihood of welfare problems that can ultimately be fatal if not adequately detected or acted upon. In our case we only had one instance of clearly increased mortality after a crowding event compared to the week before crowding (cage 9 in the January crowding event). This cage was crowded to level 4. In statistical terms one increase in mortality of nine is often considered an outlier, but in the industry, it might be just these outliers that are the problem. However, proving a 1 in 9 (we carried out nine level 4 crowding events in total, 3 occasions in triplicate) probability of elevated mortality would require a much larger dataset. In an industry situation the risk associated with increased crowding intensity must be balanced against the need for getting the fish into the pump and limiting the total holding time of the fish.

There was also no evidence of differences in growth between the fish crowded at the different levels in our study. All in all, we therefore find it reasonable to state that the crowding events carried out in the current study were not detrimental for long-term animal welfare, but still caution that this might not be the case for similar scenarios in the industry where there will typically be 10–30 times as many fish. The risk severity of crowding fish to level 4 under commercial situations should therefore be investigated in future studies before concluding that level 4 is safe, especially due to the absence of other detrimental factors such as subsequent handling in our study. It must also be underlined, that given that the crowding scale is inherently subjective, it might be that we have been too careful and used a weak level 4, while a strong level 4 could have had more detrimental effects on the fish in our study.

4.3. Which of the indicators are suitable as OWIs and LABWIs for monitoring crowding?

The third goal of this study was to audit the utility of a range of existing and emerging welfare indicators to be used in OWI- or LABWI-toolboxes for monitoring the crowding of salmon in sea cages. Firstly, it immediately became clear during the October crowding that monitoring surface behaviour is not sufficient for safeguarding fish welfare during a crowd, and that complementary subsurface observations are also needed. This was not only because the fish did not display surface activity before suddenly displaying level 4 activity with no prior warning, but also because underwater camera monitoring can minimize welfare risks that are difficult to ascertain from the surface, such as the formation of net pockets or due to fish being pressed against the net by the crowd. Fish that are stuck in the group, or against the net, are obviously at risk of physical injury, potential asphyxiation and have no freedom to escape a potentially poor environment, for example a locally formed water volume with poor oxygen conditions. Whilst in this study we never documented situations involving detrimental oxygen conditions, they might occur in real case scenarios, where the number of fish will be far greater. Although, we did not detect any increase in the frequencies or severities of injury based OWIs due to crowding, we still recommend the continued use of the injury-based OWI documentation tools, such as the LAKSVEL OWIs (Nilsson et al., 2022) for documenting injuries. Any appearance of such injuries would be a clear sign that the crowding has been rough enough to cause injury to the fish. If the farmers observe changes in injury status and the appearance of fish with new injuries, they should identify the source, rectify, or terminate the crowd operation. Similarly, if unresponsive, unconscious, or dead fish are registered, this is also a clear warning that the crowding is creating poor welfare, and that either something has gone unnoticed, or that the health status of the fish is too poor to tolerate the operation. In addition to the real-time monitoring of the fish from above and below the surface to adjust the crowd, we also tested out a qualitative set of behaviours and observations based on camera recordings. These generally performed quite well, and since they were relatively easy to carry out and don't require any special equipment, they have potential to be included in an OWI-toolbox. However, as descriptions of behaviours were ad-hoc, and since it was possible to guess the crowding levels from the films, the assessments therefore were not blinded, and the methodology must be considered as a first try, and not ready for general use. A fixed term Qualitive Behaviour Assessment (QBA) similar to those already proposed for Atlantic salmon by Jarvis et al., (2021) or Wiese et al., (2023) may also have utility in these situations. It is interesting to note that even though surface activity for level 4 was judged as very high from the recordings from cameras positioned above the surface, the ROV-recordings showed that the fish were swimming relatively calmly below the water line. This is probably not a discrepancy, but the result of that a water surface filled with moving fish backs and fins is assessed as high surface activity even when the fish are moving slowly. It is also likely that individual fish that become forced by the crowd against the surface will try to escape, creating splashes and showing their white sides, while the overall school remains relatively unperturbed. To summarise, an OWI-toolbox to monitor crowding should build upon that proposed by Noble et al., (2018) and include both surface and underwater observations, and at least document oxygen saturation and changes in the prevalence and severity of external damages. These are all observations that easily can be done by a farmer.

LABWIs are not tools for everyday situations and it cannot be expected that farmers take blood, muscle and skin samples during or after every crowding operation. However, they offer utility to both researchers and fish health professionals, especially when trying to understand the impact of a crowding event on fish welfare and move beyond indicator based welfare audits and towards diagnostics (Djordjevic et al., 2021; Erikson et al., 2022; Madaro et al., 2022; Patel et al., 2022). In our study, the plasma cortisol welfare indicator performed relatively well with values that increased between crowding level 2 and 4 at all crowding events. It had similar values for the January and May crowding events where we were able to crowd fish according to the crowding intensity scale as planned. The lower values for the October crowding, where we had to rely on the underwater observations, can indicate that this crowding was gentler, especially for crowding level 3. The reason for the poor performance of the other plasma parameters may be because the fish were not only pulled up into varying crowding intensities, but also varying water qualities, e.g., salinities and temperatures. Further, even though plasma cortisol successfully distinguished between all crowding intensity levels at the October crowding event, there were no difference in cortisol between level 3 and 4 at the January and May crowding events. This may be because the fish were swimming slowly amongst each other, even at a higher intensity at level 4, and this behavioural control may have meant these fish were not more stressed than those at the lower crowding intensity. Cortisol values were also similar for the January and May crowding events, which were executed in a similar manner, while they were slightly lower for the October crowding event where the crowding had to be done based on underwater observations. In the study carried out by Erikson et al. (2016) who appear to have crowded the fish to level 4, according to our audits of their crowding pictures in the article, cortisol levels reached 266 ng ml^{-1}, slightly higher than the average cortisol values measured for level 4 here. In addition, the Speilberg et al. (2018) study on sedation during crowding, crowded the fish to level 2, and the controls expressed mean cortisol levels of around 136 ng ml^{-1}, which also is similar to our values for level 2 at the January and May events. Although cortisol seems to be the most promising plasma parameter to be included in our LABWI-toolbox, cortisol has its weaknesses. Plasma cortisol levels may be affected by a host of other factors in addition to crowding, including temperature (Madaro et al., 2018), the maturation status of the fish (Couch et al., 2022), infections (Fjelldal et al., 2020; Hvas et al., 2017) and more. It might also be difficult to get reliable cortisol baseline values as illustrated by the May crowding event. However, all in all, plasma cortisol does appear to be a promising LABWI for monitoring stress levels in fish in connection with crowding.

The parameters from the neural network analysis did not correlate with crowding intensity as well as expected. This can be explained by the epidermis being damaged or absent in many of the skin samples. This was mostly related to the October and January samplings. The results were however better for the May crowding. Due to covid-19 restrictions there were limitations on the number of people who could be at the farm at the October and January crowding events, while at the May crowding a histology expert was able to join the sampling and be dedicated to only doing this task. This implies that this technique requires high quality samples, and that it therefore might not be suitable to sample without specialist training beforehand. In contrast, the manual histology scoring showed clear differences between fish that had been crowded to intensity level 2 and fish that had been crowded to level 4 for the skin sample sets taken at all three crowding events. These results were also supported by increasing reductions in the migration capacity of the keratocytes with increased crowding intensity. Thus, the integrity of the epidermis looks like a promising LABWI candidate for documenting the health and welfare effects of crowding. Especially since the skin histology revealed increasing level of skin damage in tandem with crowding intensity that were not visible to the naked eye.

In conclusion an OWI-toolbox for crowding should include both surface and underwater observations to make sure that the fish are able to move among each other and that both the crowding process and the net itself retain a smooth and pocket free structure. Measuring oxygen to ensure the prevention and avoidance of hypoxic conditions, and using morphological injury data to steer decisions and act upon conditions of welfare risk will help prevent problems and mortalities. In addition, qualitative assessment of fish behaviour, plasma cortisol, and skin histology can be included in a LABWI-toolbox if more in-depth information on the effects from the crowding is wanted.

This work has audited the utility of the widely recommended range of existing OWIs for documenting fish welfare during crowding in sea cages, in addition to some emerging candidate behavioural OWIs and skin based LABWIs. The lists of OWIs and LABWIs tested here is of course not exhaustive, and existing and new OWIs and LABWIs may also be suitable for inclusion in toolboxes for monitoring crowding operations. These could be digital tools that automate the monitoring of some of the behavioural indicators to improve their accuracy, precision and range of auditing capabilities compared to the list of behavioural parameters we have suggested. Crowding has also been documented using heart rate monitors for a range of species including Atlantic salmon (e.g., Warren-Myers et al., 2021) and whilst the data they generate has real utility as a LABWI, their surgical implantation and subsequent recovery from a large group of fish farmed at commercial scales may challenge their commercial applicability. Another possible OWI is the latency of return of appetite after the crowding event. However, as the fish in our study were fed according to tables, as provided by the feed producer and as per standard on our research facility, it was not possible to record latency of return to appetite after the crowding events in this study. For it to work as a welfare indicator it would also be necessary to ensure that the personnel controlling the feeding are not aware of which cages had been crowded to the different levels. However, in the industry, feeding is increasingly controlled from remote feeding centres, where the ones judging the appetite of the fish do not necessarily have to know which treatment the fish have undergone. It is therefore possible that latency of return of appetite could prove a good OWI for the industry. This should be explored in future studies.

CRediT authorship contribution statement

Angelico Madaro: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Gerrit Timmerhaus:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Elisabeth Ytteborg:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **David Izquierdo-Gomez:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Chris Noble:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization. **Jonatan Nilsson:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Lars Helge Stien:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lars Helge Stien and Chris Noble reports financial support was provided by Norwegian Seafood Research Fund. David Izquierdo-Gomez, Elisabeth Ytteborg, Gerrit Timmerhaus and Angelico Madaro 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

Data will be made available on request.

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Appendix A

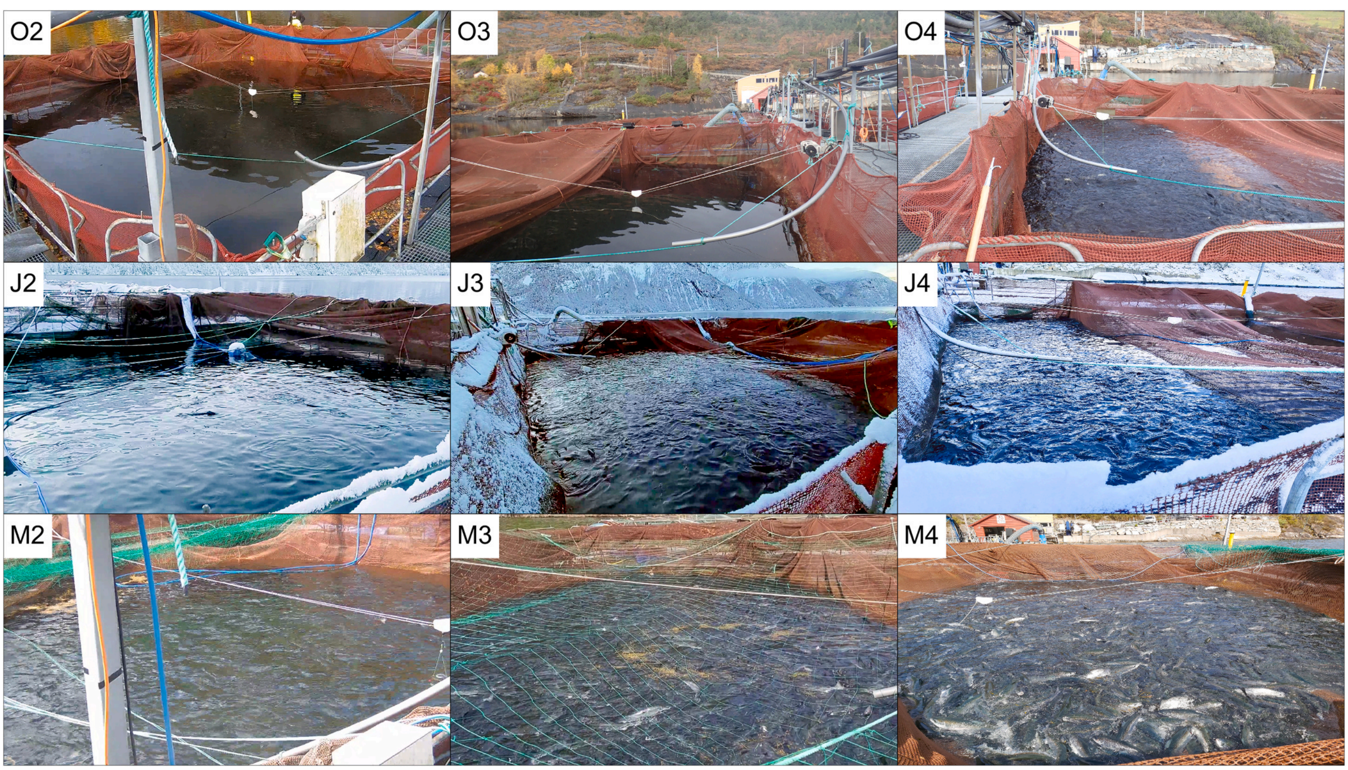

Fig. A.1. Example screenshots from the surface cameras from the October (O2–4), January (J2–4) and May (M2–4) crowding events at crowding levels 2, 3 and 4.

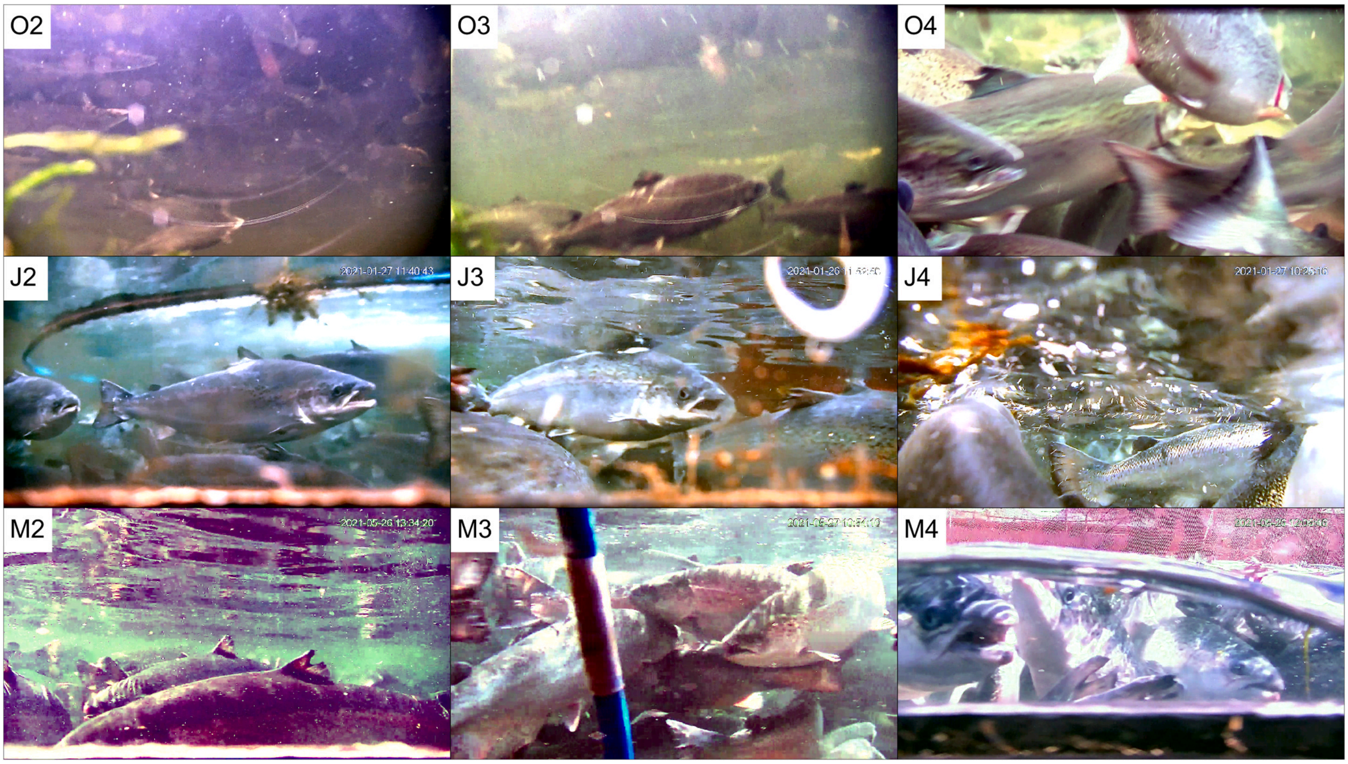

Fig. A.2. Example screenshots from the subsurface winch cameras filming just beneath the surface from the October (O2–4), January (J2–4) and May (M2–4) crowding events at crowding levels 2, 3 and 4.

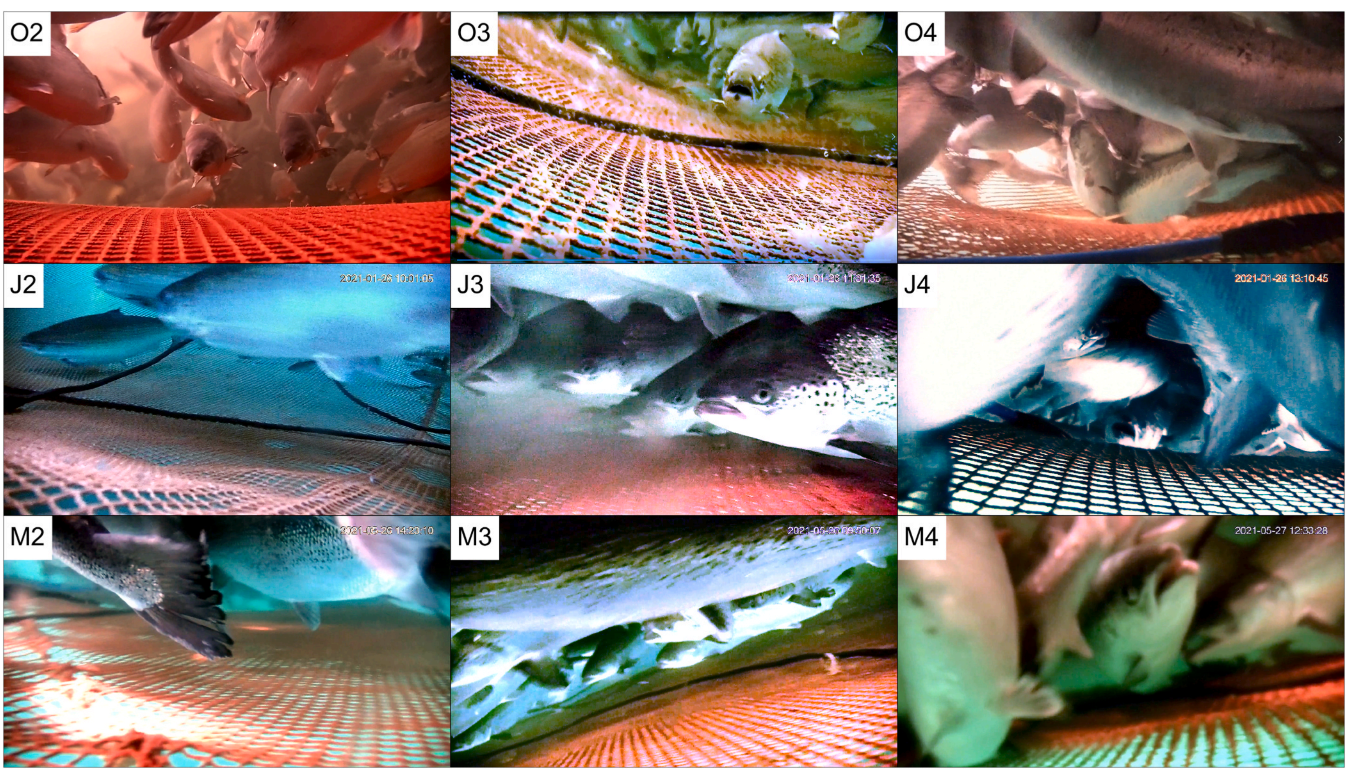

Fig. A.3. Example screenshots from the subsurface winch cameras filming close to the bottom of the net from the October (O2–4), January (J2–4) and May (M2–4) crowding events at crowding levels 2, 3 and 4.

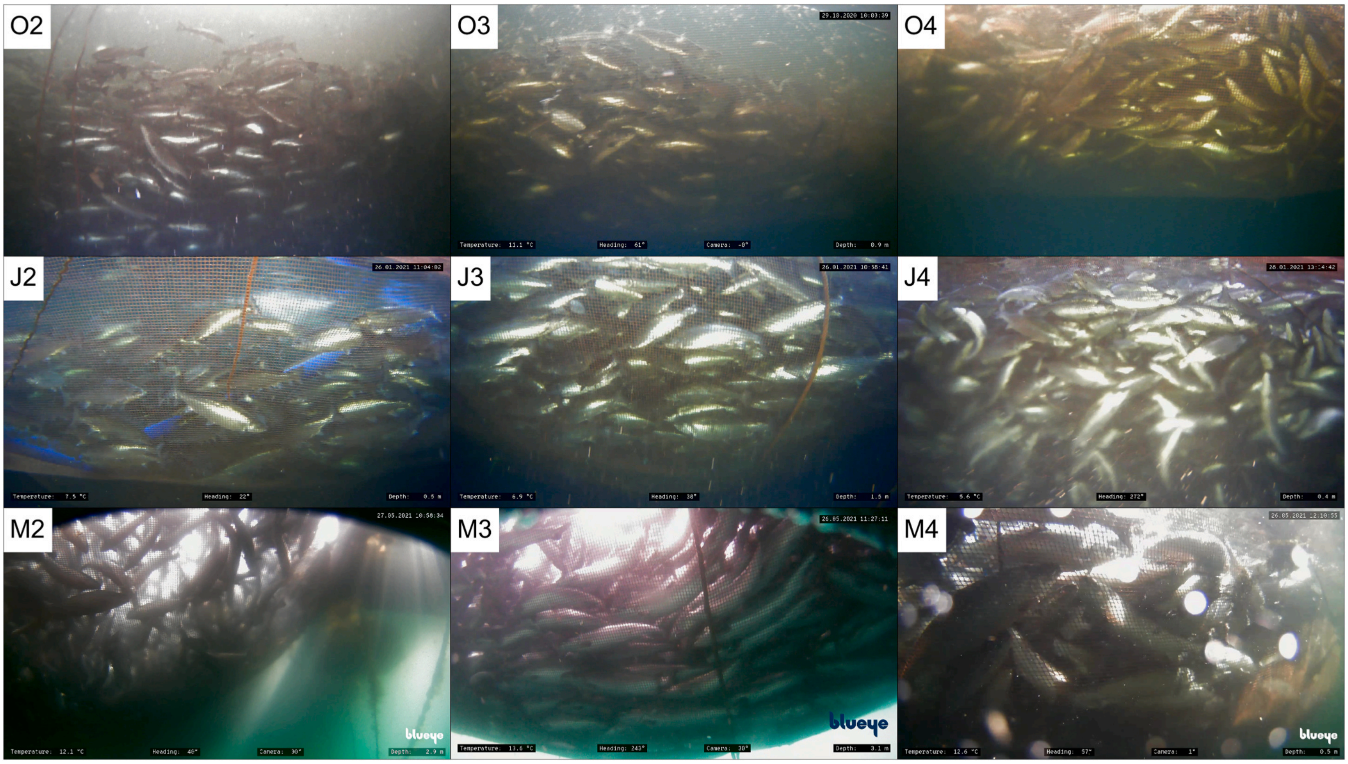

Fig. A.4. Example screenshots from ROV films from the October (O2–4), January (J2–4) and May (M2–4) crowding events at crowding levels 2, 3 and 4.

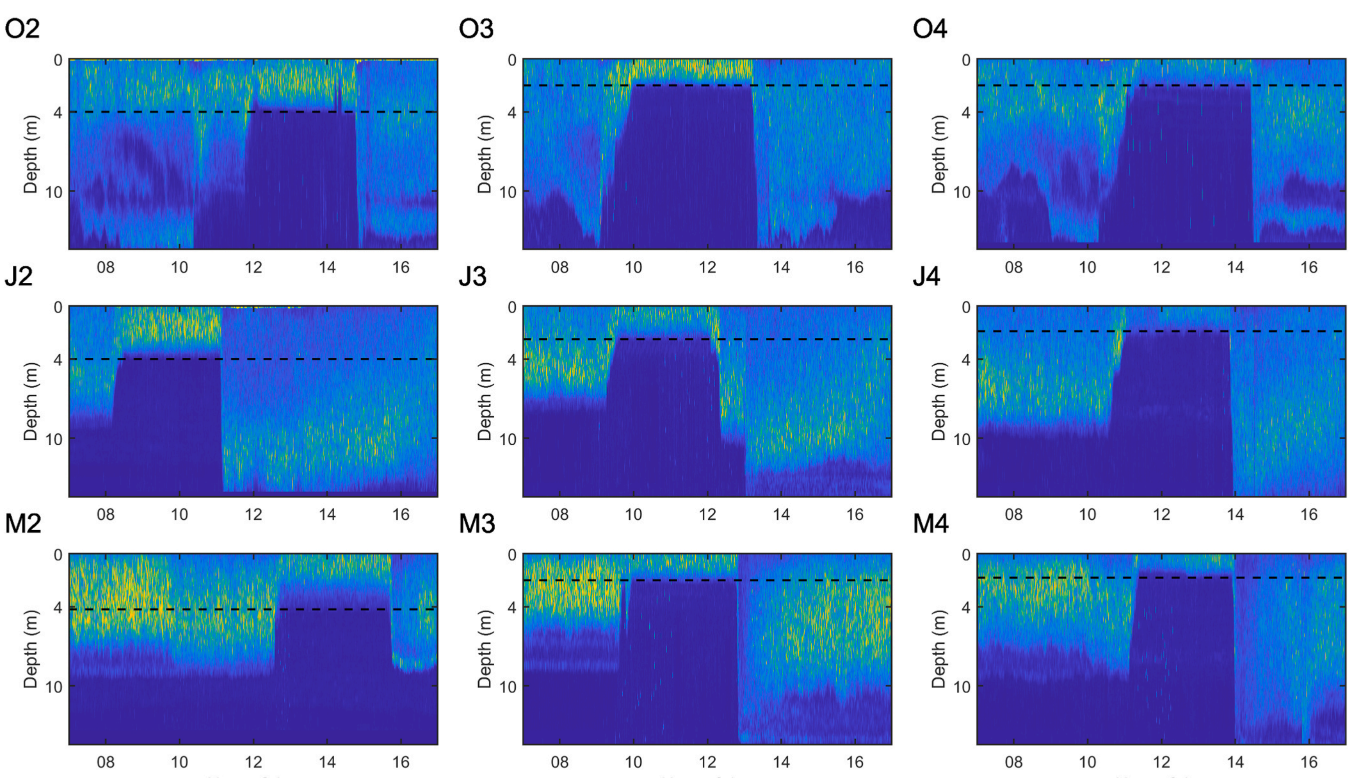

Fig. A.5. Echo sounder data from cages crowded in October (O2–4), January (J2–4) and May (M2–4), at crowding intensity level 2,3 and 4. The superimposed dashed lines indicate the approximate maximum depth of each crowd.

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