UiA University of Agder

Master

Behavioral reactions to H_2S in farmed Salmon

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Abstract

In recirculating systems, the production of hydrogen sulfide (H_2S) can lead to mass death of salmon. There is little knowledge on how fish reacts to H_2S in lower concentrations. Automatic interpretation of behavior by AI and behavioral monitoring is in progress as a tool to identify positive and negative production conditions. In this thesis, the levels of H_2S which evoke avoidance in post smolt Atlantic salmon (*Salmo salar*) were examined. This was done by monitoring the position of the salmon in two laminar waterflows, while the H_2S levels in one of the flows were increased. Behavioral studies were performed in a two-choice tank system (Loligo inc.) consisting of a 32 cm x 40 cm test chamber with two parallel laminar flows. The percentage of time the fish spent in each flow-zone was quantified for each 15 minutes period after H_2S was added. Each fish was monitored for a total of 90 minutes. Avoidance to H_2S was calculated as the percentage of time the fish spent in the zone where H_2S was not added. The results show that as salmon were subjected to increasing levels of H_2S, the fish started spending less time in the H_2S flow. The results also indicated that the H_2S-concentration which salmon detect and react to lies between 1 to 2.7 μM. It has recently been shown that H_2S-concentration above 1.8 μM causes respiratory problems in post smolt Atlantic salmon. This demonstrates a potential application of behavior changes associated with increasing H_2S for generating warning signals before toxicity is reached. Thus, behavioral reactions to acute H_2S toxicity can potentially be a part of a monitoring system for warning signals of harmful H_2S levels in RAS. The behavioral response detected in this study together with mitigation measures could offer a strategy to decrease mortality linked to increased H_2S in aquaculture.

Sammendrag

I resirkulerende akvakulturanlegg kan dannelse av hydrogensulfid (H_2S) føre til akutt massedød av laks. Det er begrenset med kunnskap om hvordan fisk reagerer på H_2S ved lave konsentrasjoner. Automatisk tolking av adferd og adferdsovervåking ved hjelp av AI er i fremmarsj som et verktøy for å identifisere positive og negative produksjonsbetingelser. I dette masterprosjektet undersøkes hvilke nivåer av H_2S som post-smolt laks (*Salmo salar*) unnviker. Dette ble undersøkt ved at posisjonen til laksen i to laminære vannstrømmer ble overvåket, samtidig som H_2S-konsentrasjonen ble økt i den ene av vannstrømmene. Adferdsundersøkelsen ble utført i et to-valg tanksystem (Loligo inc.) som besto av et 32 cm x 40 cm testkammer med to parallelle strømmer. Prosenten av tiden fisken tilbrakte i hver strømsone ble målt for hver 15. minutts periode etter at H_2S ble tilsatt. Hver fisk ble filmet i maks 90 minutter. Laksens unnvikelse mot H_2S ble kalkulert som prosenten av tiden fisken tilbrakte i sonen hvor H_2S ikke ble tilsatt. Resultatet viste at når laks ble utsatt for økende nivåer av H_2S, så tilbrakte fisken mindre tid i H_2S sonen. Resultatet indikerte også at H_2S-konsentrasjonen som frembrakte fluktrespons lå mellom 1-2.7 μM. Det har nylig blitt funnet at H_2S konsentrasjon over 1.8 μM frembringer respirasjonsproblemer hos post-smolt atlanterhavslaks. Dette demonstrerer en potensiell bruk av adferdsforandringer assosiert med økende H_2S nivåer for å generere varselsignaler før toksisitet blir oppnådd. Derfor kan adferdsreaksjoner til akutt H_2S forgiftning potensielt være del av et monitoringssystem for skadelige H_2S nivåer i RAS. Adferdsresponsen som ble observert i denne undersøkelsen, sammen med mitigasjonstiltak, kan være en mulig strategi for å minke dødelighet knyttet til økt H_2S i akvakultur.

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Preface

I would like to thank my supervisor Erik Höglund for providing me with guidance and writing advice. He has given me a greater understanding of the research process and shown great patience with me during the journey of writing this thesis. I want to thank you for being a great supervisor every step of the way, from driving me to, and introducing me to the people I worked with at DTU Aqua. Thanks for building the two-choice tank the salmon were in, setting up the Ethovision XT program, and for putting in the time to advise me on writing the thesis, both physically and over internet. I couldn´t have done it without you.

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Finally, I want to thank friends, family, and fellow students from UiA (University of Agder, Norway) who all have provided me with motivation, economical support and proofreading during my writing process. Thank you all.

List of symbols and abbreviations

Adenosine triphosphate (ATP)

Analysis of variance (ANOVA)

Closed containment system (CCS)

Flow-through (FT)

Recirculating aquaculture system (RAS)

Sulfate-reducing archaea (SRA)

Sulfate-reducing prokaryotes (SRP)

Technical University of Denmark (DTU)

Glossary

Acute: Present or experienced to a severe or intense degree.

Avoidance behavior: Type of activity, seen in animals, in response to adverse stimuli. Efforts are made to remove themselves from the adverse stimuli.

Bradycardia: A slower than normal heartrate.

Chronic: Persisting for a long time or constantly occurring.

Critical H_2S levels: Concentrations of H_2S above which adverse effects occur.

Frequency: The rate at which something occurs over the course of a set time period, or in a given sample.

Gasotransmitter: This is a class of neurotransmitter that functions as gaseous signaling molecules.

Homeostasis: Is a self-regulating process by which an organism tends to maintain stability while adjusting to conditions that are best for its survival.

Hypoxia: A state in which there is not enough oxygen at the tissue level to maintain adequate homeostasis.

Sublethal exposure (toxicology):

Exposure: The amount of an agent that reaches a target organism, system or population in a specific frequency for a defined duration.

Sublethal: Less than lethal.

Threshold concentrations: The amount of a substance the target can be exposed to, before experiencing adverse health effects.

Toxic concentrations: The amount of a toxic substance in a defined space.

Toxic effect: An adverse change in the structure or function of an experimental animal as an effect of exposure to a chemical substance.

Toxic substances: A substance that can cause adverse health effects.

Toxicity: The degree in which a chemical substance or a particular mixture of substances can damage an organism.

1.0 Introduction

1.1 Production cycle of Atlantic salmon

It is important to take the natural life cycle into account to optimize production of Atlantic salmon (*Salmo salar*) (Song et al., 2019). In nature, salmon larvae develop into parr after hatching in the spring, and feed and grow in freshwater for the next 1-3 years. Thereafter, they smoltify, a process where they prepare for sea migration. After feeding and growing in the sea for 2-3 years, salmon return to their natal river to spawn.

In traditional sea-based salmon farming, the juvenile freshwater phase lasts from 5 to 12 months (Afewerki et al., 2023), while the sea phase lasts for roughly a year (Asche & Bjørndal, 2011). There are a few downsides to sea-based aquaculture. When salmon is farmed in the sea, the salmon risk exposure to factors such as sea lice and contagious diseases (Misund, 2019). There is also the threat of salmon escaping from their sea-cages, mingling and potentially breeding with wild salmon populations and affecting them with any diseases and sea lice they accumulated in the cages (Misund, 2019). Sea-based aquaculture also has some detrimental effects on the environment (Grefsrud et al., 2019). Organic material from the sea-based fish farms fall down to the bottom of the sea and interferes with the ecosystems of the ocean floor. This material includes fish feed that spill out and feces. The bacteria in the sediments on the ocean-floor break down organic material with the help of oxygen. Therefore, excess organic material will lead to increased bacterial activity, which depletes the oxygen levels faster than it would without the presence of fish farms. Over time, such anoxic conditions can lead to the production of H_2S, which is toxic for creatures living on the ocean floor (Grefsrud et al., 2019).

Recirculating aquaculture systems (RAS) on the other hand, are land-based systems, and therefore affecting both the coastal environment and the salmon differently. Generally, RAS can help reduce major impediments to industry growth, such as sea lice, escapees and diseases (Summerfelt et al., 2001; Martins et al., 2010). There has been a dramatic increase in adoption of land-based RAS facilities in Norwegian salmon aquaculture, which historically has been dependent on flow-through (FT), or partial water reuse systems (Bergheim et al., 2009; Martins et al., 2010). In contrast to FT, RAS has a limited exchange of water, consisting of fish tanks, filtration systems and water treatment incorporated into one system. The main advantage of RAS systems is that it recycles water by cleansing it of contaminants while keeping a stable level of oxygen for the fish.

The whole salmon production cycle can take place in the RAS facility. The advantages RAS has over flow-through is that it is possible to decrease water consumption and reduce nutrient outlet concentrations through technological developments (Dalsgaard et al., 2013). It is possible to control rearing temperatures and essential water quality parameters such as dissolved oxygen, carbon dioxide, nitrite, nitrate, ammonia, pH, salinity and suspended solids (Dalsgaard et al., 2013), which is an advantage over flow-through systems. Norway is one of the leading countries in Atlantic salmon production and has had a large growth in RAS production facilities (Summerfelt & Christianson, 2014). Norway is currently providing roughly 50% of the global salmon production (Iversen et al., 2020).

1.2 Biological environment in RAS

From a fish health perspective there are several threats in RAS. The physiological response to H_2S is similar to that of exposure to hypoxia for a wide range of vertebrates (Olson et al., 2006). High concentrations of gaseous H_2S (10-100μM) inhibit aerobic ATP production and cellular respiration in the mitochondria (Szabo et al., 2014). Bioaccumulation of substances in RAS could lead to impaired fish growth performance and welfare (Deviller et al., 2005; Martins et al., 2009). Accumulating fine particles (<20μm) could potentially cause detrimental effects to fish fins and gills at low exchange rates (Davidson et al., 2009), and together with solids and other constituents could create an environment where opportunistic, heterotrophic bacteria could become more prevalent and pose potential health risks for the fish (Davidson et al., 2009). In order to avoid toxic accumulation in RAS, water exchange should be increased or periodically flushed (Davidson et al., 2009). Fish farmers running low water exchange RAS should avoid copper piping and components in their system (Davidson et al., 2009). UV or ozone could be incorporated into low water exchange systems to safeguard against opportunistic bacteria. It was also found that fish health improved with incorporation of ozone and future studies could research the effects ozonation have on harmful water qualities such as metals, fine particulates and opportunistic bacteria (Davidson et al., 2009). The introduction of water treatment with a high-rate algae pond could further reduce nutrient discharge and water requirement levels (Deviller et al., 2004).

1.3 Risks associated with H_2S

There are risks of H_2S accumulating in RAS. Fish waste is a known hotspot for H_2S production (Letelier-Gordo et al., 2020). Biofilters are filters meant to remove contaminants, including

different nitrogen forms, but biofilters have also turned out to be potential hotspots for H_2S production (Rojas-Tirado et al., 2021). H_2S can be produced in freshwater systems from freshwater organic waste, but this would be in lower levels due to sulfate limitations (Letelier-Gordo et al., 2020). H_2S production in seawater is higher than in freshwater (Letelier- Gordo et al., 2020) because marine water has ion concentrations 10-1000 times higher than in freshwater (Nazaroff & Alvarez-Cohen, 2000). One of the ways H_2S is produced in seawater is by sulfate reducing bacteria (SRB), which through anaerobic respiration, utilize sulfate as a terminal electron receptor, and produce H_2S (Muyzer & Stams, 2008). Marine land-based RAS still offers a higher level of control over environmental conditions compared to traditional sea cages (Martins et al., 2010). This is because RAS reduces water consumption (Dalsgaard et al., 2013), securing animal welfare (d'Orbcastel et al., 2009), implementing environmental regulators (such as solid waste removal, nitrite-nitrogen and ammonia control, oxygenation and disinfection) (Losordo et al., 2000) and eliminating escape to the ocean as a risk thanks to RAS being land-based.

1.4 H_2S toxicity in fish

Most of the studies regarding H_2S toxicity have been done in mammals (Rubright et al., 2017) with some notable exceptions in fish (Kiemer et al., 1995; Lien et al., 2022). The mechanisms involving H_2S toxicity are partially known (Guidotti, 2010). H_2S is water soluble and at physiological pH, two thirds exist as hydrogen sulfide ion (HS^-) and one third as undissociated H_2S (Reiffenstein et al., 1992). Sulfide (HS^-) works as an endogenous signal transmitter via protein sulfhydration (Mustafa et al., 2009). At low intracellular concentrations, approximately 0.01 to 1 μM, it will donate electrons to complex II of the mitochondrial electron transport chain (As seen at #1 in Fig. 1; Módis et al., 2013; Szabo et al., 2014). This will stimulate ATP production (As seen at #2 in Fig. 1; Szabo et al., 2014). Complex II is a group of proteins that serve as a secondary entry point into the electron transport chain. When the concentration becomes 3 to 30-fold higher, sulfide then becomes toxic by binding itself to and inhibiting cytochrome C oxidase in complex IV (As seen at #3 in Fig. 1; Goubern et al., 2007; Módis et al., 2013; Szabo et al., 2014). This is the last complex in the electron transport chain, prior to ATP synthesis by complex V (or F_0F_1 ATP synthase as it is known). In other words, sulfide´s inhibition of complex IV of the mitochondrial electron transport chain might cause cellular toxicity through reduced ATP production and/or generation of oxidative stress (Fig. 1, Jiang et al., 2016).

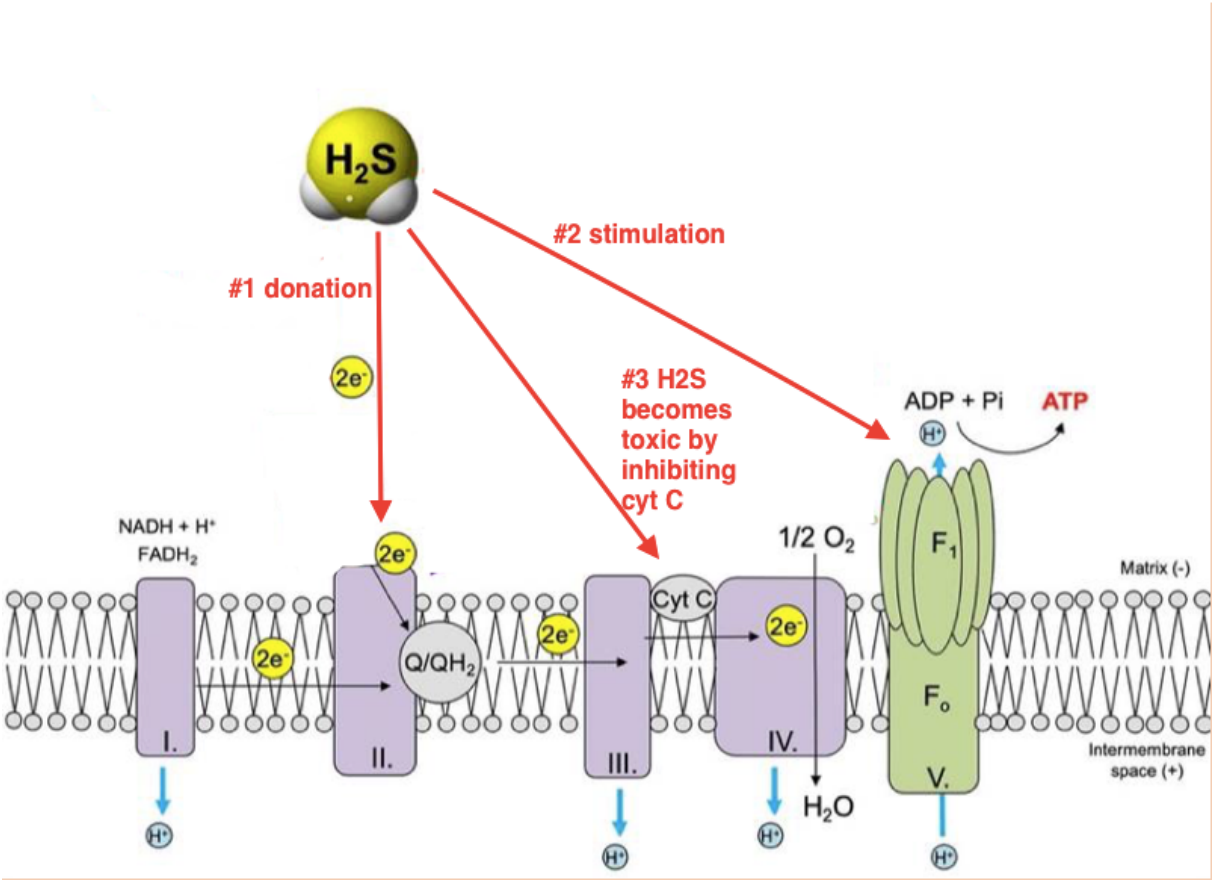

Figure 1: Sulfide functions as an endogenous signal transmitter via protein sulfhydration (Mustafa et al., 2009). At approximately 0.01 to 1 μM, it will donate electrons to complex II of the mitochondrial electron transport chain, as seen at #1 (Jiang et al., 2016; Módis et al., 2013; Szabo et al., 2014). This will stimulate the ATP production, as seen at #2 (Szabo et al., 2014). When sulfide reaches concentrations of 3 to 30-fold higher, then sulfide becomes toxic by binding itself to and inhibiting the cytochrome C oxidase at complex IV at #3 (Goubern et al., 2007; Módis et al., 2013; Szabo et al., 2014). The inhibition of cytochrome C oxidase at #3 leads to reduced production of ATP at F_0F_1 ATP synthase (sometimes called complex V). Figure modified from Szabo (2021).

There are large knowledge gaps of the H_2S impacts on fish. One of the most dramatic effects of H_2S in farmed fish, including salmon, is mass mortality (Sommerset et al., 2020). Unlike other known physio-chemical variables in an aquaculture environment, knowledge about the sensitivity and susceptibility to H_2S of many farmed fish species is almost non-existent. Still, there was a study performed by Kiemer et al. (1995) where the physiological impact of H_2S on Atlantic salmon was studied. In the latter study, Atlantic salmon smolts were exposed to two

regimes of H_2S. In the first regime, fish were subjected to chronic yet sublethal concentrations of H_2S (0 - 7.8 μM) over a timeframe of 18 weeks. Sulfide was injected into the tank for 10 minutes every 6 hours. Immediately after injection the concentration in the tank was 7.8 μM. The H_2S concentration dropped to 0 μM after 3 hours. In the second regime, salmon were exposed to a single acute, sublethal dose (22 to 29 μM) and monitored over 14 days. The results from the first regime showed that chronic exposure induced gill damage that peaked between 6 and 8 weeks after exposure. However, after 16 weeks of exposure gill tissue appeared normal, suggesting an adaptive response. Moreover, liver damage, such as diffuse hepatic necrosis and vacuolar degeneration, increased to 80%. There were no signs of regenerative hepatocytes (liver cells that can regenerate liver tissue) or neoplasia (the formation of new abnormal growth of tissue), which there often is a risk for after liver damage. In the second regime, a single acute exposure resulted in liver damages that appeared 3 days after exposure (Kiemer et al., 1995).

1.5 Avoidance as endpoints for detecting toxicity in fish

Avoidance responses are typically evoked by chemicals which fish recognize as harmful (Tierney, 2016). Generally, long-term contamination leads to hypoactivity where the fish are abnormally inactive, while short-term contamination produces higher than normal level of activity in organisms often reflecting an active avoidance behavior (Araújo & Blasco, 2019). Following this, fish behavioral responses have been used as indicators of the magnitude and duration of exposure to potential toxic substances (Little & Finger, 1990). It has been noted that the concentrations causing toxic effects usually exceed the threshold concentrations that trigger avoidance (Araújo & Blasco, 2019). Nevertheless, concerning the thresholds at which toxicity and avoidance occur, there exists two possibilities. An avoidance response will ideally occur at a concentration lower than that of toxicity. This means avoidance will occur before harm and would therefore be protective. However, toxicity may occur before avoidance. Such situations can appear when the toxic agent adversely affects sensory neurons, attenuating the avoidance response thus increasing the threshold concentration for avoidance (Fig. 2) (Tierney, 2016).

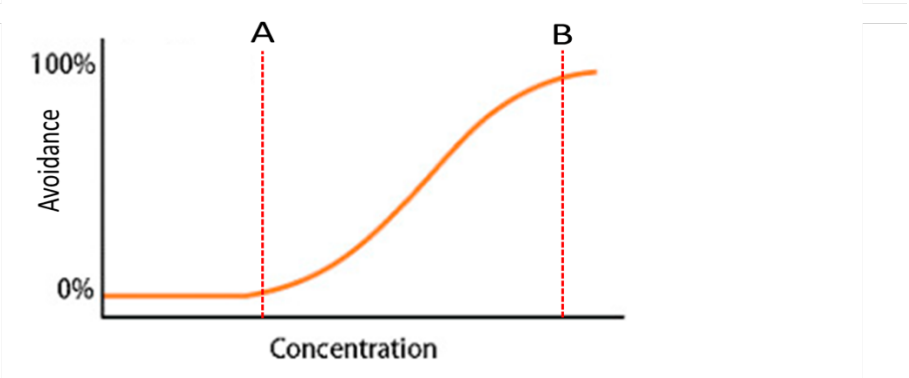

*Figure 2: Hypothetical model demonstrating how a substance can affect avoidance. Avoidance behavior at subtoxic concentrations **(A)** or avoidance behavior above toxic concentrations **(B)**. In the latter case the sensory organ might be damaged or desensitized by the substance tested. Figure modified from Tierney (2016).*

Information regarding avoidance behavior and sensory detection of H_2S in fish is limited. It has been shown that hypoxia increases mitochondrial H_2S in cardiomyocytes which are cells that are responsible for contracting the heart, thus suggesting a role for the gasotransmitter in oxygen sensing (Arndt et al., 2017). H_2S is also involved in long-term oxygen sensing (continuously active oxygen sensing mechanism) during chronic hypoxia, in addition to short-term oxygen sensing (briefly active oxygen sensing mechanism) during acute hypoxia (Olson et al., 2020; Paul et al., 2021). There have been several studies (Olson et al., 2006; Olson et al., 2008; Olson et al., 2020) researching and formulating a theory of metabolization of H_2S being used as an oxygen sensor. The mechanisms behind this correlation is largely unknown (Olson et al., 2006) but some things are known. In fish, much of the external O_2-sensitive chemoreceptors are sited at the first pair of gill arches (Olson et al., 2008). In an experiment investigating the role of H_2S in respiratory responses, it was shown that H_2S delivered to the buccal cavity resulted in bradycardia and increased ventilatory frequency (Olson et al., 2008). Moreover, removing the first gill arches attenuated this respiratory response to H_2S. The removal of gill arches is relevant because in fishes, chemoreceptors are found on the gills (Burleson et al., 1992). The chemoreceptors on the first pair of gill arches are homologous to the chemoreceptors found on the mammalian carotid body (Milsom & Burleson, 2007), and has a similar sensitivity to pO_2 (Partial pressure of oxygen, this is the amount of dissolved oxygen in the blood) (Burleson et al., 1992). This made the authors suggest that H_2S is involved in hypoxia sensing (the ability to sense and respond to changes in oxygen) of the environment in fish (Olson et al., 2008). Still,

the involvement of the chemoreceptors in the two first gill arches in evoking behavioral responses in fish is unknown (Olson et al., 2008).

Avoidance responses to H_2S have been studied in three species living in highly sulfidic mangroves; *Kryptolebias marmoratus*, *Poecilia orri*, *Gambusia* sp. and *Dormitator maculatus* to investigate whether these fishes persist because they are exceptionally tolerant to H_2S or because they can leave the H_2S-rich water (Rossi et al., 2019). All could cope with H_2S concentrations up to 1,1 μM, but at higher levels emersion responses (the act of emerging as a response to stimuli) were observed. This made the authors suggest that physiological tolerance and avoidance behavior are complementary strategies that are used together (Rossi et al., 2019).

1.6 Applications in aquaculture

Generally, artificial Intelligences (AI) can be used to draw conclusions from larger dataflows. Accordingly, real-time monitoring of system parameters by AI in an aquaculture production unit can be used in risk assessment and to optimize production (Chang et al., 2021; Yue & Shen, 2022). Following this, water quality parameters, waterborne hormones, and fish behavior can be used to safeguard health and welfare of farmed fish (Chang et al., 2021; Jothiswaran et al., 2020; Yue & Shen, 2022). RAS offers a rearing environment where many water quality parameters can be monitored and controlled (Chang et al., 2021). Still, sensors monitoring H_2S is in development and data on behavioral reactions which can be utilized for preventing acute H_2S toxicity in farmed fish is missing.

2.0 Aim

The aim of this master thesis was to investigate at which concentration farmed Atlantic salmon show avoidance behavior to H_2S.

3.0 Material and methods

3.1 Pre-treatment of fish used in the experiment

The study was carried out from November 2021 to March 2022 at DTU Aqua in Hirtshals, Denmark. The salmon used in the experiment were 80-100g post-smolt Atlantic salmon (*Salmo salar*) obtained from BioMar Denmark (Caledonia StofnFiskur, a strain from Benchmark Genetics Iceland HF). Before the start of the experiments, fish were kept in a seawater flow-through tank of 1 m x 1 m x 0.5 m (length, width and height) and were handfed 3 mm pellets of EFICO Enviro 940 at a ratio of approximately 1% of their body weight per day.

3.2 Experimental setup

Behavioral studies were performed in a two-choice tank system (Loligo inc.) consisting of a 32 cm x 40 cm test chamber with two parallel laminar flows (Fig. 3). One of the flows was added H_2S, whilst nothing was added to the other. Each parallel flow was individually recirculated. The water temperature was 10-13 ^oC during the experiments. The two-choice tank system was illuminated by UV light from beneath to prevent light-reflection interference with behaviour analyses. A plexiglass covered the choice area (the stippled area at Fig. 3) to prevented fish from jumping out of the test area.

Prior to the initiation for the experiment, the salmon fasted one day before being transferred to the two-choice tank system where it was allowed to acclimatize for 12 hours, after which the experiment started. The first 15 minutes of the experiment no H_2S was added. The H_2S levels were then increased every 15 minutes by adding 64, 1.28, 2.56, 5.12, 10.2 mL H_2S stock solution (9.6 gram (0.4M) of $Na_2S \bullet 9H_2O$ in 100 ml MilliQ water). The experiments lasted a total of 90 minutes. The experiments were run sequentially for each salmon. The H_2S levels were continuously monitored in the two parallel flows by H_2S sensors (Unisense + amperometric sensors). In addition, 5 control fish were inserted in the two-choice tank system without adding H_2S in any of the flows. Each control salmon was in the two-choice tank system for 90 minutes each. Fish behavior was recorded by video cameras with UV-filters.

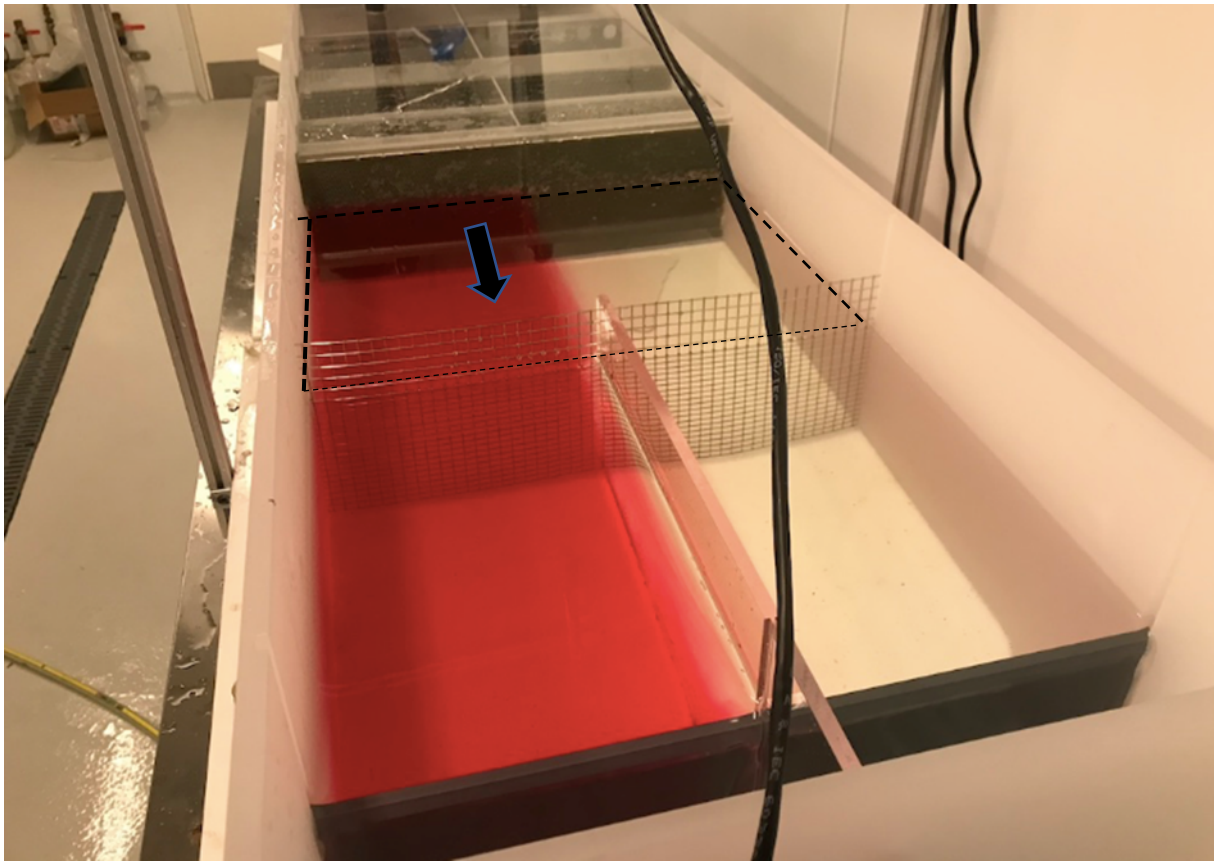

Figure 3: The Loligo Choice Tank (Loligo Inc) with two parallel laminar flows. Red colour was inserted in one of the flows to check potential mixing between the flows. The arrow indicates the choice area inside the dotted lines where the test post-smolt salmon could swim freely.

3.3 Behavior analysis

Behavior analysis was done using the video tracking system Ethovision XT. This is a software that tracks and analyses the behavior of the animal being filmed. The two parallel flows were defined as two different zones in an arena (choice area; Fig. 3). The percentage of time the fish spent in each zone was quantified for each 15 minute period after H_2S was added. Each fish was monitored for a total of 90 minutes. Avoidance was calculated as the percentage of time the fish spent in the zone where H_2S was not added. The behavioral parameters analysed for the five control fishes were velocity, avoidance and the frequency of shifting between the two zones in the arena.

3.4 Statistics

Avoidance was analyzed by a multiple comparison Kruskal-Wallis analysis of variance (ANOVA) with 15 minutes periods as an independent factor.

4.0 Results

4.1 H_2S concentrations

After adding stock solution to one of the parallel flows every 15 minutes for 90 minutes, the H_2S concentration ended up at $2.7^{\pm}0.12$ µM (Table 1). The H_2S in the other flow increased slightly, ending up at H_2S concentration of 0.047 ± 0.01 µM (Table 1).

*Table 1: H_2S added and measured in two parallel laminar flows in a two-choice system. In one of the flows (**Seawater + H_2S**) the H_2S levels were increased every 15 minutes for 5 times (after 15 min, 30 min, 45 min, 60 min, and 75 min) by adding 64, 1.28, 2.56, 5.12 and 10. 2 mL H_2S - stock solution, respectively (9.6 gram (0.4M) of $Na_2S \bullet 9H_2O$ in 100 ml MilliQ water). No H_2S was added in the other flow (**Seawater**).* Values are mean$^{\pm}$S.E.M, n=10.

Minutes	**Seawater [H_2S] (µM)**	**Seawater + H_2S [H_2S] (µM)**
15	$0.00046^{\pm}0.0003$	$0.0022^{\pm}0.0021$
30	$0.0032^{\pm}0.0009$	$0.034^{\pm}0.012$
45	$0.0088^{\pm}0.001$	$0.29^{\pm}0.040$
60	$0.017^{\pm}0.003$	$0.48^{\pm}0.075$
75	$0.038^{\pm}0.005$	$1.5^{\pm}0.20$
90	$0.047^{\pm}0.01$	$2.7^{\pm}0.12$

4.2 Behavior

4.2.1 Control fish

The median velocity measured in the 15 minute intervals for the 5 control fishes through the 90 minute experiment varied from around 1 cm/s to 2.5 cm/s (Fig. 4). The maximum velocity of the control fishes reached close to 5 cm/s in 4 of the 6 time intervals, while the minimum velocity for most of the time intervals was close to 0 cm/s.

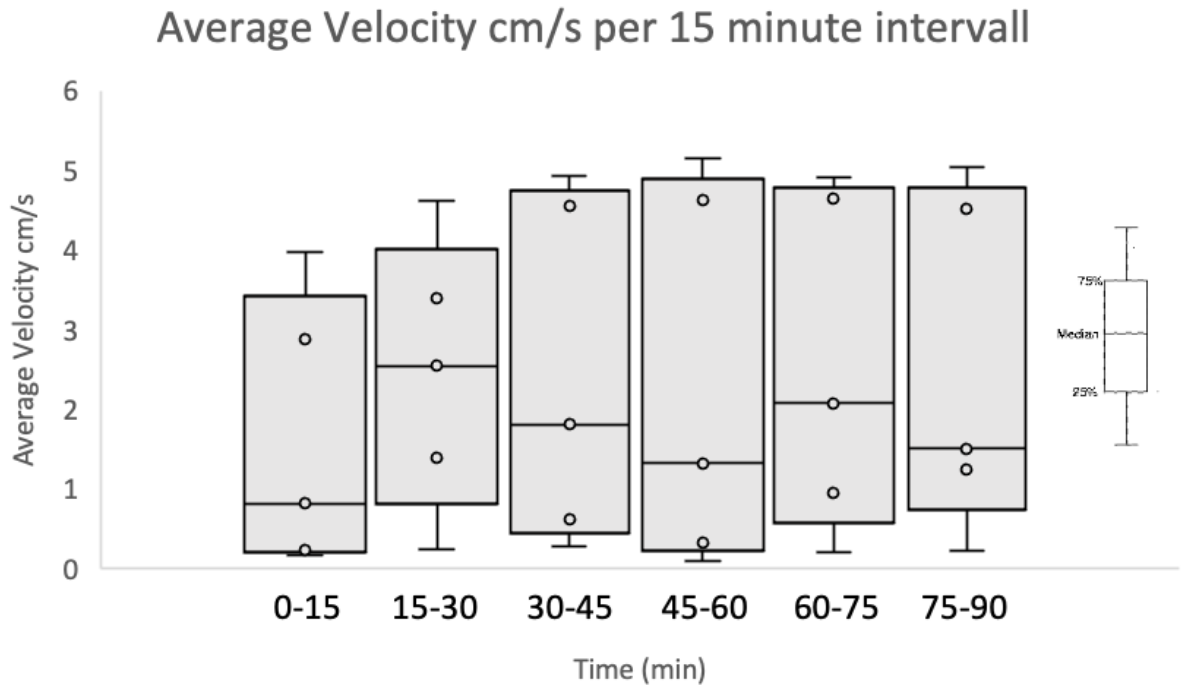

Figure 4: Velocity of post smolt salmon during 15-minute intervals. This was done in the two-choice system with two laminar flows, without H_2S added. Data was collected from 5 individuals over the course of 90 minutes. Circles represent individual salmon that are not the minimum or maximum velocity of the group.

The number of times crossed over measured in the 15 minute intervals for the 5 control fishes through the 90 minute experiment varied from around 1 to 5 times (Fig. 5). The maximum number of times crossed over varied considerably from 12 to 35 times (Fig. 5).

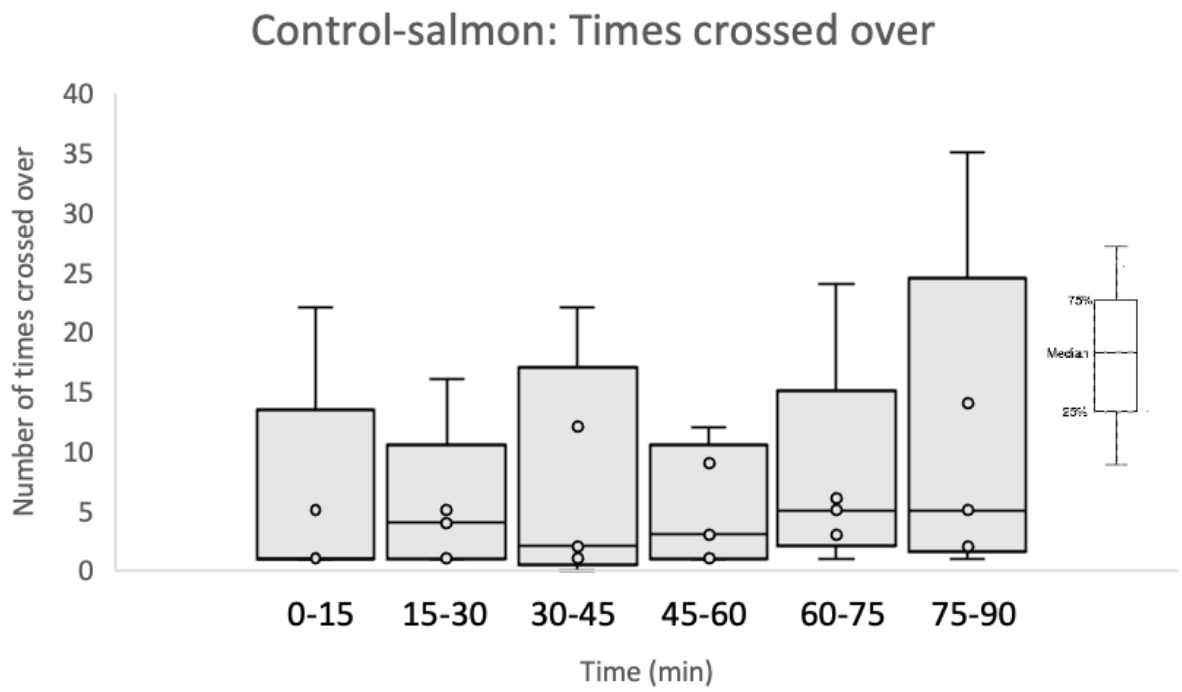

Figure 5: Number of times post smolt salmon crossed between flows during 15-minute intervals. This was done in the two-choice system with two laminar flows, without H_2S added. Data was collected from 5 individuals over the course of 90 minutes. Circles represent individual salmon that have not crossed over the minimum or maximum times of the group.

The percentage of time the 5 control fish spent in the "Seawater + H_2S" zone was registered for each 15 minute interval for the 90 min the experiment lasted. The median for each 15 minute interval varied from roughly 20% to 35% (Fig. 6). Out of the 6 medians, 4 of them was roughly between 25% and 30%. The maximum preference for each 15 minute interval was close to 100%, while the minimum preference for most of the 15 minute intervals almost reached 0 % (Fig. 6).

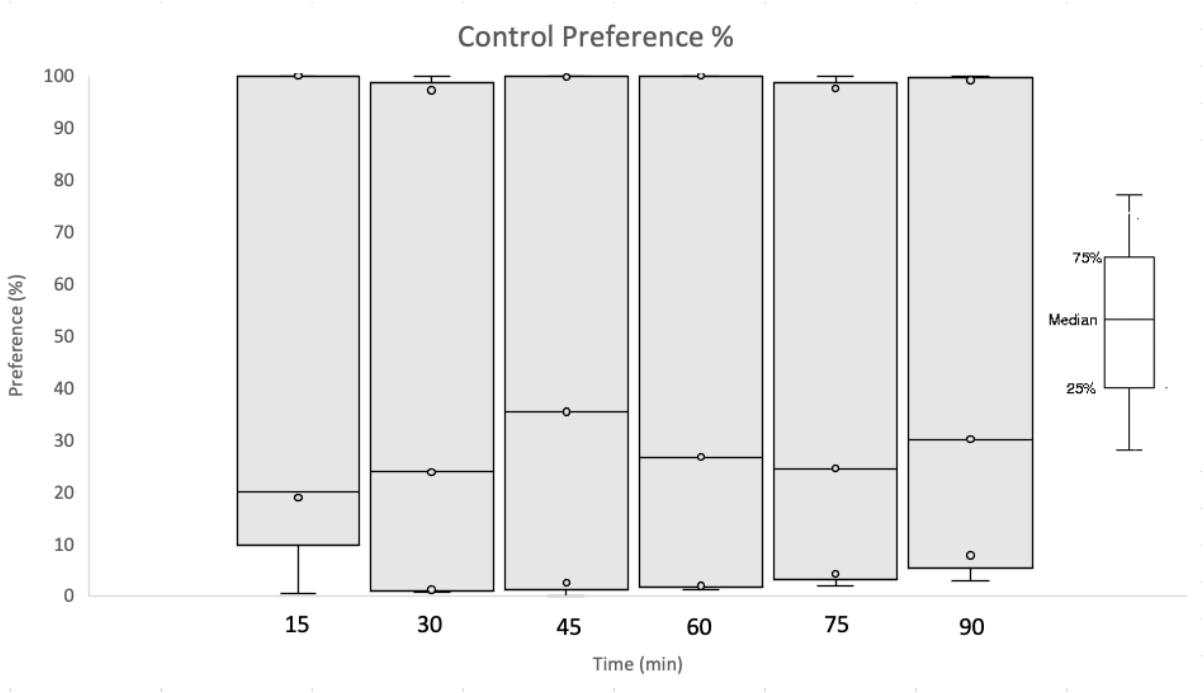

Figure 6: Time spent in the "Seawater + H_2S" zone, compared to the "Seawater" zone. Time was measured as percentage of time spent in the "Seawater + H_2S" zone. Data was collected from 5 control-fish. Circles represent individual salmon preference (%) that are not the minimum or maximum of the group.

4.2.2 Avoidance to H_2S

Avoidance to H_2S in the 10 experimental post-smolt salmon varied from medians of 45 % to 90 % (Fig. 7). Avoidance changed over time (Kruskal-Wallis ANOVA, $H=11$, $P<0.05$), and showed significantly higher values at the 75-90 min intervals with a H_2S concentration of 2.7 ± 0.12 µM compared to 0-15 min intervals when no H_2S was added ($P< 0.05$). There were no significant differences between the 0-15 min time interval and the other 15 min time intervals ($P < 0.23$).

The minimum avoidance for each 15 minute interval became increasingly higher, going from 0 % at 0-15 to roughly 99% at 75-90 (Fig. 7).

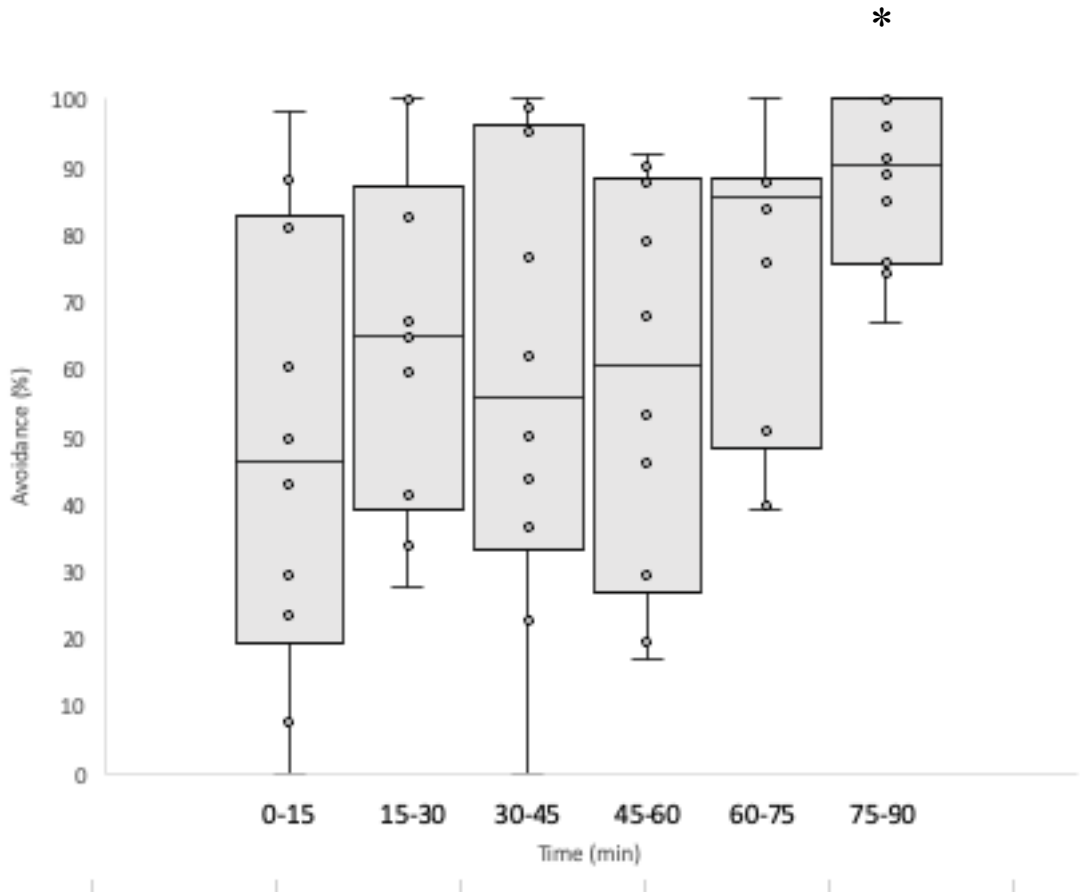

Figure 7: Avoidance to increasing H_2S levels in Atlantic salmon post-smolts. H_2S concentration was increased every 15 minutes by adding 64, 1.28, 2.56, 5.12 and 10. 2 mL H_2S -stock solution (9.6 gram (0.4M) of $Na_2S \bullet 9H_2O$ in 100 ml MilliQ water) in one of two parallel flows in a two-choice system. For concentrations see table 1. Avoidance was quantified as % of time a fish spent in the flow where H_2S levels were not increased. An Asterix indicates significant difference ($P<0.05$) to the first (0-15) 15 min interval, n=10.

Avoidance (%) to H_2S among individual Atlantic salmon post-smolts was highly variable but increased as the H_2S concentration (µM) increased (Fig. 8). While avoidance in H_2S concentrations from 0 to around 0.7 µM, the avoidance varied from 0% to 100%, the avoidance displayed by the fishes after critical H_2S concentration (c. 1.8 µM; Bergstedt & Skov, 2023) was reached, varied between 70 % to 100% (Fig. 8).

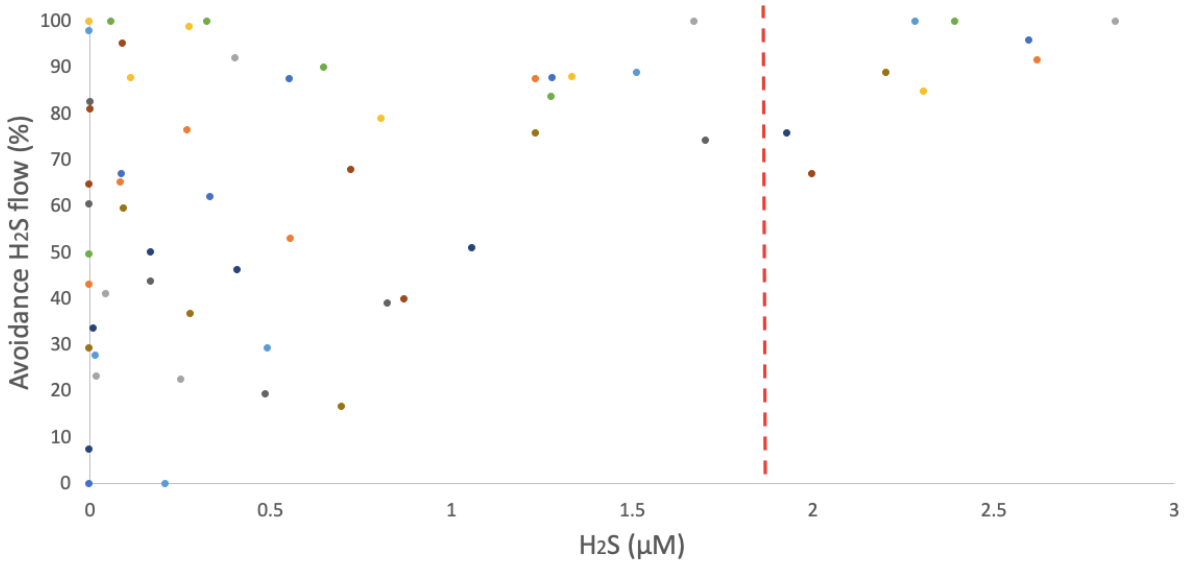

Figure 8: Avoidance to increasing H_2S levels in individual Atlantic salmon post-smolts. H_2S were increased every 15 minutes by adding 64, 1.28, 2.56, 5.12 and 10. 2 mL H_2S -stock solution (9.6 gram (0.4M) of $Na_2S•9H_2O$ in 100 ml MilliQ water) in one of two parallel flows in a two-choice system. Avoidance was quantified as % of time a fish spent in the flow without H_2S addition. H_2S concentrations monitored continuously and are presented as averages for each 15 min interval. An arrow indicates critical H_2S concentrations (1.78±0.39 µM) at which fish cannot withhold metabolic functions (Bergstedt & Skov, 2023). Each color represents individual fish.

5.0 Discussion

5.1. Methodological considerations

The relatively high interindividual variation in velocity in post-smolt salmon (between almost 0 and 5 cm/s; Fig. 4) was reflected by some individuals not changing sides during several 15 min behavioral analyzation periods (Fig. 5). Large interindividual changes were found also in a study of wild salmon where radio-tagged adult salmon voluntarily swam against water stream velocities of 1.32 to 2.85 m/s reaching burst swimming speeds of 4.13 m/s (Colavecchia et al., 1998). The velocity 4.13 m/s is roughly three times the speed of 1.32 m/s (Colavecchia et al., 1998), while a speed of 5 cm/s is 5 times the speed of 1 cm/s (Fig. 4). The high interindividual variation in my study could be a result of running tests on a relatively small amount (5) of control salmon. Out of the 30 different 15 minute intervals (five fishes x six time intervals), the salmon crossed over once or less in 11 of those 30 time intervals (Fig. 5). Still, in H_2S exposed fish, 15 min periods between H_2S additions resulted in a significantly smaller amount of time spent in the part of the flow where H_2S was added in the highest concentration (Fig. 7). It is possible that longer periods between H_2S additions would increase the probability of a fish being present in the flow without H_2S addition. This increased chance for the fish to spontaneously change side in a 15 min interval, by for example adding H_2S every 30 minutes and monitoring each salmon longer than 90 minutes, would have increased the statistical power of my study. Moreover, it is important to note that variation in concentrations was not included in the non-parametrical statistical model of avoidance behavior. A scatterplot of the actual concentrations at each 15 min interval indicated that avoidance may occur at lower concentrations (1-1.5 μM; Fig 8) compared to what the non-parametrical method suggested (1.5-2.7 μM).

There was a slight increase in H_2S concentration in the flow where no H_2S was added. This resulted in the H_2S concentration reaching 0.047 μM in the last observation period. It cannot be excluded that this affected the choice between the flows. However, the threshold for detection and reaction to H_2S was somewhere between 1 - 2.5 μM. Thus, this suggests a limited impact of H_2S in the flow where no H_2S was added. The movement of the relatively big fish (≈12-15 cm) compared to the size of the behavior arena (32 x 40 cm) could be a contributing factor to the seepage of H_2S between the flows.

5.2 Avoidance

Our study indicates that avoidance appear at H_2S concentrations 1.0-2.7 μM in Atlantic salmon post-smolts. Interestingly, studies in the H_2S tolerant tropical fishes *Kryptolebias marmoratus*, *Poecilia orri, Gambusia sp.* and *Dormitator maculatus,* indicate avoidance to similar H_2S concentrations (1,1μM) (Rossi et al., 2019) as in my study. A recent investigation regarding the respiratory responses to increasing H_2S levels showed that post-smolt salmon could not withhold respiration necessary for supporting basal functions in concentrations > 1.8 μM (Bergstedt & Skov, 2023). Assuming an ideal situation, when the avoidance response occurs at a concentration lower than the toxicity threshold (Araújo & Blasco, 2019), then the study performed by Bergstedt & Skov (2023) suggests that avoidance occurs at H_2S levels <1.8 μM in my study (Fig. 8). However, it is important to note that the study of Bergstedt & Skov (2023) was performed in water temperatures of 14 ºC, while the temperature was 10-13 ºC in my study. Considering that H_2S at toxic levels interferes with mitochondrial oxygen uptake (Jiang et al., 2016) and that seeking colder temperatures is a general response to hypoxia in several fish species (Petersen & Steffensen, 2003), it is possible that a lower temperature decreases the H_2S concentration that evokes an avoidance response in post-smolt Atlantic salmon. The fact that a H_2S exposure lowered the preferred temperature in zebrafish (Skandalis et al., 2020) lend some support to this assumption.

5.3 Behavior

As salmon were subjected to increasing levels of H_2S, the fish started spending less time in the H_2S flow. As the levels got higher and higher, salmon started trying to jump out of the testing area. This is because certain levels (1.78±0.39 μM) of H_2S is toxic to salmon (Bergstedt & Skov, 2023) and it evokes an escape reaction from them. The highest velocity reached by the salmon was 5 cm/s, whether or not this is a notable velocity, was not a focus of my study. Colavecchia et al (1998) researched the swimming speed of wild adult salmon and found out the highest burst swimming speed was 4.13 m/s or 413 cm/s. The research of Colavecchia et al (1998) was done on adult salmon (48.3 to 54.8 cm long), while my salmon were 80-100g post-smolt salmon (roughly 10-15 cm long). By comparing the velocity of the research done by Colavecchia et al (1998) with the velocity my study researched, it can be inferred that my salmon did not reach very high speeds compared to their size. By this I mean that the burst speed (413 cm/s) of adult salmon (48.3 cm long) was roughly 10 times the body length of the salmon (Colavecchia et al., 1998). My salmon (10-15 cm long) swam at most 5 cm/s, which

would be 2 times or 3 times the salmon body length. It should be noted my salmon were much smaller than the salmon of Colaveccchia et al (1998). When exposed to high levels of H_2S, the maximum number of times the salmon switched sides between flows were 35 times (Fig. 5). The next highest number of times switching sides was 24 times (Fig. 5), which could be inferred to be a large difference, but I have found no research to compare this assumption with.

5.4 Applications in aquaculture

My data indicate that salmon detect and react to H_2S at concentrations of 1-2.7 μM, which might be at subtoxic levels. These results can potentially be applied in the aquaculture industry, specifically for generating early warning signals for increasing H_2S levels. Automatic behavioral pattern detection by computer vision is an emerging field in aquaculture (Yang et al., 2021). Thus, potentially, computer vision can be used to monitor behavioral pattern associated with increasing H_2S levels for and initiating mitigation measures against this issue. Interestingly, Bergstedt et al (2022), showed that H_2O_2 was an efficient water treatment technology for H_2S removal. The mechanisms behind this is that H_2S is oxidized in an aqueous solution, forming the unharmful products; sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), and sulfate (SO_4^{2-}). Moreover, H_2O_2 decomposes into water and oxygen, and is considered environmentally safe (Bergstedt et al., 2022). Thus, the behavioral response detected in my study together with mitigation measures might offer a strategy to decrease mortality linked to increased H_2S in aquaculture. However, it is important to note that I only studied behavioral reactions of single fish in a situation where the fish was able to escape from increased H_2S levels, and in an aquaculture setting there are no refuges from H_2S and fish are in big groups.

If subtoxic levels of H_2S are reflected in avoidance behavior in my study, then this raises questions about the physiological impact of this perturbance. It could be speculated that subtoxic levels of H_2S might act as a chronic stressor, which together with other potential suboptimal environmental factors in RAS, such as hypercapnia (Höglund et al., 2023) might affect the welfare and growth performance of fish (Höglund et al., 2023).

5.5 Future studies

As pointed out in 5.2 the behavioral reaction and toxicity thresholds to H_2S might be temperature dependent. Thus, further studies are needed to investigate how toxicity and behavioral reactions to increasing H_2S levels are affected by temperature. Especially, the

relation between behavioral reactions and toxicity need to be further studied for verifying avoidance as a tool for initiating mitigation measures against increasing H_2S levels in RAS. It should also be noted that my results that show behavioral response to H_2S still needs to be verified in an aquaculture setting. This is because fish are held in high densities and there are no refuges from H_2S in an aquaculture setting.

It is also important to note that fish in RAS might be exposed to other environmental stressors. Generally, the low water exchange in RAS provides culture environment that generally contains higher concentrations of waterborne metabolites, nutrients, bacteria and dissolved metals (Davidson et al., 2009; Martins et al., 2009; Xue et al., 2017). Accordingly, studies of interaction effects between H_2S and other accumulating substances in RAS, such as carbon dioxide, ammonia, ammonium, nitrate, nitrite, residual oxidants, particles and metals (Davidson et al., 2009; Martins et al., 2009; Xue et al., 2017), are needed to reveal potential negative interaction effects on fish performance and welfare.

6.0 Conclusion

My research indicate that salmon detect and react to H_2S at concentration levels between 1-2.7 μM. Together with the study performed by Bergstedt and Skov (2023), which show acute toxicity at 1.8 μM, this demonstrates a potential application of behavioral changes associated with increasing H_2S for generating warning signals before toxicity is reached. However, further studies are needed for verifying the behavior reactions to increasing H_2S levels in an aquaculture setting, where fish are kept in high densities and there are no refuges from high H_2S levels.

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