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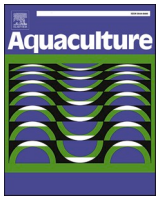

Early warning through video monitoring: Dissolved hydrogen sulphide (H_2S) affects Atlantic salmon swimming behavior in recirculating aquaculture systems

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ABSTRACT

Hydrogen sulphide (H_2S) poses a major threat in marine land-based recirculating aquaculture systems (RAS) leading to acute mortality in sensitive fish species such as Atlantic salmon (*Salmo salar*). To date, little is known about the effects of H_2S on the physiology and behavior of the species. The present study analyzed Atlantic salmon swimming behavior in response to H_2S in a controlled trial. The setup consisted of two Recirculating Aquaculture Systems (RAS) in parallel. The control RAS comprised of one fish tank (800 L, 10 kg fish/m^3 (≈ 70 fish)), while the exposure RAS included two fish tanks (800 L; 10 and 30 kg fish/ m^3 (≈ 70 and 200 fish)). Swimming behavior was monitored via a submerged custom-built stereo camera system and an overhead surveillance camera. Fish (smolt, ≈ 114 g) were exposed once a day for 10 consecutive days to increasing H_2S concentrations, from ≈ 1- up to ≈ 68 μg/L (2 μM). Continuous measurements of dissolved H_2S, O_2 and CO_2 were taken using a real-time monitoring system. Three swimming parameters were extrapolated from video recordings using machine learning algorithms: i) speed, ii) pattern (representing whether the fish swim in a straight or zigzagging direction) and iii) dispersion (indicative of schooling behavior). The results showed that fish reacted rapidly to H_2S, with a stress response characterized by faster swimming speed, erratic pattern, and loss of schooling behavior. The response was concentration-dependent, increasing linearly up to 30–40 μg/L, above which a clear threshold was observed. Notably, concentrations around 40–50 μg/L, induced significantly greater behavioral changes compared to lower concentrations, and further increases in H_2S did not lead to additional changes in behavior. Swimming parameters quickly returned to basal levels, comparable to the one's prior exposure, once H_2S was no longer present in the water. This study provides new insights on the sensitivity of Atlantic salmon to acute H_2S exposure and highlights the potential behind the use of machine vision as an early warning tool for poor water quality in RAS.

1. Introduction

Hydrogen sulphide (H_2S) poses a major threat in marine land-based recirculating aquaculture systems (RAS) causing acute mortality in different fish species including salmonids (Hjeltnes et al., 2019; Sommerset et al., 2020). Incidents were also reported in systems using brackish and seawater for post-smolt salmon farming, where the H_2S production is increased by the high sulphate concentration in the water (Letelier-Gordo et al., 2020). The presence of H_2S in RAS and its toxicity on fish species is connected with its chemo-physical properties.

Sulphide can be present as three chemical species in aqueous solutions: The toxic H_2S gas, or the relatively harmless ionic forms, hydrosulphide (HS^-) or sulphide (S^{2-}) (Smith et al., 1977). Temperature, pH and ionic strength of the solution influences the fraction of each species (Millero et al., 1988). Dissolved H_2S can easily diffuse from the ambient water across the gill epithelium and biological membranes (Hunn and Allen, 1974). The toxic effects of H_2S are exerted at the level of mitochondria by inhibiting cellular respiration and blocking aerobic

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adenosine triphosphate (ATP) production (Cooper and Brown, 2008; Szabo et al., 2014). The sensitivity to H_2S varies between species, probably due to physiological adaptations to specific habitats, and critical concentrations leading to acute mortality (LC_{50}) range from 0.25 to 53 μM (Bagarinao and Vetter, 1989; Smith and Oseid, 1974). Limited information is available regarding toxicity threshold in Atlantic salmon. Physiological studies showed that H_2S impairs metabolism, by decreasing oxygen uptake below the standard metabolic rate, from a concentration of 1.78 ± 0.39 μM (60.7 ± 13.3 μg/L) (Bergstedt and Skov, 2023). While concentration threshold and recovery time were independent of fish size, the excess oxygen consumption during recovery was greater for smaller salmon, indicating a more severe impact on smaller animals.

The production of H_2S in RAS is mainly carried out from sulphate-reducing bacteria (SRB) via a process known as dissimilatory sulphate reduction which use sulphate as terminal electron acceptor when degrading organic matter (Muyzer and Stams, 2008). Such bacterial communities can often accumulate in biofilters under anaerobic conditions (Rojas-Tirado et al., 2021) during which H_2S can reach lethal level inducing sudden mass mortalities (Sommerset et al., 2020). Despite the potential danger, there is currently a lack of early warning tools to signal the presence of H_2S in RAS. Detecting low concentrations of dissolved H_2S remains challenging, even with the latest state-of-the-art sensors available today, and the industry has yet to offer a reliable and user-friendly system (Lien et al., 2022). Furthermore, any system would provide H_2S readings at the specific locations where the sensors are placed. Therefore, the use of multiple sensors is essential to obtain a reliable representation of H_2S levels in the RAS.

Video monitoring systems coupled with artificial intelligence, or machine learning algorithms, might bypass the technical challenges for the detection of H_2S by directly observing sign of distress from fish behavior. This might represent a promising integration to fish health monitoring in RAS. Such tools have been used for fish species identification, counting and behavior analysis (Yang et al., 2021). Most of the automated image and video analysis work in aquaculture has been dedicated to estimate fish size and biomass (Costa et al., 2006; Naiberg and Little, 1994). Traditionally, swimming behavior has been observed from farm operator to judge how well fish respond to management practice and environmental changes (Brown et al., 2006). In captive environments, behavioral responses may be varied (Brännäs et al., 2001). Rapid burst in swimming speed and aggressive behavior may occur as a response to stocking density or competition for feeding (Kadri et al., 1991). Reduction in swimming speed and response time are often a result of poor health condition (Furevik et al., 1993). Schooling behavior may vary as a response to stocking density (Juell and Westerberg, 1993), feeding regime (Ang and Petrell, 1998; Juell et al., 1994) or photoperiod (Juell, 1995). A software analyzing Atlantic salmon swimming behavior (speed and direction) was developed from video recording of commercial sea cages (Pinkiewicz et al., 2011). The changes in swimming behavior were associated with daily shift in tidal cycle. Indeed, video recording and image analysis techniques might offer useful information on fish health status and provide early warning tools for poor water quality.

Suboptimal water quality (including the presence of sublethal H_2S concentration) might induce stress in teleosts, therefore impairing growth, reproduction, immune system and ultimately fitness of the animal (Ellis et al., 2012). Cortisol has proven to be a reliable indicator of stress and is considered the major stress hormone (Sadoul and Geffroy, 2019). Initially principally measured in blood or scales, non-invasive cortisol measurement methods includes water and feces sampling (Sadoul and Geffroy, 2019).It is hypothesized that the presence of dissolved H_2S in RAS water induces a stress response in Atlantic salmon, leading to significant alterations in their swimming behavior. The aim of the here presented research is to identify changes in swimming behavior of Atlantic salmon related to the presence of H_2S in RAS to form a solid base for the development of cost-effective early warning tools in RAS.

2. Material methods

2.1. Ethical statement

The experiment was performed according to EU regulations concerning the protection of experimental animals (Directive 2010/63/EU). Appropriate measures were taken to minimize pain and discomfort. The experiment was approved by the Norwegian Food and Safety Authority (FOTS id. 29,143).

2.2. Experimental setup

The experiment was conducted at the Recirculating Aquaculture Systems research facility at LetSea AS, Bjørn, Norway (Nordland; 66°05′ N 12°31′ E). The setup was composed of two RAS in parallel as seen in Fig. 1.

The control RAS consisted of one fish tank (800 L) and one biofilter, while the exposure RAS included two fish tanks connected to one biofilter with a water reservoir. Atlantic salmon smolt (*Salmo salar,* Salmobreed strain; mixed sex; average weight 114 g) were acclimatized to the setup for one week prior experiment start. The control and one of the exposure tanks were stocked with a density of 10 kg/m^3 (≈ 70 fish) The second exposure tank was stocked with 30 kg/m^3 biomass (≈ 200 fish). Photoperiod was kept at constant light. Fish were fed daily in the evening. Temperature was logged automatically every 10 min using a dedicated probe (OxyGuard, Denmark). Salinity, total ammonia nitrogen (TAN), nitrite and pH were measured daily (before H_2S exposure) with Photometer 7100 (Palintest, UK). The mean values (± SD) were as follows: Temperature 9.31 ± 0.59 °C; pH 7.4 ± 0.2 and salinity 19.56 ± 1.65 PSU.

Exposure to H_2S was conducted once a day at the same hour (approximately 8 AM) for 10 consecutive days. Sodium sulphide (Na_2S) was released in the reservoir tank by a peristaltic pump to produce H_2S according to the reaction $Na_2S + 2H_2O \rightarrow H_2S + 2NaOH$. Air bubbling and water exchange were interrupted for the duration of the exposure to avoid a rapid loss of H_2S out of the system. The target H_2S concentration was doubled every day, up to a final concentration of ≈ 65 μg/L or 1.9 μM (Fig. 2; Table 1). Dissolved H_2S, O_2 and CO_2 were continuously measured using Aquasense real-time monitoring system (SeaRAS Bergen, Norway) using a sensor located in the treatment tank with video recording. Target O_2 saturation (> 90%) was maintained throughout the whole trial period (Supplementary fig. 1). The instrument collected and analyzed water samples every second reporting periodical average reads every minute.

2.3. Video recording

Two video recording systems were used in the low-density tank in the exposure RAS (Supplementary fig. **2**). A custom built submerged stereo camera system recording at 15 frames per second (FPS) and 1080 × 1920 resolution filming horizontally across the tank (Supplementary video 1; Supplementary video 2) and a surveillance camera system (RLK8-800B4, Reolink, Hong Kong) recording at 25 FPS and 2560 × 1440 resolution vertically from above.

The recordings from the stereo camera system were analyzed using a modified version of Stereo RCNN (Li et al., 2019) to detect the fish-pairs in the left- and right images. The swimming trajectory in 3D (x, y, z [mm]) was derived using OpenCV v4.4.0 (Bradski, 2000) for stereo calibration and 3D positioning. The recordings from the surveillance

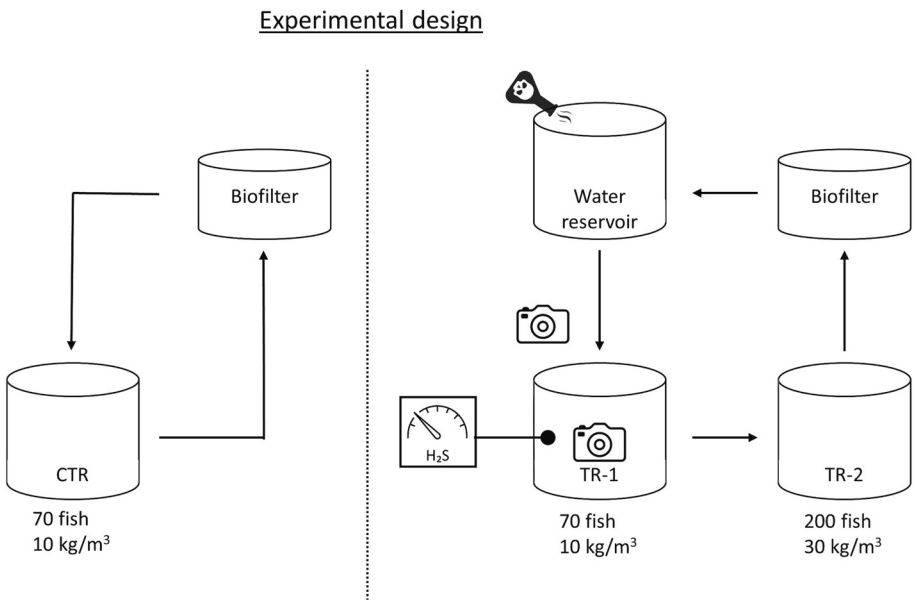


Fig. 1. Experimental setup. The control RAS was composed of a biofilter and a fish tank (800 L; 70 fish; ≈ 10 kg/m^3). The exposure RAS was composed from: a biofilter connected to a reservoir tank where the Na_2S was pumped to form H_2S; a first fish tank (70 fish; ≈ 10 kg/m^3) with the video recording system and the H_2S probe; a second fish tank with higher stocking density (200 fish; ≈ 30 kg/m^3).

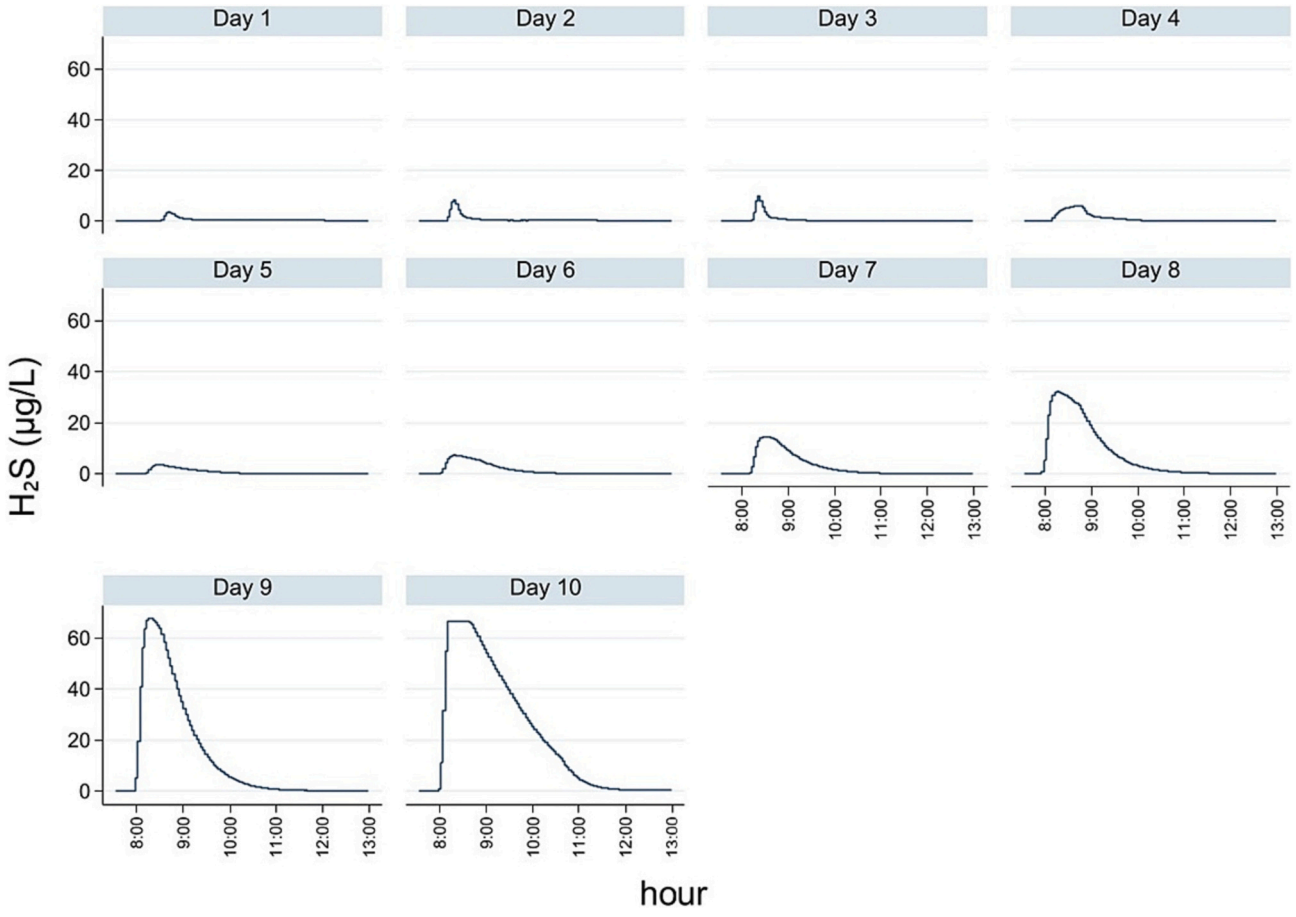


Fig. 2. Dissolved H_2S concentration measured with Aquasense real-time monitoring system. Increasing concentration of Na_2S were added daily around 8 AM.

Table 1
H_2S dynamics. The table summarizes, foe each experimental day, the max H_2S concentration, the time necessary to reach the peak, the average increment in H_2S concentration per minute; the time necessary for the system to revert to normal, and the average decrease of H_2S concentration per minute. The time was calculated using a threshold $H_2S = 0.2$ μg/L to overcome measurement noise. At day 10, the concentration took 5 h 17 min to go down to 0.3 μg/L and 8 h 33 min to go below 0.2 μg/L H_2S.

Day	Max [H_2S]		Time to peak	ΔH_2S	Time to norm.	ΔH_2S
	(μg/L)	(μM)	(m:s)	(μg/L*min)	(h:m:s)	(μg/L*min)
1	3.54	0.10	9:03	+0.39	3:41:09	−0.02
2	8.13	0.24	10:47	+0.75	3:22:07	−0.04
3	9.72	0.29	9:39	+1.01	1:20:56	−0.12
4	5.96	0.17	37:25	+0.16	1:26:07	−0.07
5	3.55	0.10	15:23	+0.23	1:45:38	−0.03
6	7.27	0.21	17:01	+0.43	2:13:44	−0.05
7	14.45	0.42	20:58	+0.69	2:29:15	−0.10
8	32.15	0.94	20:47	+1.55	3:17:01	−0.16
9	67.72	1.99	20:32	+3.30	3:46:19	−0.30
10	66.43	1.95	11:56	+5.57	5:17:44 (8:33:55)	−0.21

camera were analyzed with DDR-Net (Hong et al., 2021) to detect the fish in each image. The swimming trajectory in 2D [x, y (pixels)] was derived using Norfair v2.0.0 (Joaquín Alori et al., 2023).

From the trajectory data, time-series [$p_n(t)$] of three parameters were extracted to describe fish swimming behavior:

1. Swimming **Speed**.

The swimming speed of each individual fish [$v_n(t)$] was calculated as follows:

$$v_n(t) = \frac{p_n(t) - p_n(t-1)}{\Delta t}$$

Where $p_n(t)$ indicates the position of fish "n" at time "t". From this, the average swimming speed [$\bar{v}(t)$] of the population was calculated according to the following formula:

$$\bar{v}(t) = \frac{\sum_{n=0}^{n} v_n(t)}{n}$$

Where "n" indicates the number of individuals in frame.

Average swimming speed is a continuous non-negative variable where higher values indicate higher swimming speed. It is measured in pixels per second (2D video) or mm per second (3D video).

2. Swimming **Pattern**

Average swimming pattern [$\bar{p}(t)$] indicates whether fish swim in straight lines (values closer to 1) or in random directions (values closer to 0).

Swimming pattern was calculated as a moving average of a time windows of 5 s (or 150 frames [T] at 30 FPS) according to the following formula:

$$\bar{p}(t) = \frac{\sum_{n=0}^{n} \frac{\sum_{T=1}^{150} p_n(t-T) - p_n(t-1-T)}{p_n(t) - p_n(t-150)} - 1}{n}$$

3. Swimming **Dispersion**

Swimming dispersion [$\bar{d}(t)$], indicative of fish schooling behavior, was calculated as follows:

$$\bar{d}(t) = \frac{\sum_{n=0}^{n} \frac{p_n(t) - p_n(t-1)}{\Delta t} - \bar{V}(t)}{n}$$

Where $\bar{V}(t)$ indicates the average velocity vector of the fish population calculated, according to the following formula:

$$\bar{V}(t) = \frac{\sum_{n=0}^{n} \frac{p_n(t) - p_n(t-1)}{\Delta t}}{n}$$

This resulted in a continuous non-negative variable. Lower $\bar{d}(t)$ values indicates synchronization of swimming between fish (schooling behavior) while higher values indicate loss of synchronization.

All three parameters were reported as average values per fish in frame (FIF) in each time interval of one second.

2.4. Cortisol analysis

Water samples (250 mL, in triplicates per each tank) were collected with a syringe at the end of the acclimatization period, then 60 min after every H_2S exposure. Samples were pre-filtered from impurities via a syringe filter (Millipore. Darmstadt, Germany; 0.45 μm pore size, 33 mm diameter). Cortisol was extracted from water using C18 solid-phase extraction cartridges (Waters, Milford Massachusetts, USA). Cartridges were activated with 5 mL methanol (Sigma-Aldrich) and rinsed with 10 mL Milli-Q (MQ) water (Sigma Aldrich, DNase- RNase- Protease-free non DEPC treated). The pre-filtered water samples were pumped through the activated cartridges. The cartridges were then rinsed with 10 mL MQ water, and all excess was expelled by pressing air through the cartridge, before storing it at -20 °C until further use.

Cartridges were thawed at room temperature before elution with 10 mL methanol into glass test tubes (130 × 16 mm, VWR, Radnor Pennsylvania, USA). Methanol was removed in an evaporator (Reacti-Therm III heater, Thermo Scientific, Waltham Massachusetts, USA) at 25 °C in a fume hood. Water cortisol extractions were assayed using a commercial ELISA kit (Arbor Assays, Ann Arbor Michigan, USA) following manufacturer's instructions. Validation data from the producer indicated the following characteristics: sensitivity = 27.6-pg/mL; detection limit = 45.5-pg/mL; intra- and inter-assay coefficient of variance of 6.0% and 7.2% respectively. The operator was "blinded" regarding the origin of the samples [group (control vs treatment); timepoint].

2.5. Data analysis and statistics

Data were analyzed using Stata 17 (StataCorp, 2021).

Two databases were built including the swimming parameters (speed, pattern, dispersion) as averages values per second of recording from the stereo and the vertical system. As the Aquasense system reported H_2S concentration reads every minute, missing values (per second) were calculated by linear interpolation of available measurement. Different regression analysis were applied to assess statistical significance according to the scientific question and the type of data, as discussed in the dedicated paragraphs below.

In all models, the distribution of the dependent variables were visually assessed by histogram using the "gladder" function in Stata (Supplementary fig. 3, Supplementary fig. 4). This function searches a subset of the ladder of powers (Good, 1990) for a transformation that converts the variable into normally distributed, displaying histograms of the transformations. When necessary, variables were transformed selecting the method that yielded the best results after running test diagnostics of the final models, specifically the inverse or the log transformations. The independent variables were screened for multicollinearity by correlation matrix and exclusion of predictors with correlation $\geq |0.65|$. Multicollinearity was tested once more in each final model by evaluating the Variation Inflating Factors (VIF ≤ 5).

2.5.1. Regression analysis

Regression models were built to assess the effect of dissolved H_2S on swimming behavior. "speed" and "dispersion" were included as dependent variables in linear regression models, while a beta regression was used for "Pattern" (suitable for continuous variable ranging between 0 and 1).

The effect of dissolved H_2S on swimming parameters was investigated both alone, in univariable models, and adjusting for other parameters such as dissolved O_2, CO_2 and the number of fish in frame (FIF) in multivariable models. The independent variables were screened in univariable models applying a liberal cut-off of $p < 0.2$. Finally, multivariable models were built to assess the effects of filtered predictors. For the final models, the cut-off for statistical significance was set to $p < 0.05$. The most stable model was selected according to AIC-BIC values and LS test. Linearity and Homoscedasticity were tested via QQ plot and scatterplot of residuals versus predicted values, respectively. Autocorrelation was evaluated via Durbin–Watson and Breusch-Godfrey tests and adjusted for using Prais-Winstern estimation. This approach uses the generalized least-squares method to estimate the parameters in a linear regression model in which the errors are serially correlated. The regression coefficients and significance were compared to the original model.

2.5.2. Analysis of variance

Swimming parameters were grouped according to H_2S concentration in increments of 10 μg/L. Given the unequal variance (Bartlett's equal-variances test) and unequal sample size between groups even of transformed data, a non-parametric Kruskal-Wallis H test followed from Dunnett's multiple comparison test (Bonferroni adjustment) was conducted to examine the differences in swimming parameters according to H_2S concentration.

2.5.3. Cortisol measurements

Regression models were built to assess the effect of dissolved H_2S on water cortisol. "H_2S", "time (days)" and "stocking density" were included as dependent variables in linear regression models. The independent variables were screened in univariable models applying a liberal cut-off of $p < 0.2$. Finally, multivariable models were built to assess the effects of filtered predictors. For the final models, the cut-off for statistical significance was set to $p < 0.05$. The most stable model was selected according to AIC-BIC values and LS test. Linearity and Homoscedasticity were tested via QQ plot and scatterplot of residuals versus predicted values, respectively.

Table 2
Database from (A) vertical and (B) stereo recording system. Each observation represent the average value per one second of registration. (n) number of observations; (SD) standard deviation.

(A) Vertical camera

Variable	n	Mean	SD	Min	Max
Fish in Frame	170,355	15.69	3.82	0.04	27.04
Speed	170,355	45.29	16.58	19.53	549.53
Pattern	170,355	0.64	0.09	0.01	0.97
Dispersion	169,224	44.60	11.51	21.90	256.73
H_2S (μg/L)	170,355	4.99	12.98	0.00	67.72

(B) Stereo camera

Variable	N	Mean	SD	Min	Max
Fish in Frame	122,597	3.70	2.43	0.07	14.00
Speed	122,597	53.56	18.10	11.92	330.86
Pattern	122,597	0.60	0.11	0.00	0.98
Dispersion	107,312	44.67	12.26	11.28	216.16
H_2S (μg/L)	122,597	5.52	13.11	0.00	67.72

3. Results

3.1. Swimming behavior

Swimming behavior was monitored daily for 1–2 h before and after H_2S exposure and recorded video sequences were subjected to machine learning algorithms for parameterization and analysis (Supplementary figs. 5–7).

3.1.1. Descriptive statistics

The amount of video recording time from the vertical and stereo system slightly differed due to technical adjustment during the experimental period and to the lack of fish in frame for different periods of time. A summary of descriptive statistics is available in Table 2.

The database from the video recording from the vertical camera was composed from 170,355 observations for speed, pattern, fish in frame and H_2S while 169,224 observations for dispersion. The lower number is due to the necessity of at least two fish in frame to calculate the parameter. The database for the stereo camera was composed from 122,597 observations for speed, pattern, fish in frame and H_2S while 107,312 observations for dispersion.

The mean number of fish in frame was much lower for the stereo camera (3.7 ± 2.4) compared to the vertical (15.7 ± 3.8). Given that the vertical camera provided more representative results, with a higher number of fish in frame and lower variation, this system was used for further statistical analysis on swimming behavior.

3.1.2. Effect of H_2S on swimming behavior

Scatterplot visualization indicated a positive correlation between H_2S, speed and dispersion and a negative correlation between H_2S and pattern (Fig. 3). Correlation matrix indicated high correlation between speed and dispersion (Supplementary table 1). Only one of this two effects was included in multivariable models based on the best results from AIC, BIC, and LS tests.

3.1.3. Speed

Linear regressions were used to test whether dissolved H_2S significantly correlated with swimming speed [inversely transformed $(-1/x)$], using both univariable and multivariable models and marginal predictions. The univariable regression was statistically significant ($R^2 = 0.19$; $F(2, 170{,}352) = 19{,}902$; $p < 0.001$) and showed a positive correlation ($\beta = 0.0002$, $p < 0.001$) between H_2S concentration and swimming speed (Table 3a). This indicates that fish tend to increase their mean speed at higher H_2S concentrations. The significant negative coefficient for the interaction term ($\beta = -0.000001$, $p < 0.001$) indicates that the effect of H_2S on swimming speed decreases at higher concentrations. When adjusting for autocorrelation (Supplementary table 2), the model showed almost identical coefficients but lower explanatory power ($R^2 = 0.02$). The marginsplot offered a visual interpretation of the model's coefficient (Fig. 4a) The plot of marginal predictions showed that H_2S increases swimming speed in a linear way up to 50 μg/L. A further increase in H_2S concentration result in a less pronounced increase in swimming speed.

The multivariable model (Table 4) was statistically significant ($R^2 = 0.4$; $F(5, 170{,}349) = 22{,}872$; $p < 0.001$) and confirmed a positive correlation ($\beta = 0.00006$, $p < 0.001$) between H_2S concentration and speed, also when adjusting for O_2, CO_2 and FIF. It was found that CO_2 was also positively correlated with speed ($\beta = 0.0003$, $p < 0.001$) while O_2 and FIF showed a negative correlation ($\beta = -0.0005$; $\beta = -0.0006$, $p < 0.001$).

QQPlot and scatterplot of residuals versus predicted values confirmed linearity and homoscedasticity of residuals of the models (Supplementary fig. 8).

3.1.4. Pattern

Beta regressions were used to analyze the relationship between H_2S

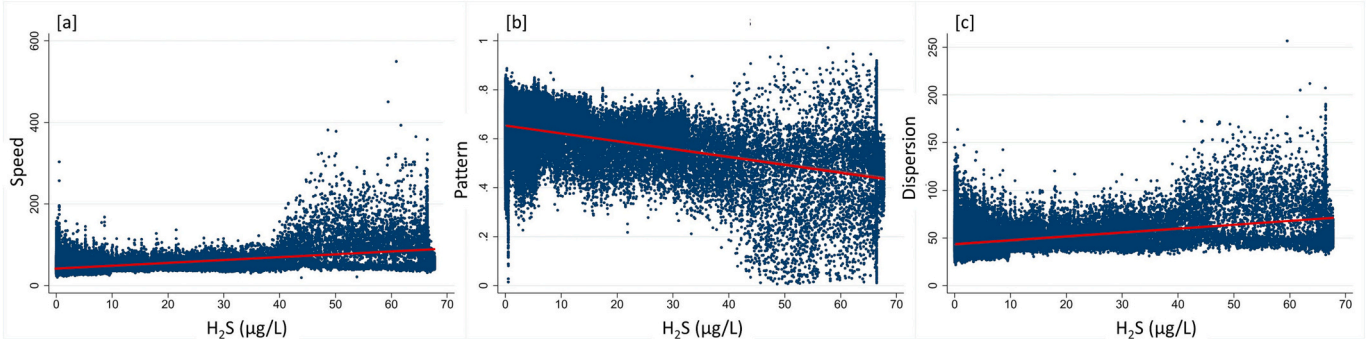


Fig. 3. Effect of H_2S on swimming parameters. The scatter plots and linear predictions (red lines) show the relationship between the measured H_2S concentration and swimming parameters for 170,355 data points. It appeared that speed [a] and Dispersion [c] were increasing while Pattern [b] was decreasing at higher H_2S concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Univariable models. Swimming parameters were included as dependent variables. Speed and dispersion were inversely transformed (−1/x) to meet test assumption (linear regression). Pattern was analyzed by beta regression. (SE) standard error (CI) confidence intervals (n) number of observations (#) interactions between variables.

Linear regression							
Speed [inversely transformed (−1/X)]							
Parameter	Coefficient	SE	p	95% CI		r^2	n
H_2S	0.000240	0.000003	< 0.001	0.000234	0.000246	0.19	170,355
H_2S # H_2S	−0.000001	0.0000001	< 0.001	−0.000001	−0.000001		

Linear regression							
Dispersion [inversely transformed (−1/X)]							
Parameter	Coefficient	SE	p	95% CI		r^2	N
H_2S	0.000214	0.000003	< 0.001	0.000209	0.000220	0.13	169,224
H_2S # H_2S	−0.000001	0.0000001	< 0.001	−0.000002	−0.000001		

Beta regression						
Pattern						
Parameter	Coefficient	SE	p	95% CI		n
H_2S	−0.008617	0.000205	< 0.001	−0.009019	−0.008216	170,355
H_2S # H_2S	−0.000087	0.000004	< 0.001	−0.000095	−0.000080	

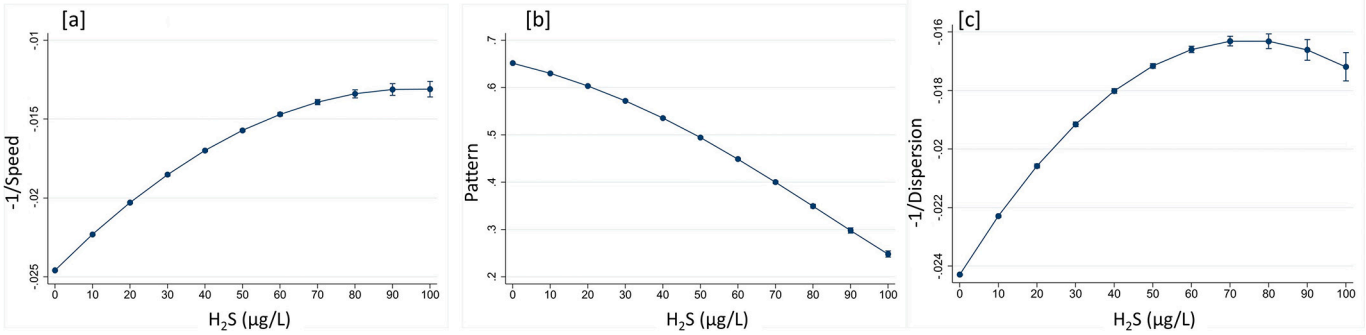


Fig. 4. Marginsplot showing Linear predictions (mean ± 95% CI) of −1/speed [a] pattern [b] and − 1/dispersion [c] according to H_2S concentration obtained from linear regression models (Table 3).

Table 4
Multivariable models. Swimming parameters were included as dependent variables. Speed and dispersion were inversely transformed (−1/x) to meet test assumption (linear regression). Pattern was analyzed by beta regression. In all models, H_2S, O_2 CO_2 and the number of fish in frame (FIF) were included as fixed effect. (SE) standard error; (CI) confidence intervals; (#) interactions between variables.

Linear regression					
Speed [inversely transformed (−1/X)]					
Parameter	Coefficient	SE	p	95% CI	
H_2S	0.000056	0.000003	< 0.001	0.000051	0.000061
O_2	−0.000546	0.000012	< 0.001	−0.000568	−0.000523
CO_2	0.000334	0.000006	< 0.001	0.000322	0.000346
FIF	−0.000595	0.000003	< 0.001	−0.000602	−0.000589
H_2S # H_2S	−0.00000012	0.00000005	0.008	−0.00000021	−0.00000003
adj. R^2: 0.40	*n: 170,355*		*F(5, 170,349): 22,871.66*		

Linear regression					
Dispersion [inversely transformed (−1/X)]					
Parameter	Coefficient	SE	p	95% CI	
H_2S	0.000050	0.000003	< 0.001	0.000044	0.000055
O_2	−0.000437	0.000011	< 0.001	−0.000459	−0.000415
CO_2	0.000325	0.000006	< 0.001	0.000313	0.000337
FIF	−0.000519	0.000003	< 0.001	−0.000526	−0.000513
H_2S # H_2S	−0.00000021	0.00000005	< 0.001	−0.00000030	−0.00000012
adj. R^2: 0.32	*n: 169,224*		*F(5, 169,218): 16,309.87*		

Beta regression					
Pattern					
Parameter	Coefficient	SE	p	95% CI	
H_2S	−0.0006	0.0002	0.002	−0.0010	−0.0002
O_2	0.0967	0.0039	< 0.001	0.0890	0.1043
CO_2	−0.0114	0.0005	< 0.001	−0.0124	−0.0105
FIF	0.1018	0.0023	< 0.001	0.0973	0.1063
H_2S # H_2S	−0.000109	0.000004	< 0.001	−0.0001163	−0.0001024
O2 # FIF	−0.0068	0.0002	< 0.001	−0.0073	−0.0063
	n: 170,355				

concentration and swimming pattern, using both univariable and multivariable models. The univariable model showed that H_2S significantly decrease the variable "Pattern" ($\beta = -0.009$, $p < 0.001$), meaning that fish tend to swim in random directions rather than in a straight line when H_2S is present. The effect of H_2S on Pattern changes at higher concentration, as showed from the negative coefficient ($\beta = -0.00009$, $p < 0.001$) of the interaction term and the marginsplot (Fig. 4b).

The multivariable model confirmed a negative correlation ($\beta = 0.0006$, $p < 0.002$) between H_2S and Pattern when adjusting for O_2, CO_2 and FIF. It was found that CO_2 was also negatively correlated with Pattern ($\beta = 0.01$, $p < 0.001$) while O_2 and FIF showed a positive correlation ($\beta = -0.097$; $\beta = 0.1$, $p < 0.001$).

3.1.5. Dispersion

Linear regressions were used to test whether dissolved H_2S significantly correlated with swimming dispersion [inversely transformed (−1/x)], using both univariable and multivariable models. The overall univariable regression was statistically significant ($R^2 = 0.13$; F (2, 169,221) = 12,878; $p < 0.001$) showing a positive correlation ($\beta = 0.0002$, $p < 0.001$) between H_2S concentration and Dispersion. This indicates that fish tend to lose their schooling behavior at higher H_2S concentration. The significant negative coefficient for the interaction term ($\beta = -0.000001$, $p < 0.001$) indicates that the effect of H_2S on Dispersion decreases at higher concentrations. When adjusting for autocorrelation (Supplementary table 2) the model showed almost identical coefficients but lower explanatory power ($R^2 = 0.013$). The marginsplot showed that H_2S increases swimming speed in an almost linear way up to 50 μg/L (Fig. 4c). A further increase in H_2S does not result in an additional rise in Dispersion, which actually tend to decline above 80 μg/L.

The multivariable model was statistically significant ($R^2 = 0.32$; F (5, 169,218) = 16,310; $p < 0.001$) and confirmed a positive correlation ($\beta = 0.00005$, $p < 0.001$) between H_2S and Dispersion when adjusting for O_2, CO_2 and FIF. It was found that CO_2 was also positively correlated with Dispersion ($\beta = 0.0003$, $p < 0.001$) while O_2 and FIF showed a negative correlation ($\beta = -0.0004$; $\beta = -0.0005$, $p < 0.001$).

QQPlot and scatterplot of residuals versus predicted values confirmed linearity and homoscedasticity of residuals of the models (Supplementary fig. 9).

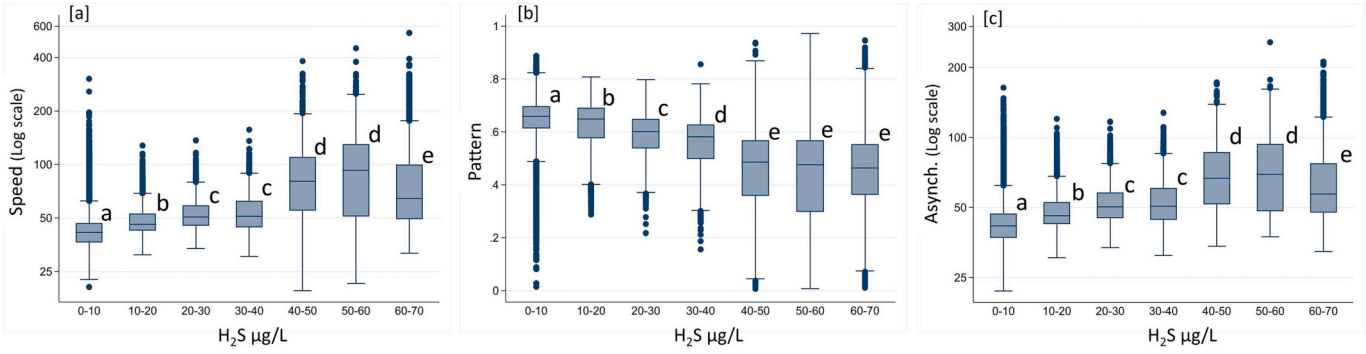
3.1.6. Overview

Regression analysis confirmed that H_2S is positively correlated with swimming speed and Dispersion, and negatively correlated with Pattern. This indicates that the presence of H_2S in the water causes a shift in swimming behavior inducing fish to swim faster in random directions independent of each other. The change in swimming behavior coincide with increasing CO_2 and decreasing O_2 levels in water.

3.1.7. Analysis of variance

Kruskal-Wallis Test was conducted to examine the differences in swimming parameters according to the H_2S concentration (in increments of 10 μg/L; Fig. 5, Table 5).

Significant differences were found for speed (χ^2: 21772.4; $p < 0.001$; df: 6) Dispersion (χ^2: 17319.6; $p < 0.001$; df: 6) and Pattern (χ^2: 16799.5;



Speed				
H_2S (µg/L)	Mean	SD	Change	n
0-10	42.68	9.48	-	150312
10-20	49.12	9.84	+15.1%	5868
20-30	53.35	11.40	+8.6%	3603
30-40	54.70	13.82	+2.5%	3341
40-50	90.28	44.70	+65.1%	1745
50-60	100.73	53.55	+11.6%	1689
60-70	81.51	45.90	-19.1%	3797

Pattern				
H_2S (µg/L)	Mean	SD	Change	n
0-10	0.6515	0.07	-	150312
10-20	0.627	0.09	-3.7%	5868
20-30	0.589	0.08	-6.1%	3603
30-40	0.559	0.09	-5.1%	3341
40-50	0.458	0.17	-18.2%	1745
50-60	0.436	0.19	-4.6%	1689
60-70	0.460	0.16	+5.5%	3797

Asynchronization				
H_2S (µg/L)	Mean	SD	Change	n
0-10	43.05	9.04	-	150289
10-20	48.83	9.48	+13.4%	5868
20-30	52.73	10.78	+8.0%	3603
30-40	53.69	12.62	+1.8%	3341
40-50	71.43	24.30	+33.0%	1514
50-60	74.55	29.42	+4.4%	1317
60-70	65.27	24.34	-12.4%	3292

Fig. 5. Boxplots of swimming parameters: speed [A], pattern [B] and dispersion [C], grouped in increments of 10 µg/L H_2S. Lower and upper box boundaries: 25th and 75th percentiles; Line inside the box: median; lower and upper error lines: lower and upped adjacent values; blue dot: outliers. A Kruskal-Wallis H test followed by Dunnett's multiple comparison test (Bonferroni adjustment, Supplementary table 3) was conducted to determine if swimming parameters were different for the seven concentration groups. Different letters denotes statistically significant differences between groups ($p < 0.05$). Number of observations and summary statistics are available in the tables below. Change indicates the percentage change of mean value compared to previous group. (SD) standard deviation (n) number of observations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Kruskal-Wallis H test was conducted to examine the differences in swimming parameters according to H_2S concentration, grouped in increments of 10 as shown in Fig. 5. (DF) degrees of freedom.

Kruskal-Wallis H test			
	$\chi 2$	p	DF
Speed	21,772.4	< 0.001	6
Pattern	16,799.5	< 0.001	6
Dispersion	17,319.6	< 0.001	6

Table 6
Dissolved cortisol Dissolved cortisol (Log transformed) was included as dependent variable in a linear regression model. Time (in days), H_2S concentration and stocking density were included as fixed effect. (SD) standard deviation; (CI) confidence intervals; (#) interactions between variables.

Dissolved cortisol (log transformed)							
Parameter	Coeff	SE	p	95% CI		R^2	n
Time (days) F(1, 75) = 109.15	−0.11	0.01	<0.001	−0.13	−0.09	0.59	77

$p < 0.001$; df: 6) among the seven concentration groups. A significant increase in both mean speed and dispersion occurred at higher H_2S concentrations until reaching a plateau between 40 and 60 µg/L. Further increasing H_2S concentration caused a decline in the response at 60–70 µg/L. Mean swimming Pattern significantly decreased at rising H_2S concentrations, until reaching a minimum above 40 µg/L.

Interestingly the change in swimming parameters in the 40–50 µg/L group, was much higher than the others, showing a change of +65% speed +33% Dispersion and − 18% Pattern as compared to the previous concentration group.

3.2. Water cortisol

Linear regressions were used to test whether dissolved H_2S significantly correlated with dissolved cortisol [log transformed] when adjusting for time(days) and stocking density. The final model (Table 6) was statistically significant ($R^2 = 0.59$; $F(1, 75) = 109.15$; $p < 0.001$) showing that dissolved cortisol decreased overtime ($\beta = -0.11$, $p < 0.001$) in all tanks (Fig. 6). Both H_2S concentration and stocking density had no effect. Cortisol levels were higher from day 0 to day 4, between 4.5 and 7.5 ng/mL, to then decrease to 2–3 ng/mL from day 5 onward.

4. Discussion

This study demonstrated via video monitoring that sublethal concentrations of dissolved H_2S can alter the swimming behavior of Atlantic salmon in RAS. Fish quickly reacted to H_2S with a stress response characterized by faster swimming speed, erratic pattern, and loss of schooling behavior. This response was clearly distinct from their normal behavior in tanks. Salmon generally swim in a circular pattern forming schools (Johansson et al., 2007) to avoid collisions with each other or the cage wall (Føre et al., 2009), swimming at what is defined as preferred speed, or standing stationarily against the current (Hvas et al., 2017; Johansson et al., 2014). Swimming behavior was represented in the present study from three parameters: speed, pattern and dispersion (indicative of schooling), which has been shown to change according to environmental, social and welfare conditions (An et al., 2021; Hvas et al., 2021). For instance, swimming speed in Atlantic salmon can be influenced from health condition (Furevik et al., 1993), competition for feeding (Kadri et al., 1991) or stress (An et al., 2021). Changes in schooling behavior can occur as a response to stocking density (Juell and Westerberg, 1993), feeding regime (Ang and Petrell, 1998; Juell et al., 1994) photoperiod (Juell, 1995) or water currents (An et al., 2021). Monitoring of fish behavior therefore represents a valuable tool in aquaculture to assess animal welfare and water quality (Brownscombe et al., 2019; Martins et al., 2011). While experienced personnel could

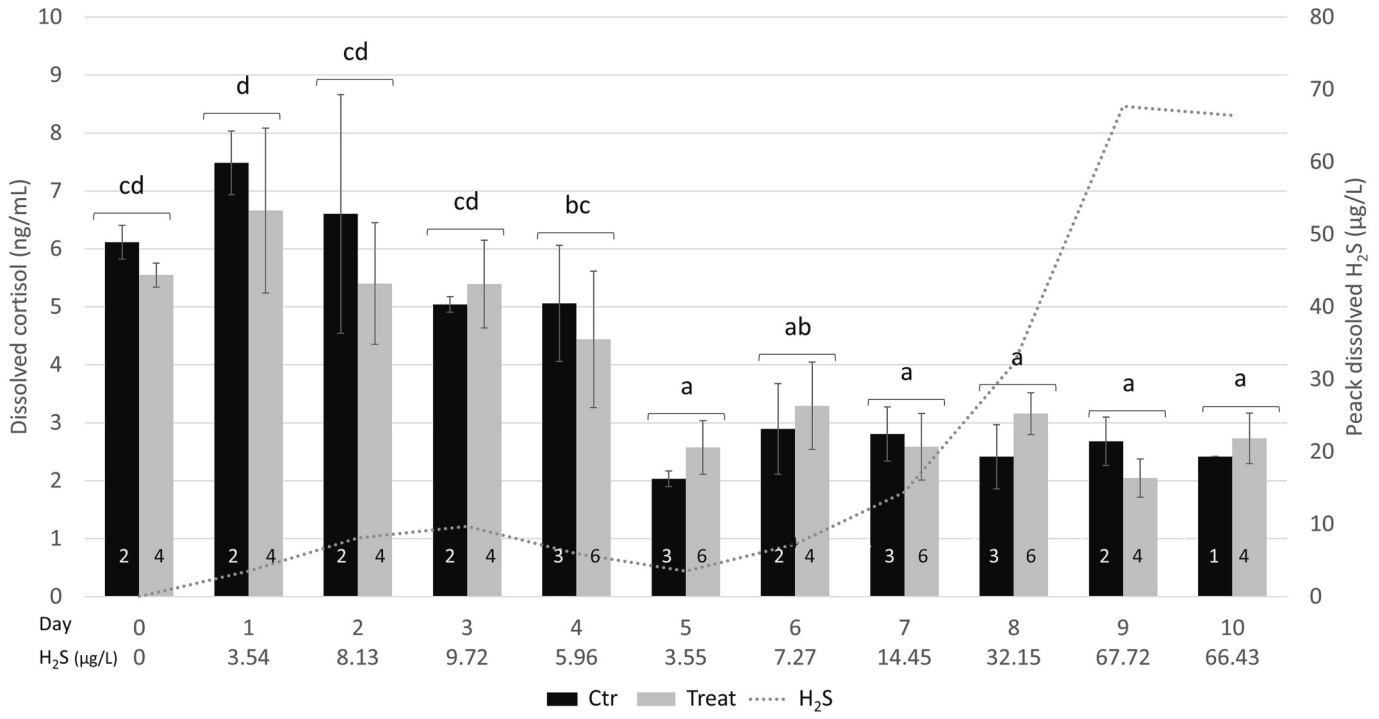


Fig. 6. Dissolved cortisol levels in experimental tanks at the end of the acclimatization period (day 0) and 60 min after each H_2S exposure (day1–10). Different letters denote statistically significant differences ($p < 0.05$) between groups (Linear regression followed by multiple comparison (Bonferroni adjustment)). No significant differences were detected between treatment and control group nor between higher or lower stocking density. Cortisol levels significantly decreased over time. Sampling from the two tanks at different stocking density in treatment group were pooled together to improve clarity. The number of replicates per group is indicated within the columns. H_2S refers to the peak concentration measured in treatment group per experimental day.

visually assess fish behavior, this time-consuming and typically not well documented process might be not sensitive enough to detect subtle but significant changes. The use of new technologies, such as the machine vision presented in the current study, holds the potential to accurately quantify and analyze fish behavior to a greater extent than possible by human observations (Duarte et al., 2009; Papadakis et al., 2012; Saberioon et al., 2016).

Machine vision tools are widely used in animal research, recently also including aquaculture, as they offer non-invasive, objective, and repeatable analysis (An et al., 2021; Saberioon et al., 2016). While swimming behavior is recognized as an indicator of welfare in captive environments (Brännäs et al., 2001), most video monitoring systems developed thus far have primarily focused on fish counting and biomass estimation (Yang et al., 2021). Only recently have protocols been developed to monitor fish behavior and welfare (Pinkiewicz et al., 2011; Soltanzadeh et al., 2020) while, to the best of our knowledge, no protocols has so far been connected to the presence of dissolved H_2S. One challenge in monitoring swimming behavior is that fish move in three dimensions within the water column, rather than on a flat surface. While most advanced tracking systems use convolutional neural networks to analyze 2D images (Romero-Ferrero et al., 2019), these parameters may lead to inaccurate representations of fish behavior in the water column. In this study, both an overhead camera, providing 2D images, and a submerged stereo system, allowing for 3D trajectory reconstruction, were employed. Although 3D mapping offers a more realistic representation, the submerged cameras captured fewer fish in frame as compared to the overhead system, especially during H_2S exposure, due to fish obstructing the view or being absent from the camera's field of vision for extended periods. Nevertheless, this experience provided valuable insights for follow-up studies to carefully consider the placement of the video monitoring system. Statistical models were built using data from both camera systems produced overall comparable results. However, since the overhead camera captured a higher number of fish for a longer period during H_2S exposure, this database was prioritized as it produced a higher quantity of data during the critical part of the experiment. This also suggests that, in a farm setup with larger tanks and higher number of fish, a simple overhead camera might represent a more suitable solution for monitoring fish behavior compared to a submerged system. Indeed, the system accurately showed how swimming behavior changed in response to dissolved H_2S.

The behavioral response to H_2S exhibited a linear increase up to a concentration of 40 µg/L. Beyond this threshold, fish showed a significantly heightened response compared to lower concentrations. Interestingly, further increases in H_2S did not result in a more pronounced response, indicating a plateau effect. Physiological studies have identified the critical H_2S concentration (H_2S_{crit}) for salmon at 60 µg/L, beyond which they start experiencing asphyxiation as their oxygen uptake drops below basal metabolic values (Bergstedt and Skov, 2023). The video recording system was therefore able to detect significant changes in swimming behavior below the critical toxic concentration of H_2S. These findings strongly support the notion that machine vision can serve as a valuable early warning tool potentially providing an opportunity to implement necessary safety measures in a timely manner. It's important to note that this study serves as a proof of concept by utilizing statistical analysis of behavioral data. The aim was to confirm the possibility of detecting changes in swimming behavior before any toxic effect of H_2S could occur. Future studies should focus on developing dedicated machine learning and softwares capable of real-time monitoring of swimming behavior, providing timely alarms for action. While our study specifically measured the effect of the chemical stressor H_2S, it's worth considering that the observed behavioral changes may also be an unspecific response to overall poor water quality. Future investigations could explore the response to different toxic compounds or indicators of poor water quality, such as low oxygen or high CO_2 levels. Additionally, it is important to acknowledge that tolerance levels to H_2S might vary among different species, warranting further research in this area.

There is a scarcity of information regarding the tolerance and physiological effects of H_2S exposure in teleosts in vivo. Most of the studies have focused on species with a high tolerance to the gas such as the California killifish (*Fundulus parvipinnis*, >20-h LC_{50} at 1.5 mM sulphide), tambaqui (*Colossoma macroponum*, LC_{50} 34 μM H_2S), goldfish (*Carassius auratus*, 96-h LC_{50} at 3.5 μM H_2S) (Adelman and Smith Jr., 1972; Affonso et al., 2002; Bagarinao and Vetter, 1989; Torrans and Clemens, 1982). Sensitive species includes the bluegill (*Lepomis macrochirus*, 96-h LC_{50} of 1.3 μM H_2S) and rainbow trout (Marvin and Burton, 1973; Smith et al., 1976). Tolerant species have generally a high resistance to hypoxia, though applying different coping strategies (Borowiec et al., 2015; Mandic et al., 2008; Scott and Rogers, 1981) and are species that might encounter H_2S in their natural environments (Bagarinao and Vetter, 1989). Comparison between studies is made difficult by the way H_2S is calculated. Many studies have reported "total sulphide" as the sum of the three sulphide's chemical species. As temperature, pH and ionic strength of the solution influences the fraction of each species (Millero et al., 1988), it's possible to calculate H_2S only by knowing water parameters. Similar complications occur also when calculating the concentration of H_2S from sulphide donors such as Na_2S and NaHS. To produce the most reliable results possible, the detector used in the present study provided in real time reads of H_2S measuring simultaneously sulphate species and water parameters.

Dissolved cortisol is a good indicator of stress in fish which do not require handling the animals, therefore avoiding additional stress to the fish (Sadoul and Geffroy, 2019). Interestingly, water samplings did not show any differences in dissolved cortisol levels between the treatment and control RAS at any point during the study even after exposure to the highest concentration of H_2S. This further highlights the sensitivity of video monitoring compared to other methods. The cortisol levels decreased in both treatment and control tanks on the fifth day of the experiment. This indicates that the one-week acclimatization period might not have been sufficient for the fish to fully adapt after being transferred to the experimental tanks. In light of these results, an extended acclimatization of 12 days would likely allow the fish to become better accustomed to the new tank conditions.

There are some technical considerations regarding the experiment setup that are worth mentioning. Due to hardware and processing time limitations, the video recording system was placed in only one of the exposure tanks, which had the same stocking density as the control tank. As a result, direct comparisons between control and treatment (and different stocking density) was not possible. To establish a baseline or control level, the treatment fish were video-monitored for several hours one day prior to the first H_2S exposure. Indeed, visual observations confirmed normal swimming behavior of the control fish throughout the experiment. Another aspect to consider is that the fish were exposed to increasing doses of H_2S for 10 consecutive days. Distinguishing between acute or chronic (cumulative) response based on the measured results may pose a challenge. Nonetheless, several factors suggest the observed behavior to be more indicative of the first rather than the latter case. Firstly, it was observed that H_2S become undetectable in the water within 10 min to 5 h from exposure, depending on the peak concentration reached. Additionally, physiological studies on salmon have indicated a recovery time of approximately 4 h after exposure to toxic concentrations above H_2S_{crit} (Bergstedt and Skov, 2023). In the current trial, 24 h intercurred between one exposure to the other, allowing enough time for the H_2S to leave the system and to the fish to recover from the previous exposure. Furthermore, H_2S_{crit} was only reached during the last two days of the experiment. As final point of discussion, visual examination of the stereo cameras also showed an increase in ventilation frequency, indicated from faster operculum and mouth movements. However, this parameter was not quantified from the machine learning algorithms. Nevertheless, ventilation is an important parameter that could serve as an indicator of stress in fish (Sharma et al., 2019) making it a promising candidate for future analysis.

5. Conclusion

Through the use of machine vision, this study demonstrated that Atlantic salmon (*Salmo salar*) smolts exhibit a behavioral stress response when exposed to dissolved H_2S in RAS. This response is characterized by increased swimming speed, erratic patterns, and a loss of schooling behavior. Interestingly, a significant response was observed at 40 μg/L, which is below the toxic threshold of 60 μg/L H_2S for salmon. These findings strongly support the idea that machine vision can be a valuable early warning tool, offering the potential to quickly implement necessary safety measures.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740201.

Authors contribution

All persons who meet authorship criteria are listed as authors. All authors certify that they have participated sufficiently in the work to take public responsibility for the content. Conceptualization: EC, BK, SG, DR, RN; Methodology: EC, BK, DR, RN; Software: BK; Investigation: SG, BK, DR, IM; Resources: SG, RN, IM; Data curation: EC, BK, DR, IM, SG; Visualization: EC; Formal analysis: EC, BJ, MS; Writing - original draft: EC; Writing - review and editing: EC, BK, MS, IM, SG, DR, RN; Project administration: DR, RN; Funding acquisition: DR, RN.

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Relationship

There are no additional relationship to disclose.

Patents and intellectual property

There are no patents to disclose.

Other activities

There are no additional activities to disclose.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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