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Effects of Environmental Enrichment on Social Cohesion, Aggressive Behaviour and Telencephalon Cell Proliferation in Atlantic Salmon Parr

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Abstract

Much is known about the life cycle and growth of the Atlantic salmon, but there has been less focus on the behaviour and neurobiology of these animals in captivity and how they interact with each other and their environment. Salmon in aquaculture rearing tanks are normally kept in confinement in a homogenous environment without any enrichments, which raises both ethical as well as welfare related questions. Therefore, this study aimed to delve into the potential of different commercially applicable enrichments to increase welfare. The study explored the hypothesis that bubbles, increased water current and stones would each contribute to improved welfare by comparison with rearing in unenriched standard rearing tanks (the control treatment), as measured by increased social cohesion, reduced aggression, and increased cell proliferation in the telencephalon. There were 30 salmon randomly distributed in each of 12, 100-litre tanks where 3 of the tanks were Controls, whilst the rest of the tanks were evenly distributed among the enrichments Bubbles, Current and Stones, all followed for 6 weeks. The enrichment treatments used were Bubbles (intermittent release of small air bubbles in the tank), Current (increased current around the wall of the tank), and Stones (structural enrichment with 6 stones at the bottom of the tank). Videos from Control and enriched tanks were made on one day per week over a 6-week period. To assess social cohesion, snapshots were taken at two time points per 15-min video (1 min after the start of the video and 1 min after the start of bubbles in the Bubbles treatment and corresponding time points in the other treatments). From each photograph, distances between the snouts of each individual in a tank were calculated to find the average distance as a measure of social cohesion. Additionally, numbers of aggressive attacks in each tank were counted during two 5-min periods per video (0-5 min at the video start, and 10-15 min at the end of the video). Telencephalon cell proliferation was measured after 6 weeks on the treatments, by running an immunohistochemistry procedure on stained telencephalon tissue sections and, thereafter, analysing the sections computationally to find the number of stained nuclei.

Our findings showed that social cohesion was similar across treatments but declined with increasing age ($p = 0.012$). Bubbles triggered more aggression than observed in the Control tanks ($p = 0.001$), whereas the other treatments had an aggression level comparable to that of the Control group. Aggression increased over the 6-week study in all treatments, but more in the Control treatment than in the others. Analysis of cell proliferation showed that there were significant differences between the brain regions, with the dorsomedial and ventral region of

the ventral telencephalon having lower stained cell counts compared to the dorsolateral region. There were also differences between the treatments in the different brain regions ($p < 0.001$). The findings support that the environmental enrichment treatments evaluated did have some effects on behaviour and cell proliferation in the telencephalon that could influence salmon welfare.

Sammendrag

Det er mye som er kjent med livssyklusen til atlantisk laks, men det er likevel lite fokus på adferden og nevrobiologien av disse dyrene i fangenskap og hvordan de interagerer med hverandre og med deres miljø. Oppdrettslaks er vanligvis satt i et homogent miljø uten miljøberikelser, noe som både stiller etiske så vel som velferdsmessige spørsmål. Derfor ønsker vi ved dette studiet å ta et dypdykk i potensialet av tilsettingen av kommersielt tilgjengelige miljøberikelser som kan øke dyrevelferden. Studien utforsket hypotesen som omhandler hvorvidt bobler, økt strøm og steiner vil hver bidra til økt velferd i forhold til oppdrett i ikke-berikede standardtanker (kontrollbehandling) målt i økt sosial samhørighet, redusert aggresjon og indikert av økt celleproliferasjon i telencephalon. Det var 30 laks som ble tilfeldig distribuert i 12 ulike, 100-liters tanker hvor tre av disse fungerte som en uberiket kontrolltank, mens resterende av tankene var jevnt fordelt med berikelsene bobler, strøm og steiner. Fiskene ble fulgt i 6 uker. Miljøberikelsene som var brukt var Bobler (intermittert utløsning av små luftbobler i tanken), Strøm (varierende strøm tvers rundt veggene av tanken), og Steiner (strukturelle berikelser av 6 steiner på bunnen av tanken). Videoer fra Kontroll- og miljøberikede tanker var tatt én dag per uke over en 6-ukers periode. For å vurdere sosial samhørighet ble skjermdumper tatt ved to tidspunkter per 15-minutter video (1 min etter start av videoen samt 1 minutt etter start av boblene i boblebehandlingen og tilsvarende tidspunkter i de andre behandlingene). Fra hvert bilde ble avstander mellom snutene av hvert individ i tanken kalkulert for å finne den gjennomsnittlige avstanden som et mål på sosial samhørighet. I tillegg ble antall aggressive angrep i hver tank telt i løpet av to 5-minutters perioder per video (0-5 minutter ved videostart og 10-15 minutter ved videoslutt). Telencephalon celleproliferasjon var målt etter 6 uker i behandling ved å gjennomføre en immunohistokjemiprosedyre på fargede telencephalonvev og deretter ble vevseksjonene analysert digitalt beregningsmessig for å finne antall fargede cellekjerner.

Våre funn viste at sosial samhørighet var likt i de ulike behandlingene, men ble redusert ved økt alder ($p = 0.012$). Bobler trigget mer aggresjon enn observert i kontrolltankene ($p = 0.001$) og de andre behandlingene hadde et aggresjonsnivå som var sammenliknbart med Kontrollgruppen. Aggresjonen økte likevel mest i 6-ukers perioden i Kontrollbehandlingen, i forhold til de andre behandlingene. Analyse av celleproliferasjon viste at det var signifikante forskjeller mellom hjerneregionene, hvor den dorsomediale og ventrale regionen av ventrale telencecphalon hadde lavere antall fargede celler i forhold til den dorsolaterale regionen. Det

var også signifikante forskjeller mellom behandlingene i de ulike hjerneregionene ($p < 0.001$). Funnene støtter at de miljøberikelsene som ble evaluert hadde noe effekt på lakseadferden og celleproliferasjon i telencephalon, som kan påvirke laksevelferd.

Abbreviations

DL: Dorsolateral

DLL: Dorsolateral left side

DLR: Dorsolateral right side

DM: Dorsomedial

DML: Dorsomedial left side

DMR: Dorsomedial right side

glmer: Generalized Linear Mixed-Effects Model

glm: Generalized Linear Model

IHC: Immunohistochemistry

lmer: Linear Mixed-Effects Model

PCNA: Proliferating cell nuclear antigen

ROI: Region of interest

VV: Ventral part of ventral telencephalon

VVL: Ventral part of ventral telencephalon left side

VVR: Ventral part of ventral telencephalon right side

Table of content

1. Introduction

1.1. Atlantic salmon life cycle

Salmonids are in the family, Salmonidae, which comprises salmon-like fish such as trout, graylings, taimens and salmon amongst others. Many salmonids are anadromous, meaning they spawn in freshwater before they migrate downstream to the sea as adults, yet there are a few examples of landlocked salmonids that only live in freshwater throughout their life cycle (Hutchings et al., 2019). In the sea, however, the salmon grow bigger and undergo sexual maturation (Friedland, 2000) before emigrating back to their home river where they reproduce and bury their eggs in the gravel of the river, safe from predators. Growth and development of salmonids varies a lot and is determined by genetics and environmental factors such as food availability, photoperiod and water surface temperatures (Björnsson et al., 2011).

The Atlantic salmon (*Salmo salar*), one of the largest members of the Salmonidae, is typically found in the northern part of the Atlantic Ocean and in the rivers and fjords connected to this (Borgstrøm & Hansen, 2000). An Atlantic salmon usually follows an anadromous life cycle (Fig. 1) starting in the river as a fertilized egg called a *redd*. Newly hatched salmon are called *alevins* and are equipped with a yolk sac with nutrients. When the sac is emptied, the fish now enters the *fry* stage and begins to swim in the river and attempt to find food for itself (Mobley et al., 2021). The *parr* stage starts when the fish is about 5 cm long, when it develops vertical black parr bands along its body. In this stage, the individuals are also very territorial, allowing them to defend an area within the water stream for feeding. They remain in the parr stage for 1-3 years in their native river before they undergo physical as well as physiological changes, in a process referred to as *smoltification*, enabling them to migrate to sea (Borgstrøm & Hansen, 2000). *Smolts* migrating to the sea no longer have the parr bands as earlier, but develop dark spots scattered across their bodies above the lateral line as well as attaining a silvery shine and streamlined body shape (Mobley et al., 2021). At sea, the brighter the silver colour of the scales and greater the size of the fat fins, the greater the fat deposits in the fish are (Borgstrøm & Hansen, 2000). And the size of the fat deposits reflects the growth abilities of the individual (Jobling et al., 2002). When returning upriver to spawn, the males develop a red colour.

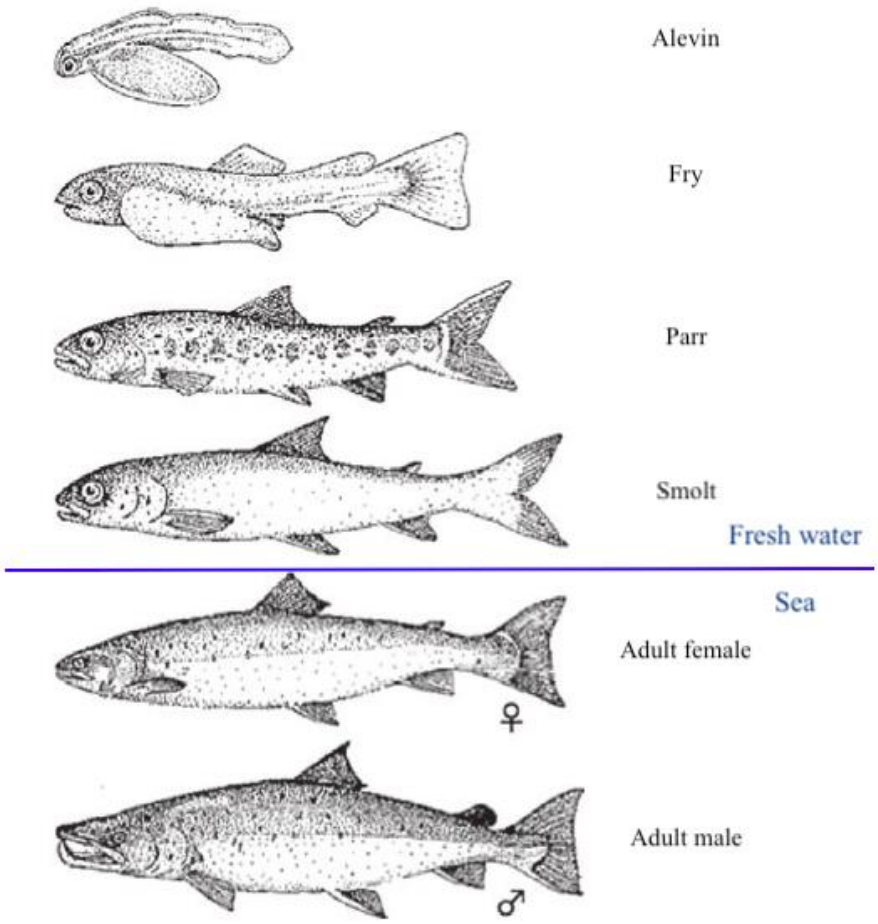

Figure 1: Atlantic salmon life stages, distinguished between fresh water and sea phases. Illustration modified from McMenamin & Parichy (2013).

Atlantic salmon farming is based on harvesting eggs and rearing juveniles in land-based freshwater facilities, followed by movement of the fish to sea water cages where the fish grow further before harvest (Taranger et al., 2014). Sea cage farming proves to be a highly effective production system requiring much lower investment and cost, compared to land-based aquaculture (Lekang et al., 2016). There are several issues with salmon farming such as parasite and predator threats in addition to escape of fish from the cages. However, a concern is the welfare of the fish in aquaculture facilities as the stocking densities are rather high and the habitats are remarkably homogenous.

1.2. The challenge

Though the Atlantic salmon is the most common salmonid species found in the wild in Norway, wild Atlantic salmon are on the "Norwegian Red List" of threatened species and the population is rapidly declining (Norwegian Biodiversity Information Centre, 2021).

Simultanously, the global demand for farmed Atlantic salmon is increasing and the production efficiency of salmon farming is growing (Asche et al., 2011). However, we also see that the welfare of farmed salmon in the aquaculture industry has significant shortcomings (Kristiansen, 2019). The farming habitat is a poor mirror of the natural environment in which the salmon evolved and the behaviour of farmed salmon is greatly constrained, which again negatively affects smolt quality and survival upon reintroduction to the sea from freshwater rearing conditions (Hjeltnes et al., 2016).

1.3. Environmental enrichment

Fish hatched in captivity are reared in a safe homogenous environment, but if released into the sea, their behaviour connected to survival is weak (Salvanes & Braithwaite, 2005). This might be due to their restrictive and homogenous rearing environment where behavioural exploration is limited by space constraints and a barren environment. Not only is the genetics of the individual an important factor in their behaviour, but we also see that the learned behaviour reflects the environment in which the animal is kept in (Odling-Smee & Braithwaite, 2003). If the environment is barren, with nothing to do, it provides little opportunity to learn to avoid danger, develop skill in finding and using valuable resources or to develop a strong, flexible physique that promotes longevity.

Environmental enrichment is a strategy for providing captive animals with these opportunities. Environmental enrichments are defined as improvements or modifications to the environment of captive animals that benefit their biological function and increase their welfare (Newberry, 1995). Enrichments reinforce learning ability as well as memory in many animals (Falkenberg et al., 1992). For young salmon, environmental enrichment might not only reduce the repercussions of stressors in the fish tanks, but also add complexity to the homogenous habitat which to some extent could mimic a natural habitat and thereby might help the salmon to adapt better if released in a natural habitat or when transferred to sea cages (Näslund et al., 2013). While there can be welfare benefits from environmental enrichment, these need to be considered in relation to practicality of implementation and expense relative to their welfare value (Newberry, 1995).

Providing environmental enrichment in captive housing has been reported to increase the survival and behavioural flexibility of hatchery-reared salmon after release in the wild (Salvanes et al., 2013). By enrichening the captive environment, the welfare of farmed salmon

may be improved due to greater opportunities for positive behaviours such as play and exploration that promote learning, as well as a reduction in fear and aggression. Many studies such as the aforementioned by Salvanes et al. (2013) focus mainly on structural enrichment factors, though there are other categories of enrichment including nutritional, occupational, sensory, and social (Bloomsmith et al., 1991).

Provision of bubbles, increased water current and stones are all potential sources of enrichment for farmed salmon during rearing in freshwater tanks that could be practical for commercial implementation. Intermittent release of bubbles may be enriching if the bubbles trigger play behaviour and increased swimming activity, resulting in occupational enrichment (Kleiber et al., 2022). Additionally, the tactile quality of bubbles might have a sensory enrichment value. In the Kleiber et al. (2022) study, bubbles were used as a temporal signal predicting the arrival of food for rainbow trout (*Oncorhynchus mykiss*) through classical conditioning, which was useful in lowering aggressive behaviour. The bubbles were on for 15 seconds, 5 times a day and the feeding began 5 seconds after the bubbles had ended. The observations were made for 6 minutes before feeding time.

During captive rearing of salmon, the water current is typically lower than that encountered by wild parr in rivers. A higher water current may not only provide a thrilling environment but also stimulate greater physical activity such as enhanced swimming activity in addition to increasing the physical ability to hide or flee from predators which is crucial for survival in nature (Thorpe et al., 1992). A study done on Atlantic salmon post-smolts with three different water velocities - slow, moderate and fast (respectively speed of 0.2, 0.8 and 1.5 body lengths s^{-1}), showed that a moderate water current velocity was best for ensuring good growth and welfare of the salmon as fish in low velocities gained more fat and those in fast velocities had high cardiac work load which could reduce production performance, whilst those in a moderate velocity had sufficient weight gain but also muscle gain (Solstorm et al., 2015).

Structural enrichment through addition of physical structures such as stones adds both complexity and shelter to the environment. Provision of shelter has been reported to lower juvenile Atlantic salmon metabolic rate, which could conserve energy and improve growth performance (Milidine et al., 2006). The addition of structures visually isolating the fish may also reduce overall stress and aggression (Näslund et al., 2013).

1.4. Social cohesion

Many fish practise schooling behaviour meaning they swim together in groups in a synchronised manner. Schooling behaviour can be beneficial for the individual, especially to detect predatory threats as well as improve prey search efficiency (Riley et al., 2014). Also, schooling fish take advantage of the hydrodynamic efficiency of moving in groups, which is more energy efficient than swimming alone (Chen et al., 2016). However, the trade-off for being in a group is that competition will increase if resources are limited, which varies in regards to resource type, distribution, ecological context and competitive abilities of the individuals in the group (Ward et al., 2006). Social cohesion is beneficial for a myriad of reasons such as attaining more information about the surroundings, invoking group hunting, and working as an anti-predator strategy as it improves predator detection and may cause predator confusion, thereby reducing the overall predation risk (Handegard et al., 2012; Miller et al., 2013).

Group decision making is determined by individual fish behaviour, and multiple factors can influence the interindividual distances between the fish in a group and, thus, the overall group cohesion (Miller et al., 2013). For example, at an individual level, fish with higher metabolic rates tend to swim at the front of the school and those with lower metabolic rates tend to stay at the rear (Killen et al., 2011). In a study done on juvenile Walleye pollock (*Theragra chalcogramma),* the degree of cohesiveness of the group was influenced by the stocking density and size of the rearing tanks, which was measured by calculating the mean distances between neighbouring fish (Sogard & Olla, 1997). In juvenile farmed salmon, increased cohesiveness could indicate that the fish perceive their environment as dangerous despite no actual risk of predation, and this anxious behaviour may negatively affect the growth of the fish. It could also indicate that, when well fed, fish are less territorial and thus better able to benefit from the energy efficiency of swimming together.

1.5. Aggression

Salmon in the parr stage are territorial in nature and this behaviour is often connected to optimizing growth and survival (Keenleyside & Yamamoto, 1962; Steingrímsson & Grant, 2008). A difference between wild and farmed fish is that the latter, which are supplied with abundant food pellets in a relatively safe environment, are generally bolder, more aggressive and more risk-taking (Adams & Huntingford, 2005; Metcalfe et al., 2003). However, though

the captive-reared fish are often larger and better nourished than wild fish, when released in the wild, they are less equipped to hunt for food and hide from predators. Also, general principles of domestication suggest that selectively bred animal should be more socially flexible, tolerant and more stress resistant compared to the wild original population. (Metcalfe et al., 2003; Price, 1999).

Rosengren et al. (2017) found that, during rearing, structural enrichment providing shelter in areas with low salmon stocking density resulted in decreased aggression. That complex habitats could be a factor to reduce aggressive behaviour is also backed up by a study done on zebrafish (*Danio rerio)* (Carfagnini et al., 2009). Nonetheless, the addition of limited structural enrichments could enhance territoriality and increase counts of aggression as the fish may compete for access to finite refuges.

1.6. Cell proliferation in the telencephalon

In mammals, cognitive stimuli are mainly perceived and processed in the forebrain cerebral cortex. This structure is not present in the same manner in teleost fish. Most of the cognitive abilities we associate with the mammalian cerebral cortex such as associative learning, memory and social intelligence are processed in the telencephalon of teleost fish (Bshary & Brown, 2014; Stewart & Kalueff, 2012).

Within the telencephalic area, the dorsolateral (DL) region is recognized to be the functional equivalent of the mammalian hippocampus and dorsomedial (DM) region as corresponding to the mammalian amygdala (Broglio et al., 2015). The ventral section of the ventral telencephalon (VV) has cholinergic neurons in teleosts and is suggested to mediate social behaviour and regulate goal-orientated behaviour, and can be looked at as the putative homologue of the extended amygdala (Brantley & Bass, 1988; O'Connell & Hofmann, 2011). These three regions can therefore be expected to be important for behavioural changes when exposed to varying environments (Mes et al., 2018).

Fish are sensitive to environmental stimuli which in turn can promote neurogenesis within certain limits, by triggering and reinforcing the neuronal circuits affecting the level of neuroplasticity and cognitive abilities (Ebbesson & Braithwaite, 2012; Mes et al., 2018). Unlike the rate in mammals, fish undergo extensive neurogenesis also in adulthood which could be connected to their capacity to regenerate injuries (Kaslin et al., 2007). Across their

life history, salmon are exposed to diverse environmental conditions, which could also explain why neurogenesis is high even in adulthood. Neurogenesis is important in both learning and stress, and includes both proliferation, differentiation, and survival of neurons (Banasr & Duman, 2007). Gliogenesis however, follows neurogenesis supporting neural functions but persists long after neurogenesis has ceased (Lee et al., 2000). A study on mice show that gliogenesis can also be influenced by environmental conditions (Steiner et al., 2004). Together, these processes of cell proliferation in the telencephalon of fish indicate neuroplasticity.

Fish have sensitive periods during development when the external environmental stimuli alters the brain in a greater manner than at other times, and when the experiences gained in these sensitive periods alters performance permanently (Knudsen, 2004). The Atlantic salmon has a unique sensitive period during smoltification which is the transformation between the parr and the smolt stage (Stefansson et al., 2007). In this period, the fish also switch from territorial to schooling behaviour and prepare to encounter new environments (Ebbesson & Braithwaite, 2012).

2. Objectives

The theme for my thesis is ***environmental enrichment for young farmed salmon.*** I wanted to explore whether different types of environmental enrichment would contribute to improved salmon welfare during rearing. I hypothesised that bubbles, increased water current and stones would each contribute to improved welfare by comparison with rearing in unenriched standard rearing tanks (the control treatment) as measured by increased social cohesion, reduced aggression and increased cell proliferation in the telencephalon. I derived the following measurable predictions.

Prediction 1: If bubbles provide a pleasurable physical sensation or attract playful swimming, the social cohesion will be higher in tanks with bubbles (i.e., the distances between the fish will be lower) compared to the control treatment during the intermittent provision of bubbles but not at other times.

Prediction 2: If young salmon prefer to swim in water with a stronger current that is more reminiscent of living in a natural river current, they will be attracted to swim in the periphery

of the tank where the current is strongest, resulting in higher social cohesion than in control tanks that have a lower water flow rate.

Prediction 3: If young salmon have access to stones at the bottom of their tank and if they swim together when active and hide between or below stones at the bottom of the tank during periods of inactivity, this will result in a lower average distance between fish in tanks with stones (i.e. social cohesion will be higher) than in control tanks.

Prediction 4: If bubbles, increased water current, and stones are attractive and stimulate positive affective states, their provision will result in less aggression than in control tanks.

Prediction 5: If parr perceive the provided food to be a limited resource which can be defended from smaller fish, social cohesion will decline, and aggression will increase, with increasing age and size, especially in unenriched control tanks as the fish will be more competitive as they grow bigger and therefore are less willing to swim close together.

Prediction 6: If the added physical and mental stimulation resulting from the provision of bubbles, increased current and stones promotes learning and memory, fish from those treatments will exhibit greater cell proliferation in different regions (DL, DM and VV) of the telencephalon indicating greater cell proliferation, than fish from the control tanks.

3. Materials and methods

3.1. Ethics

The fish tanks at the Norwegian University of Life Sciences (NMBU) in the veterinary building were operated following strict guidelines to ensure the safety, hygiene, and well-being of each fish. The setup of the fish tanks was in consonance with the ethical regulations of the 'Forsøksdyrutvalg' at NMBU in addition to approval from The Norwegian Food Safety Authority (Mattilsynet).

3.2. Aninals and management practices

We used *Salmo salar* in the early parr stage hatched from the university's hatchery at the animal care facility 'Senter for fiskeforsøk'. The tanks were located in the basement of the fish laboratory at the veterinary building of NMBU, where 30 fish were randomly distributed in each of 12, 100-litre tanks. The tanks were connected to an automatic water recirculation

system with an exchange rate of 2 L/min. Each tank was covered by a lid on one side and by a fish net on the other side to prevent the salmon from jumping out of the tank.

The oxygen levels were monitored twice a week and remained at greater than 80 % in all tanks. The water temperature in the tanks was held consistently at circa 14 °C. The fish were reared under 24 hours of dimmed lighting according to the facility's standard operating procedure. The food pellets were formulated to provide a balanced parr diet (Nutra RC, Skretting Global Operations, Stavanger, Norway) and were provided in an amount corresponding to 1 % of the fish mass. The food pellets were automatically dropped into the tanks every day between 11:45 and 12:45. And the tanks were cleaned twice a week for the first month of the experiment, thereafter daily to avoid build-up of waste.

3.3. Experimental Design

We assigned each treatment to 3 rearing tanks according to a randomised block design for a period of 6 weeks (Fig. 2).

Control: No enrichment was added. Only an air stone was present, and the water exchange rate was 2 litres per minute. All other treatments were the same with the following exceptions.

Bubbles: Air bubbles from the air stone were used as a sensory stimulus given twice a day for 5-minutes (09:00-09:05 and 15:00-15:05). Although all tanks contained an air stone to maintain structural similarity, it was only in the tanks assigned to the bubbles treatment that the air stone functioned to produce bubbles when activated.

Current: The water exchange rate in these tanks was set at 8 litres per minute to heighten the swimming exercise of the individuals. At the centre of the tank, the water flow was lower than adjacent to the tank wall, enabling the fish to choose their swimming movement and momentum by adjusting their location in the tank.

Stones: 6 stones were placed on the bottom of each tank.

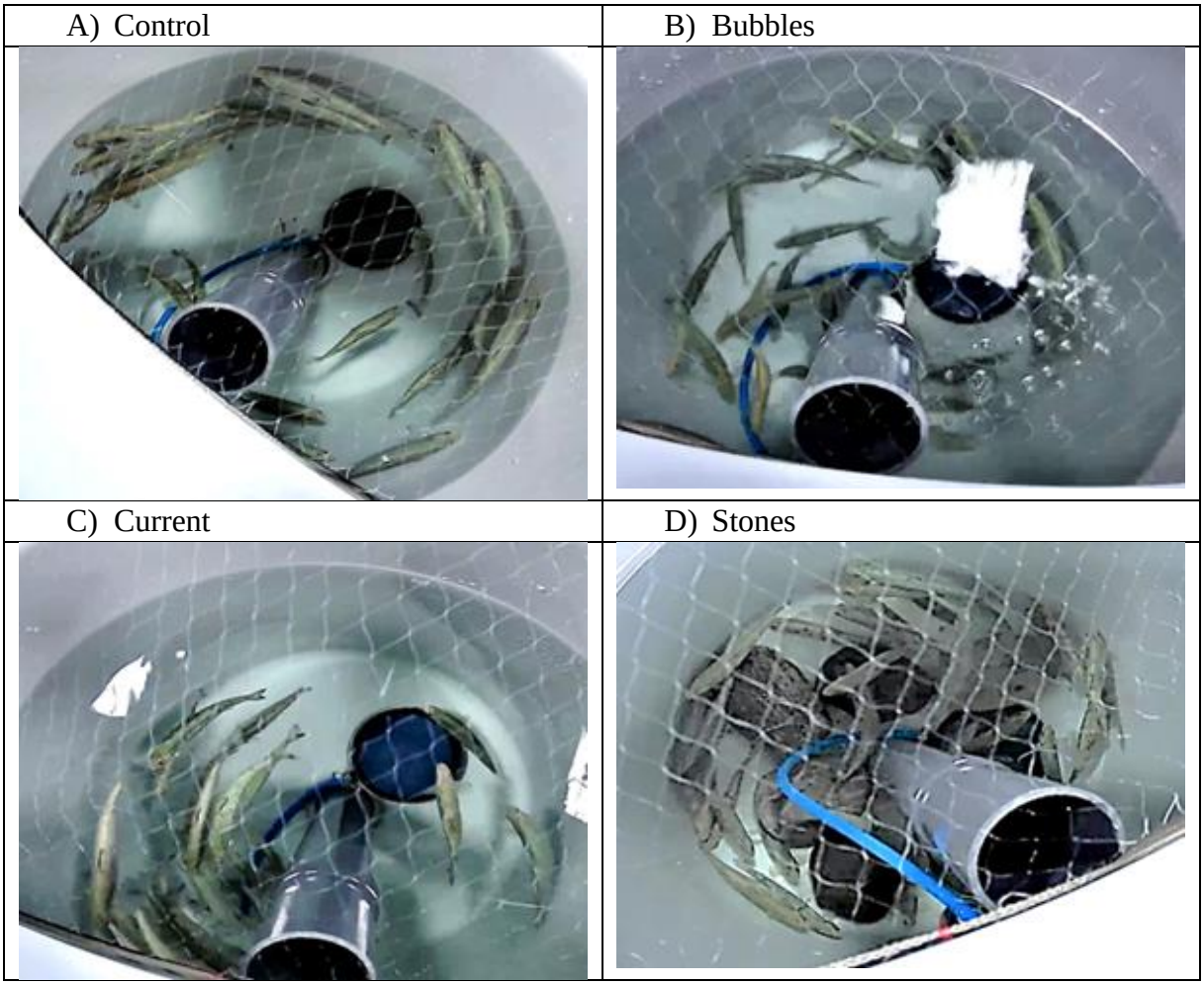

Figure 2: Example pictures of all categories of tanks including control. A) control, B) bubbles, C) current and D) stones.

3.4. Video analysis

Videos were collected from approximately 08:55-09:10 and 14:55-15:10 once weekly using a 4 megapixel HD Foscam G4P outdoor WiFi surveillance camera (Lindit AS, Sandefjord, Norway) over each tank.

3.4.1. Social cohesion

Social cohesion was determined by measuring the average distance between the snout of each fish with each other fish in a tank. Screen shots of the videos were taken 1 min after the video start of the morning and afternoon videos, and again approximately 6 min after the start of the videos, corresponding to about 1 min after the start of the air bubbles in tanks with bubbles. Because fish or bubble activity could distort the water surface and make it difficult to see the fish, the precise time of each picture varied by ±10 s as needed to obtain the clearest possible

picture. There were slightly fewer pictures from the afternoon (n = 288 from the morning, n = 240 from the afternoon) as it was not possible to get videos from the afternoon in Week 6. The lighting and contrast of the original pictures were adjusted as needed to enhance visibility of the fish in the pictures.

Image programming package Fiji in ImageJ (Schindelin et al., 2012) was used to analyse the pictures for measurement of social cohesion. To extract the coordinates of each fish, the *multi-point* tool was used to add a small, numbered mark on each parr snout (at the distal tip of the upper jaw) on the screen shot (Fig. 3). Then the *measuring* tool ('ctrl+m'-command) was used, causing a pop-up window to appear with the x- and y-coordinates of each fish in pixels. Thereafter, the coordinates were copied and pasted into an Excel-file. Information about date, time, video filename, treatment, tank, and camera number were added in their respective columns.

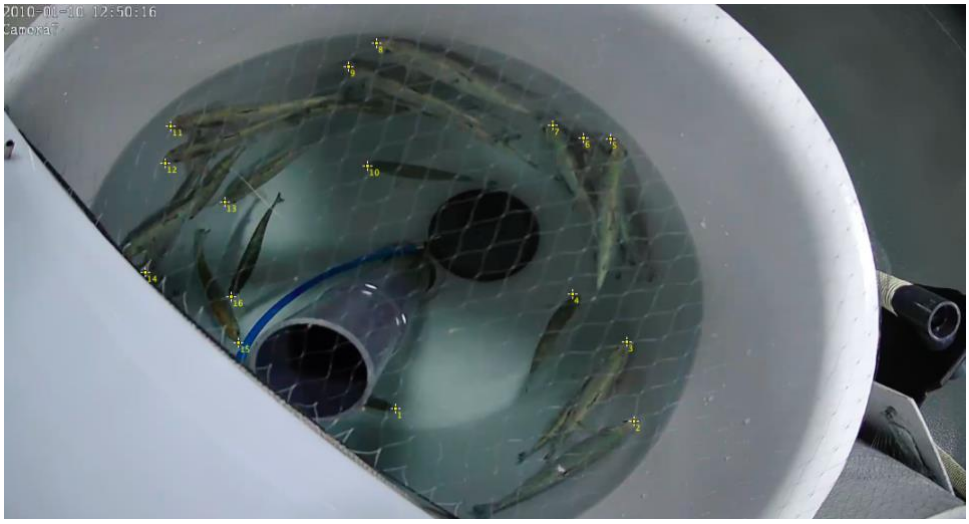

Figure 3: Example of tank screen shot with marked nose tips ("300721 CAM7 08.55.38 #M")

3.4.2. Air stone measurements

There was some variation in the angle of the camera filming each tank. To standardise the measurement of the distance between each fish snout and provide a scale, the *oval*-brush selection was used in Fiji ImageJ to trace around the outer diameter of the air stone (around the black border of the stone). By using the integrated *measuring* tool ('ctrl+m'-command), the area of the oval/round shape in pixels was obtained and the value was added to an Excel data file. Excel was used to determine the distance between each fish snout and to obtain the

mean inter-fish distance per tank in pixels. The actual area of the air stone was then calculated based on physical measurement of its diameter. Constant *K* was found by taking the root of 132.7 divided by the air stone measurement in pixels. Thereafter, *K* was multiplied with the average distance in pixels to get the average distance in cm. This distance was used as the measure for social cohesion.

3.4.3. Aggression

Aggressive behaviour was defined as "one fish darting towards another fish in an attempt to bite/bump/chase it". For both the morning and afternoon videos, all incidents of aggression in the video time intervals between 0-5 min and 10-15 min were recorded on a paper data sheet, corresponding to the 5 min before and 5 min after bubbles in the Bubbles treatment, and equivalent times in the other tanks. The aggression measurements were made by two observers who each collected half of the data. The data from the two observers were balanced across treatments and weeks.

3.5. Lab work

The fish were sampled at the end of the 6-week observation period. Two randomly chosen fish from each tank (meaning 8 fish from each treatment and 32 fish all together) were sampled. Each fish brain was sectioned to give approximately 100 slides with 8-12 brain slices on each slide (resulting in roughly 1000 slices per brain). The slides of each brain were grouped in 4 series (marked 1-4) of 6 slides (A-F). For the immunohistochemistry (IHC) of the telencephalon, sections from the first series (A) were chosen. (See Appendix A.)

3.5.1. Immunohistochemistry (IHC) – telencephalon

The immunohistochemistry protocol required two consecutive days and the work was done at the laboratory at the Veterinary building at NMBU Ås, with a laboratory technician present. To detect cell proliferation in regions of interest (ROIs) of the brain samples, *protein proliferating cell nuclear antigen* (PCNA) (Santa Cruz Biotechnology, Dallas, USA) was used as it is a nuclear cell protein that binds to the DNA in the S-phase of the cell cycle (Candal et al., 2005). (See Appendix B.)

Day 1

Slides with brain tissue were taken out of the freezer and left at room temperature for an hour. They were placed on two trays in the oven at 65°C for 10 minutes. The slides were then soaked in *phosphate buffered saline* and *tween* (PBS-T) in Couplin staining jars. After 10 minutes of soaking, the slides were soaked in 2N *hydrogen chloride* (HCL) at 37°C for half an hour. The slides were afterwards washed in PBS-T 3 times, before they were soaked again in PBS-T in the Couplin jars for 5 minutes in 3 sessions. After the third round of soaking, the borders of all slides were marked with a *PAP-pen* (Abcam, Cambridge, United Kingdom) creating a hydrophobic barrier to restrain the liquid within the slide.

The slides were then shifted to a humid chamber with some room temperature water at the bottom of the container to ensure that the slides would not dry out. Blocking solution was prepared and consisted of 6 % *Normal Goat Serum*, 0.3 % *Triton* and 1 % *dimethyl sulphide (DMSO)* in PBS-T. 200 µL of blocking solution was carefully dropped on each slide and these were incubated for 1 hour at room temperature. The liquid was disposed and the 200 µL primary antibody – *antimouse PCNA* (Santa Cruz Biotechnology, Dallas, USA) diluted in PBS-T, was pipetted onto the slides. Slides with the primary antibody were now incubated over-night at room temperature in a dark humid chamber.

Day 2

Each slide was washed 3 times with 1000 µL PBS-T to rinse off the antibody. Thereafter the slides were again soaked in PBS-T for 10 minutes in Couplin jars in 3 sessions. After the third round of soaking, the border of the slides was carefully dabbed against some regular tissue paper to re-activate the *PAP-pen* barrier, and the slides were put back again into the dark humid chamber. There, 200 µL of the secondary antibody – *antimouse* PCNA diluted in PBS-T was pipetted on each slide. Thereafter, some room temperature water was added to the bottom of the chamber, and it was closed so the slides would incubate in the dark at room temperature for 2 hours.

Each slide was then washed 3 times with 1000 µL PBS-T to rinse off the secondary antibody followed by a soaking in Couplin jars with PBS-T for 10 minutes in 3 new sessions. On a tray, the slides were mounted by pipetting 30 µL of *Vecta Shield* (Vector Laboratories. Inc, Newark, USA) with the fluorescent staining agent *4'6-diamidino-2-phenylindole* (DAPI) on each slide. Nail polish was used to seal the cover slides all around and then the slides were

left to dry at room temperature for 10 minutes on the lab bench. Finally, the finished prepared slides were stored in a refrigerator at 4°C.

3.5.2. IHC quantification – telencephalon

The slides were sent to a scanning facility at the University of Oslo, where high-resolution images were taken and sent back to NMBU where they were inspected by Carl Zeiss microscopy at the lab in the Veterinary Institute at NMBU. ZEN Lite Blue software was then used to inspect the now CZI formatted pictures (microscopy file format developed by Zeiss) and adjust them for contrast and brightness before re-saving them as TIFF.-files.

The TIFF.-files of stained cells on the slides were transferred to FIJI ImageJ. By using the extension-tool IHC toolbox, the labelled and stained cells were quantified. First, by using a brain atlas (Vindas, 2022 (unpublished)), the ROIs were identified (Fig.4). The regions were dorsolateral pallium – left (DLL), dorsolateral pallium – right (DLR), dorsomedial pallium – left (DML) and dorsomedial pallium – right (DMR), in addition to ventral part of ventral telencephalon – left (VVL) and ventral part of ventral telencephalon – right (VVR) (Vindas et al., 2017). Secondly, a random clear sample picture of telencephalon was used to train a model which could be run on all the regions, with the purpose of precisely recognising the colouration of stained immunoreactive cells and counting these. By using the *training* tool in the IHC toolbox in ImageJ, a model was created after about 7 rounds of training.

By using the *rectangle* tool in ImageJ, the ROIs were individually picked and hand-operated with the aforementioned brain atlas as a guide. The selected area was then duplicated from the original picture – this was to ensure that the original material was retained. Thereafter, the model was run on the selected picture, the *colour* function altered the picture to black and white, and by using *gland* and *analyse particles*, the number of stained nuclei was counted. The area specific picture was saved as a separate TIFF-file and the number of stained nuclei was saved in an Excel-file, including information about the fish number, tank, treatment, slide number and nuclei count for each ROI. Slides with damaged tissues or heavily unclear pictures were excluded from analysis. Also, areas that were blurry or ambiguous in any way, or were not present in the slide, were not included but were marked as not available (“NA”) in the data sheet.

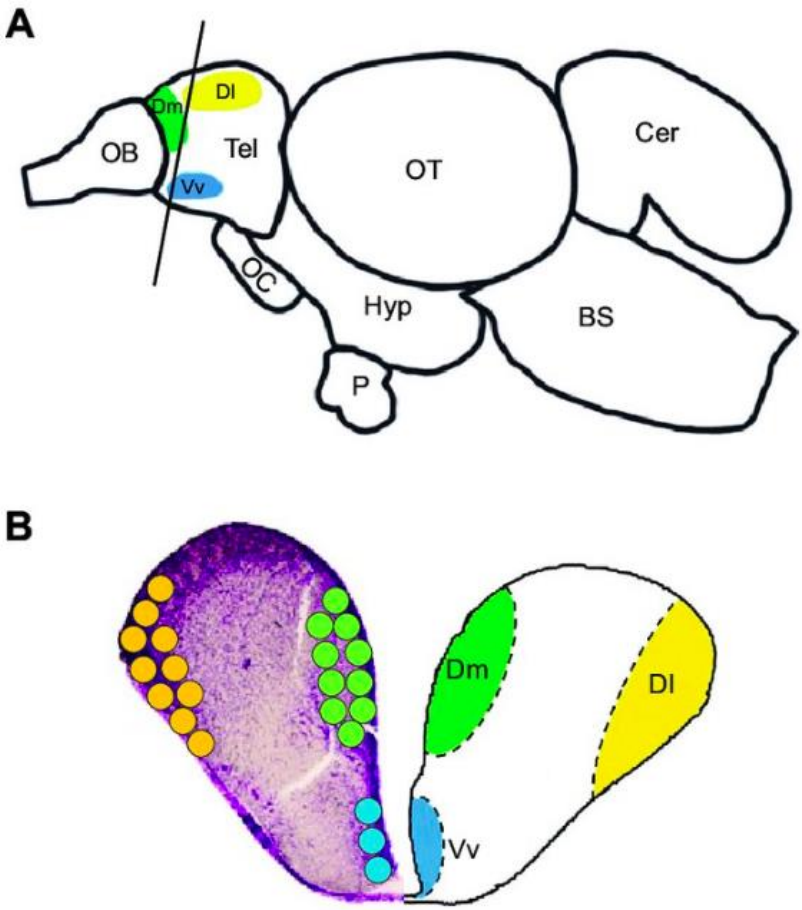

Figure 4: A) Sagittal overview of Atlantic salmon brain. From the left: olfactory bulb (OB), dorsomedial (Dm), dorsolateral (Dl), ventral part of ventral telencephalon (Vv), telencephalon (Tel), optic chiasm (OC), optic tectum (OT), hypothalamus (Hyp), pituitary (P), cerebellum (Cer), brainstem (BS). B) Transverse telencephalic view. From the left: dorsomedial (Dm), dorsolateral (Dl), ventral part of ventral telencephalon (Vv).
Illustration modified from Vindas et al. (2017).

3.6. Statistical analysis

All statistical analysis were done by using the R Studio software (R version 4.1.2, 2022 Posit Software, PBC). The "*readxl*" package was used to read in the Excel xlsx. data file and the packages "*dplyr*", "*tibble*" and "*tidyverse*" were installed for data wrangling. Afterwards, "*lme4*" and "*lmerTest*" packages were installed to set up for using the linear mixed-effects model (lmer), generalised linear mixed-effects model (glmer) and the general linear model (glm). The "*car*" package was used for conducting analyses of variance. To analyse pairwise comparisons, the package "*emmeans*" was used, and the *"DHARMa"* package was used to examine model fit. For plotting and arrangement of graphs, the "*ggplot2*" package was used.

The alpha-value was set to 0.05, meaning that a p-value < 0.05 was considered statistically significant.

3.6.1. Social cohesion

A linear mixed-effects model was used to analyse social cohesion between Atlantic salmon parr in each tank. As a response variable, the average distance in cm. (distance between snout tips of all visible individuals in tank) was used. However, this was not normally distributed (Shapiro-Wilks test, W = 0.866, $p < 0.05$). Therefore, the data were transformed to natural logs prior to analysis.

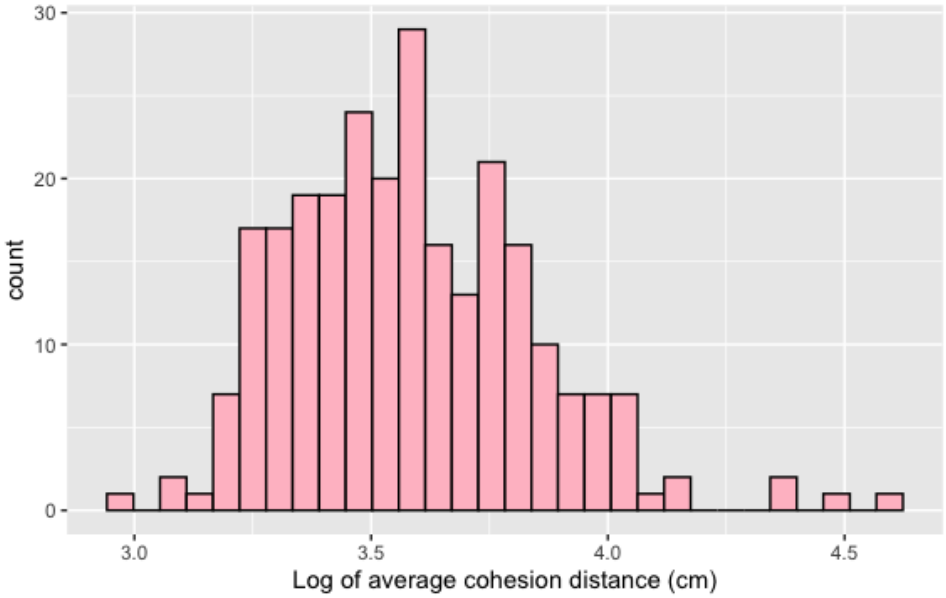

Figure 5: Histogram of natural log-transformed average cohesion distances (cm) showing a more normal distribution.

The explanatory variables in the model were Treatment (Control, Bubbles, Current, Stones), Week (1-6 weeks from the start of the experiment), Phase (before bubbles, after bubbles) and Fish count (number of visible individual fish included in the mean social cohesion distance per tank). The interactions between Treatment and Week, and Treatment and Phase, were also included in the model. Tank was assigned as a random factor to control for repeated measures in the same tank. The Control Treatment in the After Phase was set as the reference against which the other explanatory variables were contrasted.

3.6.2. Aggression

A generalized linear mixed-effects model was used to analyse aggression between parr in each tank, with Poisson distribution. The response variable was the total count of aggressive events in each tank per 5-min video observation session. The explanatory variables were Treatment (Control, Bubbles, Current, Stones), Week (1-6 weeks), Phase (before bubbles, after bubbles) in addition to the interaction between Treatment and Week, and the interaction between Treatment and Phase. Tank was used as a random factor. All model terms were compared to the Control After Phase. Emmeans was used for pairwise comparisons.

3.6.3. Telencephalon cell proliferation

The Wilcoxon signed ranks matched pairs test was used to compare stained cell counts from the right and left side of the telencephalon of each fish for each ROI. The combined counts from the right and left side of each ROI were analysed using generalized linear mixed-effects models with Poisson distribution. The explanatory variables were Treatment (Control, Bubbles, Current, Stones), Brain region (DL, DM and VV) and their interaction, with the Control DL set as the reference for comparison. Emmeans was used for pairwise comparisons.

4. Results

4.1. Social cohesion

The ANOVA test showed significance for the explanatory variables Week ($\chi^2 = 17.00$, df = 1; $p < 0.05$), Phase ($\chi2 = 21.36$, df = 1, $p < 0.05$) and Fish count ($\chi^2 = 21.31$, df = 1, $p < 0.05$) whereas Treatment and the interactions of Treatment with Week and Phase were not significant. The model estimates for Week (mean±SE: 0.004±0.002, df = 239.9, t = 2.53, p = 0.012) and Phase Before bubbles (0.017±0.005, df = 239.0, t = 3.393, p = 0.001) were positive indicating that the average distance (cm) between fish increased (i.e. social cohesion declined) over weeks (Fig. 6) whereas the fish were closer together after, than before commencement of the bubbles in the Bubbles treatment and equivalent times in the other treatments (Fig. 7). The negative estimate for Fish count (-0.002±0.0004, df = 247.0t = -4.62, $p < 0.05$) showed that the distance increased as the count of visible fish decreased.

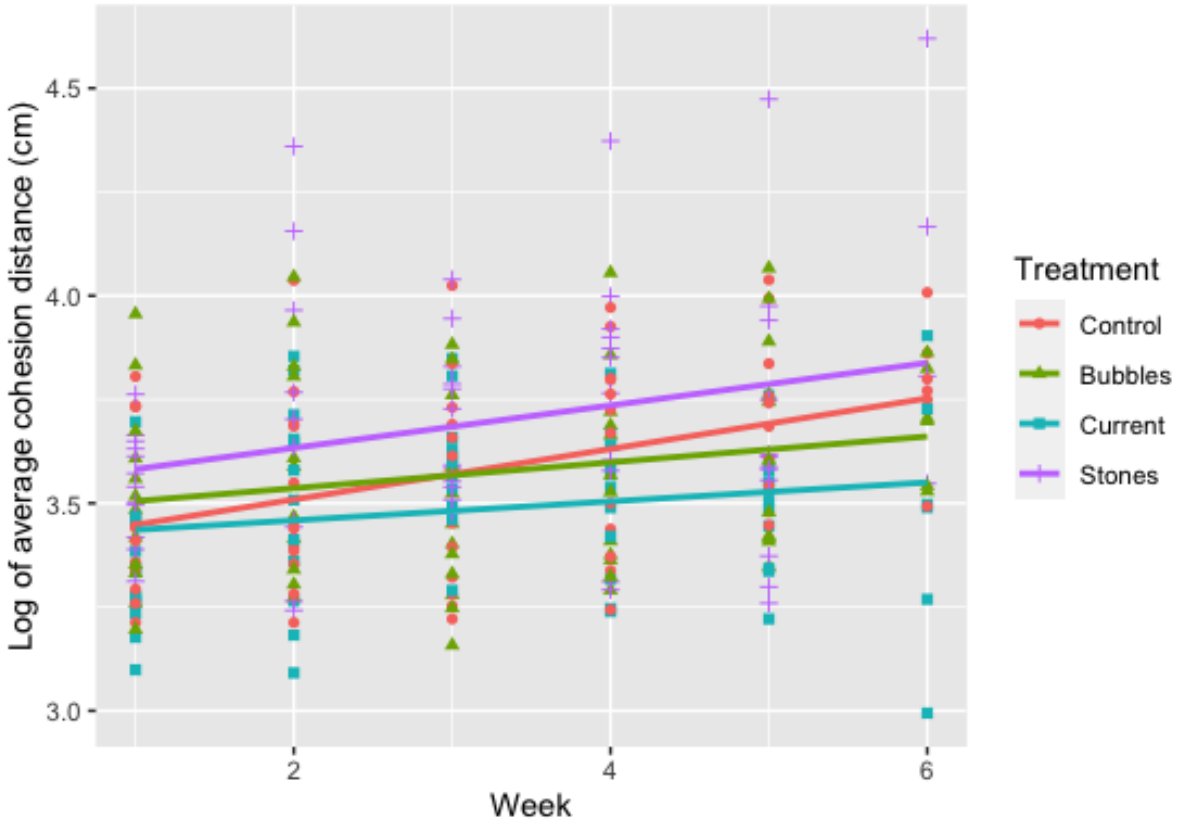

Figure 6: Scatterplot of raw data showing the average distance between visible fish (cm) per analysed snapshot of each tank across weeks of observation. The linear trendline for each treatment across weeks is also shown. The distances increased over weeks regardless of treatment.

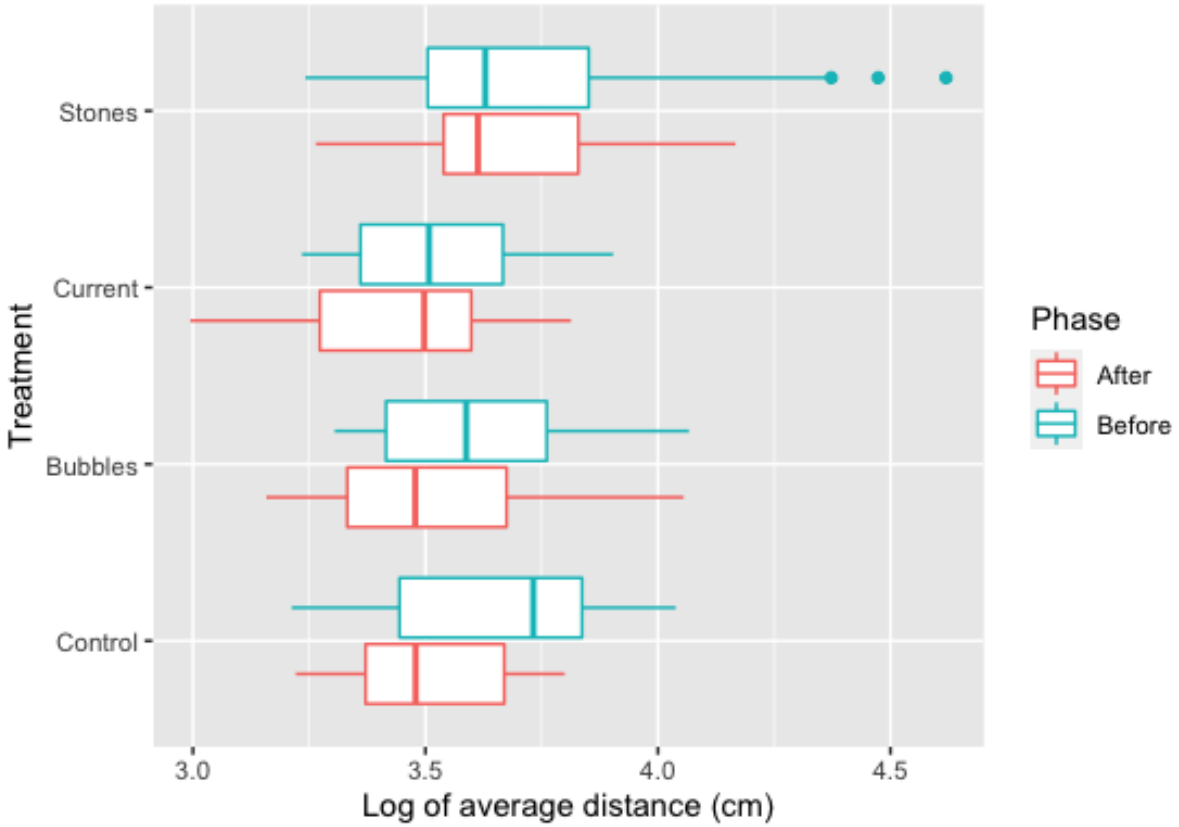

Figure 7: Box plot based on model estimates showing the median (± interquartile range) of the tank log average distances between visible fish (cm) per analysed snapshot of each tank 1 min after the start of each video (Before) and 1 min after commencement of 5 min of bubbles in the Bubbles treatment and at equivalent times in the other treatments (After). The dots show outliers. The distances were higher before than during the bubbles. This Phase effect did not differ between treatments.

4.2. Aggression

Aggression was significantly influenced by explanatory variables Treatment (χ^2 = 11.583, df = 3; p = 0.009), Week (χ^2 = 30.238, df = 1; p < 0.001), the interaction between Treatment and Week (χ^2 = 20.896, df = 3; p < 0.001) as well as the interaction between Treatment and Phase (χ^2 = 37.715, df = 3; p < 0.001). The positive model estimate for Bubbles (mean±SE: 0.917±0.252, z = 3.64, p < 0.001) indicate that the overall counts of aggression were higher in the Bubble tanks compared to the Control tanks (Fig. 8).

The estimates were negative for Bubbles x Week (-0.181±0.051, z = -3.577, p < 0.001), Current x Week (-0.178±0.052, z = -3.399, p = 0.001) and Stones x Week (-0.226±0.053, z = -4.252, p < 0.001), indicating that the increase in aggression over weeks was lower in the

enriched tanks than in the Control tanks (Fig. 8). According to the results from the pairwise comparisons, while there was no difference between treatments in the Before Phase, there was higher aggression in the Bubbles treatment compared to the Current ($p < 0.005$) and the Stones treatment ($p < 0.001$) in the After Phase (Fig. 9). In addition, within the Bubbles treatment, there was more aggression observed After than Before the Bubbles ($p < 0.001$).

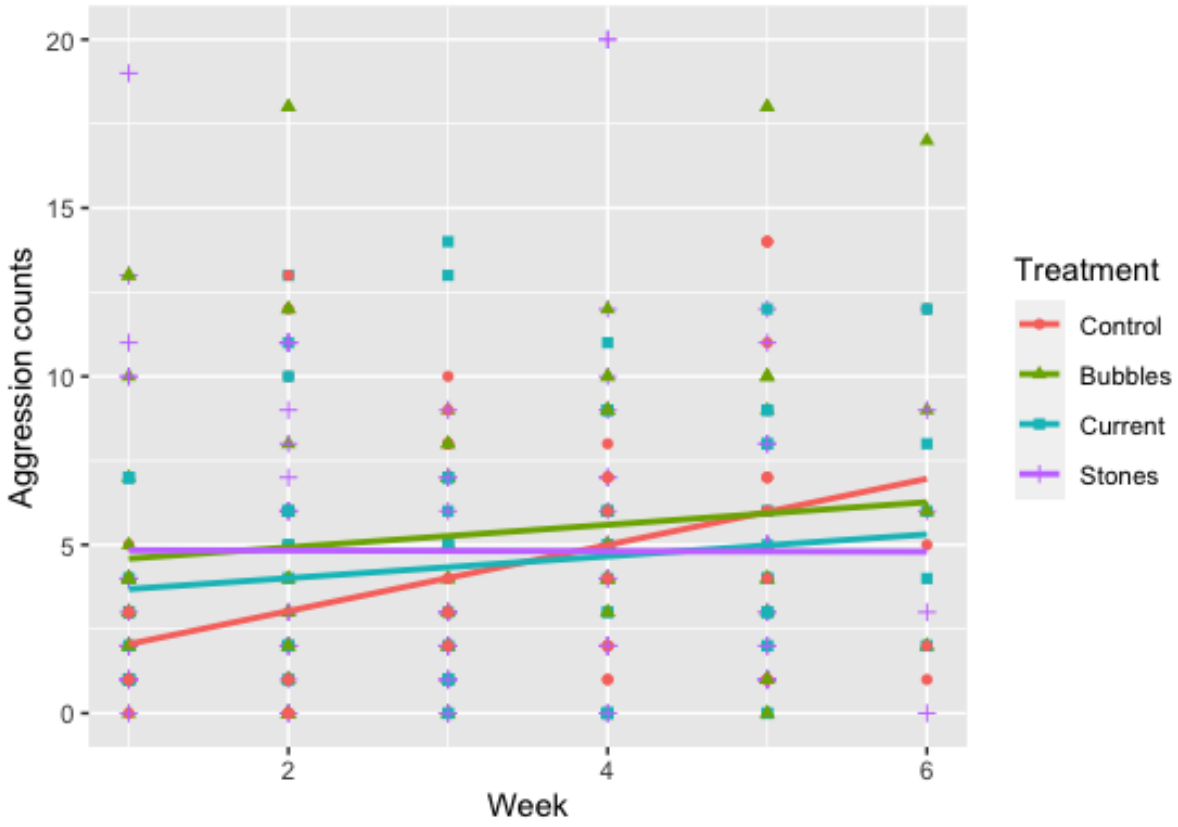

Figure 8: Scatterplot of average counts of aggression across weeks, with trend lines for the different treatments. Aggression increased over weeks, especially in the control treatment.

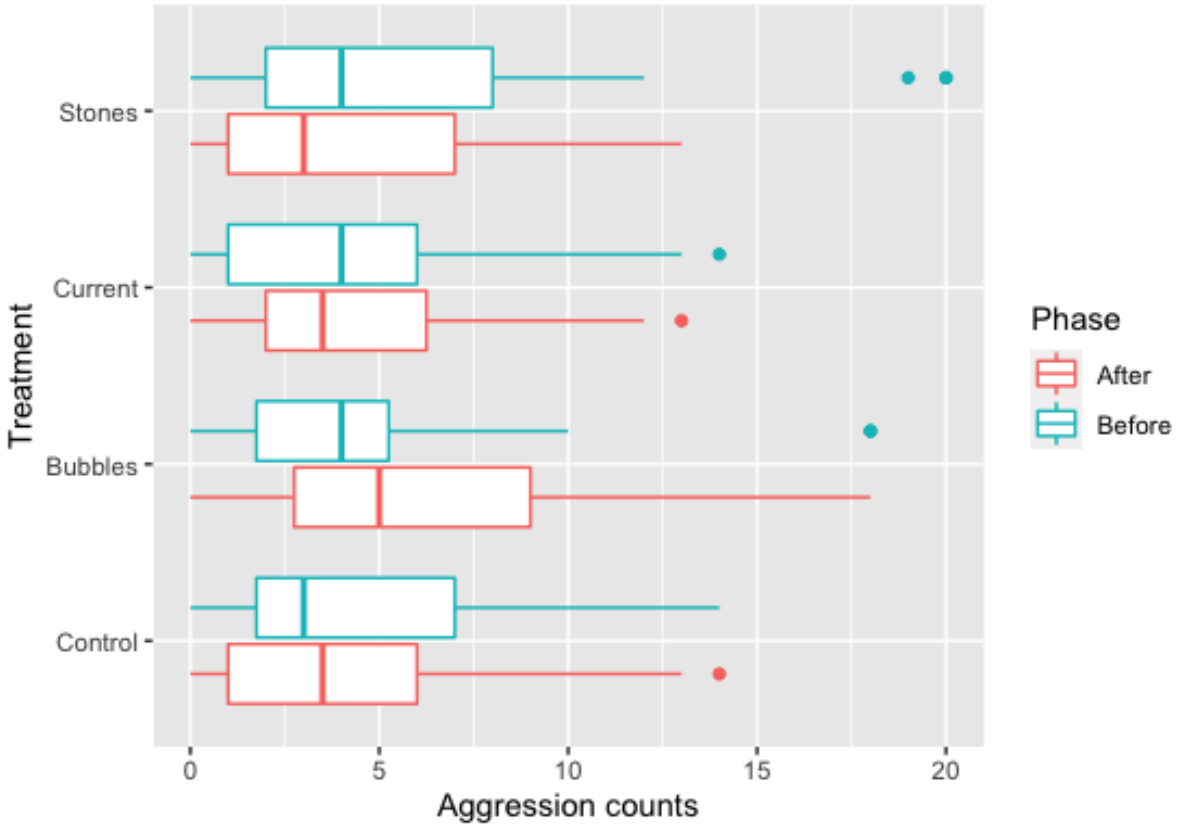

Figure 9: Box plot showing the median (±interquartile range) number of aggressive events per 5-min observation before vs. after the bubbles in the Bubbles treatment and equivalent times in the other treatments. There was a Treatment by Phase interaction, with more aggression occurring in the Phase After than the Before Phase in the Bubbles treatment. The dots show outliers.

4.3. Telencephalon cell proliferation

The stained cell counts on the left and right hemisphere were comparable in all the ROIs (DL: $p = 0.838$; DM: $p = 0.126$; VV: $p = 0.674$) (i.e., there was no indication of laterality; Table 1). Therefore, the results for the left and right counts within ROI were combined prior to further analysis.

Table 1: Average stained nuclei counts per region of interest in the telencephalon for each hemisphere.

	Dorsolateral telencephalon	Dorsomedial telencephalon	Ventral part of the ventral telencephalon
Left hemisphere	34.4	26.0	12.1
Right hemisphere	33.2	23.0	15.3

The combined stained cell nuclei counts were influenced Brain region ($\chi^2 = 70.356$, df = 2; $p < 0.001$) and there were significant pairwise differences between the Brain regions DL-DM ($p < 0.001$), DL-VV ($p < 0.001$) and DM-VV ($p < 0.001$), whereby DM had a significant negative estimate (mean±SE: -0.245±0.093), as did VV (-0.982±0.118), indicating that they both had lower counts compared to DL.

The interaction between Treatment and Brain region was also significant ($\chi 2 = 393.687$, df = 6; $p < 0.001$; Fig. 10). Results from pairwise comparisons showed that there were significantly higher counts of cells in the DM compared to VV in the Bubbles treatment ($p = 0.022$), Current ($p < 0.001$) and Stones ($p < 0.001$), also there was higher cell counts in Current-tanks in DM ($p < 0.001$) and DL ($p < 0.001$) both compared to VV. No significant treatment differences within brain regions were detected ($p > 0.05$), but there was a trend for fish on the Stones treatment to have higher cell counts in the DL than fish on the Bubbles treatment ($p = 0.069$).

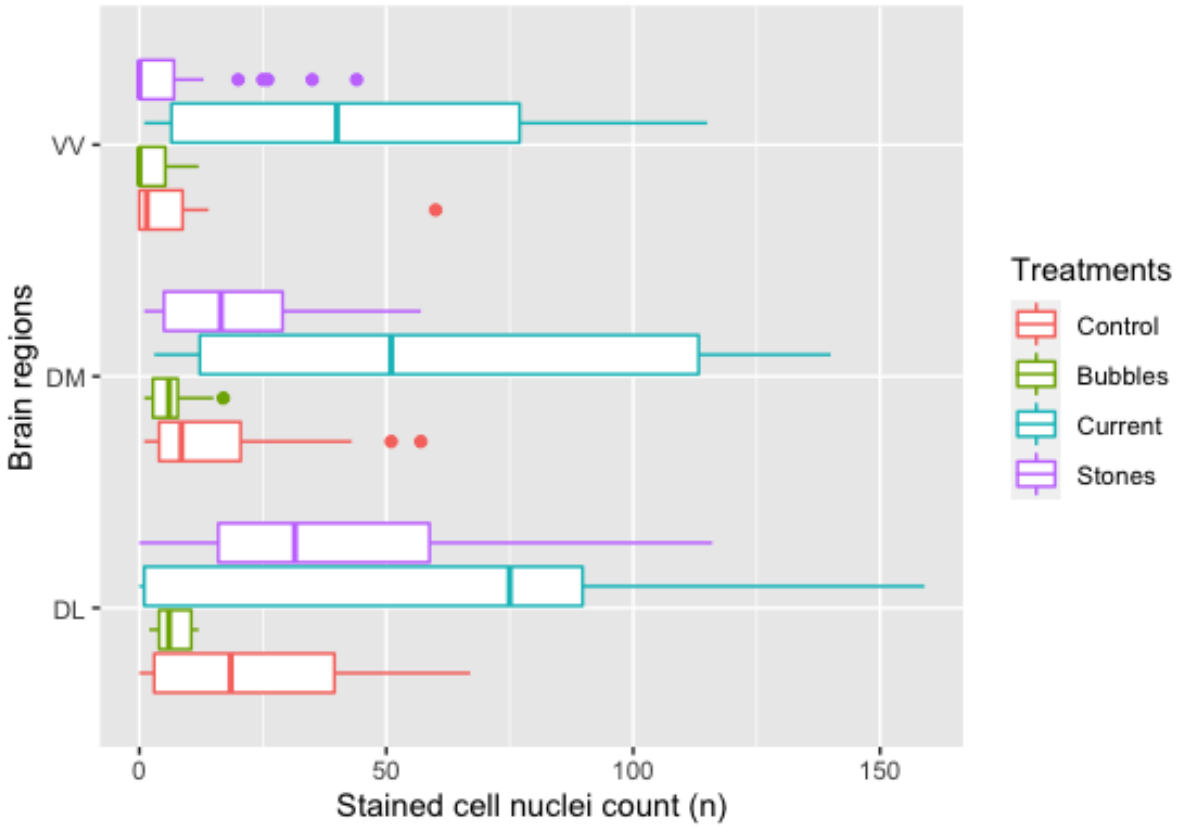

Figure 10: Box plot showing the median (±interquartile range) number of stained cell nuclei count in each of the different brain regions – dorsolateral (DL), dorsomedial (DM) and the ventral region of the ventral telencephalon (VV) per treatment. The dots show outliers.

5. Discussion

5.1. Overview of the results

The main hypothesis was that bubbles, increased current and stones would each contribute to improved welfare by comparison with rearing in unenriched standard rearing tanks (the Control treatment). We predicted that the social cohesion would increase in Bubble, Current and Stone enriched tanks, but found no significant treatment effects. Yet, we saw that Treatment did affect aggressive behaviour. When looking at cell proliferation in the telencephalon region, we observed that Treatment had a significant effect as well as the interaction between Treatment and Brain regions, especially in the DL region, however there seemed to be no significant Treatment differences within the same Brain regions.

5.2. Discussion

5.2.1. Social cohesion

Young salmon display territorial behaviour and as they mature. Also, schooling behaviour is shown, which is often connected to an anti-predator strategy. In case of danger or threats, being a part of a large group comes with benefits such as protection, shelter, and swimming hydro efficiency (Chen et al., 2016; Näslund et al., 2013). Social cohesion increased significantly across Weeks and was higher in the Phase before than during bubbles, whilst the statistics for Fish count implied that higher distances between fish were recorded when the number of visible fish was lower. Week was also a measure for age, and since the fish were fed regularly and sufficiently, we could assume that they grew steadily during the 6 weeks of the study. Taking growth into consideration, a reason for the decline in visible fish as distances increased could be due to the larger size of the fish making it more difficult to distinguish one from one another. Another reason could be that they placed themselves vertically in the tanks making it harder to separate the individuals from each other when filmed from above. In adult Atlantic salmon vertical placing has been suggested to be connected with an anti-predator strategy as well as prey-search strategy (Plantalech Manel-La et al., 2009). As the parr grow and become familiarized with the environment, they could also prefer a schooling-strategy which in turn makes differentiation between individuals on camera challenging, thereby possibly explaining the increase in distance and decrease in Fish count.

Another reason connected to the fish' familiarity with their environment could be that the general social cohesion declined as they might have been more acquainted thus relaxed with each other. However, it could also be argued that as the fish grow larger in size they consequently occupy more physical space and therefore are difficult to distinguish between the individuals in a group due to the two-dimensional pictures where the view is limited.

The social cohesion was lower before bubble start which was approximately one minute after video start, than during the bubbles, irrespective of the actual treatment group. Other research results suggest that bubbles may both be occupational as well as sensory enrichment factors and that they may have a positive effect on fish welfare by stimulating fish cognitive abilities (Kleiber et al., 2022) or even encouraging play behaviour (Arechavala-Lopez et al., 2021). Indicating that even though there were no direct Treatment effects on the average social cohesion as predicted, there was a positive Phase effect implying that the average distances between individuals were higher before the bubbles start (or equivalent time in other enrichment tanks and Control) signifying an increase in social cohesion in the tanks After the Bubbles phase. The increase in social cohesion in the Phase After start of bubbles could be because the aeration startled the fish, causing them to swim closer together. This response occurs as an anti-predator response to perceived danger. Alternatively, the fish could also prefer being close to the air stone (aeration of bubbles) resulting in a higher concentration of fish near this area. Studies on rainbow trout (*Oncorhynchus mykiss*) show that the display of bubbles are highly attractive for fish (Kleiber et al., 2022). For the other tanks (Control, Current and Stone) perhaps the Bubble treatment in neighbouring tanks created some audible disturbance, alarming the individuals of the non-bubble tanks causing them to clump together also.

5.2.2. Aggression

Aggression was influenced by most factors that were tested such as Treatment, Week, the interactions between Treatment and Week as well as the interaction between Treatment and Phase. In Bubble-tanks a higher number of agonistic interactions were observed compared to the Control tanks, which was contradictory with the (fourth) prediction, of aggression being lower in tanks with Bubbles, Current and Stones. An explanation for the increased aggressiveness in these tanks could be that the sensory and occupational stimulus caused excitement which could lead to bursts of aggressive behaviour. The aggression is also higher in the Phase After the bubbles which could suggest that the bubbles encouraged fish to

perform more social or play-behaviour which could visually for the observer be categorized and viewed as aggressive per our definition ("one fish darting towards another fish in an attempt to bite/bump/chase it"). However, we saw that the interactions between enrichment treatments (Bubbles, Current and Stone) and Week were significant with a negative estimate. Thus, implying that though aggression was somewhat increasing over time and with the age of the parr, the increase was higher in the Control tank, compared to the enriched tanks which is supported by the results of a study done on Atlantic salmon parr indicating that conspecific aggression was lower in tanks with structural shelter enrichment (Rosengren et al., 2017).

Also, aggression generally increased over time especially in the Control as per our (fifth) prediction stating that aggression would increase with increased size measured by growth over weeks. As earlier established salmon parr are territorial, though they after certain stages show more of schooling behaviour closer to adulthood, which could explain why the aggression did not increase as much in Bubble-tanks over Weeks compared to the Control. A study on growth and aggression in juvenile Atlantic salmon showed results suggesting that fast-growing salmon are more aggressive than slow-growing fish, yet they are more vulnerable to conspecific attacks which could also be explained by our results of increased social cohesion as an anti-predator strategy (Nicieza & Metcalfe, 1999). As the access to food in the tanks in this study were plentiful, we could assume that the fish growth was solid.

Specifically in Stone-enriched tanks, the individuals may rather prefer to hide beside or between the stones if alarmed or in need of shelter (Näslund et al., 2013). In tanks with Current, the force of currents around the wall of the tank varies, even though presumably many individuals prefer areas with higher force, some individuals might settle for other areas with slightly lowered current - either way supporting increased exercise for several individuals in the tank eliminating the need for competition and aggression connected to this for the opportunity of being activated (Waldrop et al., 2017). However, competition could lead to aggression if enrichment resources are limited – such as only 6 stones in a single tank, as in this study design. As earlier discussed, the social cohesion was higher in the Phase After bubbles, simultaneously we observed that the aggression was higher in the same phase suggesting that swimming close together might increase opportunities for aggression thereby increasing the counts of these.

5.2.3. Telencephalon cell proliferation

Both Brain regions and the interaction between Treatment x Brain regions had an effect on cell counts. Here, we could observe that fish from Current-tanks in both DM and DL had significantly higher cell counts compared to VV which was in agreement with our (sixth) prediction anticipating higher nuclei count in enriched tanks. Additionally, there are plenteous of results establishing that exercise (such as simulated in the Current-tanks) increases brain plasticity and thereby cell proliferation (Abreu et al., 2019; Mes et al., 2020).

The cell counts were overall lower in both the DM-region and VV-region compared to DL. The interaction between Treatment and Brain regions were also significant. In DL there were pairwise significant differences between the Bubbles and Stones. When looking at stained nuclei cells across the brain regions (Fig.10) we observed that there were large variations of cell counts from different fish within the same treatment suggesting that cell proliferation could be influenced by other factors, perhaps such as growth rate, movement rate or inter-fish interactions.

Telencephalic cell proliferation, as determined by counting stained cell nuclei, seemed to be notably influenced by the limited number of fish contributing data points for analysis. In our (sixth) prediction we suggested that there would be an overall increase in cell proliferation in enriched tanks. A study on sticklebacks (*Pungitius pungitius*) showed results demonstrating that the environment did have an effect on the brain sensory neural centres, supporting our predictions regarding environmental enrichment and its effects on the brain (Gonda et al., 2009). An important note is that brain cells are highly connected, and therefore, results from the same fish were not statistically independent. For this reason, fish was included as a random effect in the statistical analyses of the brain data.

5.3. Limitations and future research

For this study, we have focussed on social cohesion and how the salmon parr in a restricted area (such as in a rearing tank) interacted with each other and how different environmental enrichments influenced this. As discussed earlier, salmon shift from being territorial to prefer schooling and tighter groups and, therefore, social cohesion as well as aggression is likely to differ over different stages of the life cycle. Thereby, it would be interesting to see how cohesion and aggression may differ in groups of salmon from different life stages.

Further elaboration of different enrichment factors and even the combination of these should also be considered as this may in a larger degree mirror the complex natural habitat of a salmon. In the tanks, vertical physical structures could also provide interesting information about the behaviour as these in a larger extent than i.e., stones, can alter water flow and might interfere with the swimming directionality as well as have an effect on aggression and territoriality. Also, visual, olfactory and audible enrichment types could be of interest as they too might influence behaviour.

Sadly, some data were lost due to inadequate staining and visualization of the telencephalon slides. Therefore, it should be highly considered to develop and improve the staining methods making sure that the targeted cells are coloured in the correct manner, as well as refining the laboratory processes to ensure high quality and minimize spoiled slides. Additionally, the quality of microscopic pictures taken and converted to a computer-friendly file should be inspected to ensure satisfactory quality. Further progression of digital models used to count stained cells should also be of concern as this may greatly influence the acquired number of cells. It would also be interesting to evaluate cell proliferation in regard to fish size.

Generally, to attain statistical power and especially in behavioural studies, it is important to consider the sample size needed to detect effects if they exist. As personality affects behaviour, and there are individual differences between fish within the same tank, sufficient fish need to be sampled to detect behavioural trends at population level. To detect differences between treatments is difficult when the sample size is small, unless the differences are huge and consistent across fish within the same treatment. A greater sample size together with larger enriched tank sizes could provide important insights into how environmental enrichment affects behaviour in salmon and their overall welfare.

6. Conclusion

The aim of this study was to look at how commercially applicable environmental enrichments could affect behaviour in raring tanks, measured by social cohesion, aggression, and brain cell proliferation, and thereby influence salmon parr welfare. We predicted that enrichment treatments would boost the overall welfare if the social cohesion was increased, aggression lowered and cell proliferation in the telencephalon was enhanced. A fascinating finding backing our hypothesis was that the social cohesion increased in the phase after bubbles

started. However, the cohesion generally decreased over time (age). Interestingly, we also observed that aggression increased both in the phase after bubbles started as well over time (age).

Welfare is a complex concept and our chosen enrichment treatments can affect this in different ways due to the individual's perception and influence by the treatment. Our results do not support our predictions that the chosen enrichment treatments individually improve welfare. However, as several studies suggest that they do (Abreu et al., 2019; Brown et al., 2003; Carfagnini et al., 2009; Mes et al., 2020; Salvanes et al., 2013), - perhaps a larger study with combined treatments would signify otherwise. The findings all together support that the environmental enrichment treatments evaluated did have some effects on behaviour and cell proliferation in the telencephalon that could influence salmon welfare, and therefore should be further investigated.

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8. Appendices

Appendix A. Brain crytosectioning for telencephalon in Atlantic salmon parr

The brains were taken out of the sucrose solution and were mounted in Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) before freezing and storing at -80 C. A layer of Tissue-Tek was put in a silicone mold placed in a Styrofoam box with dry ice until the Tissue-Tek started to crystalize. The brain was then immediately placed horizontally on top before being filled completely with Tissue-Tek before the mold was placed back on the dry ice until frozen. Once frozen the sample was removed from the mold and placed in a small zip-lock bag labeled with sample number and stored at -80ºC for further cryosectioning.

Cryosectioning was done using a Leica CM3050 cryostat (Leica, Wezlar, Germany) set at -24ºC. The thickness of the slices was adjusted to 14 µm. All the equipment was cleaned with RNase-Zap before being placed in the machine to cool down. Tissue-Tek was poured onto a stub in order to mount the brain samples for slicing.

Previously marked glass slides (Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides) were kept outside the machine in batches of six (labelled with series number (1-4) and slide number (A-F)). Sliced tissue was then thaw mounted on the glass slides by gently pressing the slide on the tissue section. The first slice on the first glass slide (A), the second slice on the second glass slide (B) and so on until all six slides were full. Then six new slides (series 2) were marked, and the procedure repeated. When the whole brain was sliced, all the glass slides were placed in an oven at 65ºC for 10 minutes and stored at -80ºC for further analysis by immunohistochemistry (IHC). For each fish (brain) 3 to 4 series were cut (labelled 1 to 4), each series contained 6 slides (A – F), 20-28 brain tissue sections (slices) on each slide.

Appendix B. Immunohistochemistry protocol

1st day:

1. Take the slides out of the -80 freezer and leave them for one hour at room temperature and then 10 min in the oven at 65°C on a tray with aluminum foil.
    a. Start the oven early as it needs time to heat up.
2. Soak in PBS-T until the white marker tip of the slide for 10 minutes in a Couplin staining jar.
    a. Keep a jar/box close by to throw away the PBS-T.
    b. If a completely full Couplin jar is used, remember to place the last slide with face towards the next to last slide (not towards the jar end).
3. To unmask epitope soak for 30 min in HCL 2N at 37°C in the cabinet.
4. Wash in PBS-T with pipette for 3 times (1000 µl x 3) and then soak in PBS-T for 5 min x 3 times in the Couplin jar. Immediately after mark the boarder of the slides with a PAP-pen to create a hydrophobic barrier to contain the liquid.
5. Blocking in the humid chamber: pipette 200 µl per slide of blocking solution **PBST- NGS-TX-DMSO** and incubate for 1 hour at room temperature.
    a. Add some H2O in the chamber to make it humid.
    b. Make sure that the tissues on slides are all covered with the solution.
6. Discard the liquid by dabbing it lightly on some paper and add the primary antibody (the primary antibody is diluted in 200 µl x slide of **PBST- NGS-TX-DMSO,** the dilution is 1:100 of antimouse PCNA, Santa Cruz SC-56 in fridge), by pipetting.
    a. Make sufficient PBST-NGS-TX-DMSO, remove 1/100 µl, and add 1/100 µl of PCNA.
        i. Use a clean pipette to mix the liquid.
        ii. Wrap in foil as the primary antibody might be light sensitive.
    b. The primary antibody is found in the local 4°C fridge. Remember to take out the correct type (antimouse).
7. Incubate over-night at room temperature (make sure the slides don't dry up) in the humid chambre.

2nd day:

1. Use at least 1000 µl PBS-T x 3 times to rinse off antibody holding the slide vertically in a Couplin staining jar.

2. Let soak for 10 min x 3 times in PBS-T.
    a. Keep a jar/box close by to throw away the PBS-T.
    b. If a completely full Couplin jar is used, remember to place the last slide with face towards the next to last slide (not towards the jar end).
3. Take slides out one at the time and dab the borders of the slide lightly on a paper to activate the PAP-pen barrier.
4. Put the slide in the humid chamber and add the secondary antibody by pipetting (the secondary antibody is diluted in 200 ul x slide of **PBS-T,** the dilution is 1:1000)
    a. Make sufficient PBS-T, remove 1/1000 µl, and add 1/1000 µl of secondary antibody PCNA.
        i. Use a clean pipette to mix the liquid.
        ii. Wrap in foil as the secondary antibody is light sensitive.
    b. The secondary antibody is found in the local 4°C fridge. Remember to take out the correct type (antimouse) wrapped in a paper bag.
5. Incubate for 2 h in the dark (make sure the slides don't dry up).
6. Use at least 1000 µl PBS-T x 3 times to rinse off antibody and soak 3x for 10 minutes in staining jar with PBS-T.
7. Mount by evenly placing 30 µl Vecta Shiled with DAPI on the slides, and place coverslides carefully on top. Use nail polish to seal the cover slide all around the borders of the slides and let slides dry for 10 minutes on lab bench. Store finished slides at 4°C.
    a. Remember to mark the cover of storage of slides with name, date and brief explanation of content.

Buffers:

PBS pH 7,2:

Prepare the 2 buffers:

1. ❶ Na_2HPO_4: 1,4g/100mL or Na_2HPO_4 -2H_2O: 1,8 g/100mL
2. ❷ NaH_2PO_4-H_2O: 1,6g/100mL
3. 80mL❶+20ml❷+8,5g NaCl + H_2O to 1L total volume (ca. 900mL)

PBST:

1. 0.1% Tween in PBS

PBS + NGS + TX + DMSO:

1. 6% Normal Goat Serum + 0,3% Triton + 1% DMSO in PBST.
    a. 100 mL PBST + 6 mL NGS + 1 mL DMSO + 0.3 mL (300 µl) Triton = 107.3 mL
        i. Since we only need 200 µl per slide, make sure not to waste chemicals and only make as much as needed
        ii. 25 mL PBST + 1.5 mL NGS + 0.25 mL DMSO + 0.075 mL (75 µl) Triton » 27 mL

Appendix C. R-script for social cohesion analysis

```
> lmer1 <- lmer(logAverage_cohesion_distance_cm ~ Treatment + Week + Phase + Fishcount + Treatment:Week
+ Treatment:Phase + (1|Tank), data = sc.df)
> summary(lmer1)
Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']
Formula: logAverage_cohesion_distance_cm ~ Treatment + Week + Phase +
    Fishcount + Treatment:Week + Treatment:Phase + (1 | Tank)
   Data: sc.df

REML criterion at convergence: -9.5

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.4855 -0.7169 -0.0500  0.6814  3.2162

Random effects:
 Groups   Name        Variance Std.Dev.
 Tank     (Intercept) 0.009042 0.09509
 Residual             0.042485 0.20612
Number of obs: 260, groups:  Tank, 12

Fixed effects:
                             Estimate Std. Error         df t value Pr(>|t|)
(Intercept)                 3.728e+00  1.238e-01  9.439e+01  30.106  < 2e-16 ***
TreatmentBubbles            2.563e-02  1.208e-01  3.007e+01   0.212 0.833459
TreatmentCurrent           -2.318e-02  1.196e-01  2.903e+01  -0.194 0.847703
TreatmentStones             1.050e-01  1.208e-01  3.016e+01   0.868 0.391997
Week                        4.152e-02  1.640e-02  2.399e+02   2.532 0.011984 *
PhaseBefore                 1.723e-01  5.079e-02  2.390e+02   3.393 0.000809 ***
Fishcount                  -2.047e-02  4.435e-03  2.470e+02  -4.616 6.29e-06 ***
TreatmentBubbles:Week      -9.877e-03  2.283e-02  2.394e+02  -0.433 0.665628
TreatmentCurrent:Week      -1.878e-02  2.280e-02  2.394e+02  -0.824 0.410997
TreatmentStones:Week       -6.216e-05  2.319e-02  2.395e+02  -0.003 0.997864
TreatmentBubbles:PhaseBefore -2.462e-02  7.389e-02  2.397e+02  -0.333 0.739275
TreatmentCurrent:PhaseBefore -8.824e-02  7.190e-02  2.390e+02  -1.227 0.220919
TreatmentStones:PhaseBefore  -8.922e-02  7.295e-02  2.390e+02  -1.223 0.222503
```

```
> ### ANOVA
> car::Anova(lmer1, type = 2)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: logAverage_cohesion_distance_cm
                Chisq Df Pr(>Chisq)
Treatment      5.0581  3     0.1676
Week          17.0031  1  3.732e-05 ***
Phase         21.3625  1  3.801e-06 ***
Fishcount     21.3100  1  3.907e-06 ***
Treatment:Week  0.9125  3     0.8224
Treatment:Phase 2.3127  3     0.5101
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> emmeans(lmer1, list(c(pairwise ~ Treatment|Phase)), type = "response")
$`emmeans of Treatment | Phase`
Phase = After:
 Treatment emmean     SE   df lower.CL upper.CL
 Control     3.52 0.0657 11.0     3.38     3.67
 Bubbles     3.51 0.0660 11.2     3.37     3.66
 Current     3.44 0.0656 10.9     3.29     3.58
 Stones      3.63 0.0667 11.6     3.48     3.77

Phase = Before:
 Treatment emmean     SE   df lower.CL upper.CL
 Control     3.69 0.0658 11.1     3.55     3.84
 Bubbles     3.66 0.0668 11.7     3.52     3.81
 Current     3.52 0.0657 10.9     3.38     3.67
 Stones      3.71 0.0663 11.4     3.56     3.85

Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95

$`pairwise differences of Treatment | Phase`
Phase = After:
 2                estimate     SE   df t.ratio p.value
 Control - Bubbles  0.00659 0.0934 11.2   0.071  0.9999
 Control - Current  0.08443 0.0929 10.9   0.909  0.8004
 Control - Stones  -0.10475 0.0939 11.4  -1.116  0.6876
 Bubbles - Current  0.07784 0.0930 11.0   0.837  0.8358
 Bubbles - Stones  -0.11134 0.0932 11.1  -1.194  0.6424
 Current - Stones  -0.18918 0.0935 11.2  -2.024  0.2360

Phase = Before:
 2                estimate     SE   df t.ratio p.value
 Control - Bubbles  0.03121 0.0930 11.0   0.336  0.9863
 Control - Current  0.17267 0.0932 11.1   1.853  0.3008
 Control - Stones  -0.01552 0.0936 11.3  -0.166  0.9983
 Bubbles - Current  0.14146 0.0941 11.5   1.503  0.4665
 Bubbles - Stones  -0.04673 0.0945 11.7  -0.494  0.9588
 Current - Stones  -0.18820 0.0932 11.1  -2.019  0.2383
```

Appendix D. R-script for aggression analysis

```
> ## GLMER
> glmer1 <- glmer(Aggression_counts ~ Treatment + Week + Phase + Treatment:Week + Treatment:Phase
+ (1|Tank), family = 'poisson', data = aggression.df)
Warning message:
In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv,  :
  Model failed to converge with max|grad| = 0.00248716 (tol = 0.002, component 1)
> summary(glmer1)
Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) ['glmerMod']
 Family: poisson  ( log )
Formula: Aggression_counts ~ Treatment + Week + Phase + Treatment:Week +
    Treatment:Phase + (1 | Tank)
   Data: aggression.df

     AIC      BIC   logLik deviance df.resid
  1723.4   1771.0   -848.7   1697.4      273

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.5077 -1.2784 -0.4059  0.8305  7.7137

Random effects:
 Groups Name        Variance Std.Dev.
 Tank   (Intercept) 0.06933  0.2633
Number of obs: 286, groups:  Tank, 13

Fixed effects:
                           Estimate Std. Error z value Pr(>|z|)
(Intercept)                 0.69931    0.21165   3.304 0.000953 ***
TreatmentBubbles            0.91684    0.25219   3.635 0.000277 ***
TreatmentCurrent            0.46372    0.26193   1.770 0.076658 .
TreatmentStones             0.43116    0.27697   1.557 0.119539
Week                        0.25104    0.03876   6.477 9.37e-11 ***
PhaseBefore                 0.19237    0.11736   1.639 0.101189
TreatmentBubbles:Week      -0.18139    0.05071  -3.577 0.000347 ***
TreatmentCurrent:Week      -0.17809    0.05240  -3.399 0.000677 ***
TreatmentStones:Week       -0.22613    0.05318  -4.252 2.11e-05 ***
TreatmentBubbles:PhaseBefore -0.77303    0.15746  -4.909 9.14e-07 ***
TreatmentCurrent:PhaseBefore -0.28711    0.16000  -1.794 0.072742 .
TreatmentStones:PhaseBefore  0.07216    0.16181   0.446 0.655636
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) TrtmnB TrtmnC TrtmnS Week   PhsBfr TrtB:W TrtC:W TrtS:W TrB:PB TrC:PB
TrtmntBbbls -0.792
TrtmntCrrnt -0.765  0.697
TretmntStns -0.761  0.699  0.681
Week        -0.711  0.599  0.591  0.555
PhaseBefore -0.304  0.255  0.246  0.232  0.000
TrtmntBbb:W  0.537 -0.731 -0.438 -0.414 -0.762  0.000
TrtmntCrr:W  0.513 -0.434 -0.724 -0.391 -0.739  0.000  0.563
TrtmntStn:W  0.520 -0.446 -0.442 -0.699 -0.730  0.000  0.556  0.539
TrtmntBb:PB  0.226 -0.290 -0.183 -0.173  0.000 -0.745  0.000  0.000  0.000
TrtmntCr:PB  0.223 -0.187 -0.315 -0.170  0.000 -0.734  0.000  0.000  0.000  0.547
TrtmntSt:PB  0.220 -0.185 -0.178 -0.325  0.000 -0.725  0.000  0.000  0.000  0.541  0.532
optimizer (Nelder_Mead) convergence code: 0 (OK)
Model failed to converge with max|grad| = 0.00248716 (tol = 0.002, component 1)
```

```
> car::Anova(glmer1, type=2)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: Aggression_counts
                 Chisq Df Pr(>Chisq)
Treatment       11.5829  3  0.0089573 **
Week            30.2378  1  3.822e-08 ***
Phase            1.9665  1  0.1608204
Treatment:Week  20.8956  3  0.0001107 ***
Treatment:Phase 37.7125  3  3.252e-08 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> emmeans(glmer1, list(c(pairwise ~ Treatment*Phase)), type = "response") #Compares all treatment
s agains each other as well as the phases.
$`emmeans of Treatment, Phase`
 Treatment Phase  rate    SE  df asymp.LCL asymp.UCL
 Control   After  4.46 0.675 Inf      3.31      6.00
 Bubbles   After  6.28 0.688 Inf      5.06      7.78
 Current   After  4.03 0.498 Inf      3.16      5.14
 Stones    After  3.35 0.451 Inf      2.57      4.36
 Control   Before 5.40 0.794 Inf      4.05      7.21
 Bubbles   Before 3.51 0.432 Inf      2.76      4.47
 Current   Before 3.67 0.461 Inf      2.87      4.69
 Stones    Before 4.37 0.561 Inf      3.39      5.62

Confidence level used: 0.95
Intervals are back-transformed from the log scale

$`pairwise differences of Treatment, Phase`
 1                                ratio     SE  df null z.ratio p.value
 Control After / Bubbles After   0.710 0.1233 Inf    1  -1.971  0.5018
 Control After / Current After   1.106 0.2015 Inf    1   0.551  0.9994
 Control After / Stones After    1.330 0.2655 Inf    1   1.429  0.8442
 Control After / Control Before  0.825 0.0968 Inf    1  -1.639  0.7264
 Control After / Bubbles Before  1.269 0.2315 Inf    1   1.307  0.8964
 Control After / Current Before  1.216 0.2233 Inf    1   1.062  0.9644
 Control After / Stones Before   1.021 0.1996 Inf    1   0.106  1.0000
 Bubbles After / Current After   1.557 0.1871 Inf    1   3.684  0.0056
 Bubbles After / Stones After    1.873 0.2440 Inf    1   4.816  <.0001
 Bubbles After / Control Before  1.162 0.1972 Inf    1   0.883  0.9877
 Bubbles After / Bubbles Before  1.787 0.1876 Inf    1   5.531  <.0001
 Bubbles After / Current Before  1.712 0.2096 Inf    1   4.389  0.0003
 Bubbles After / Stones Before   1.437 0.1780 Inf    1   2.930  0.0667
 Current After / Stones After    1.203 0.1657 Inf    1   1.341  0.8830
 Current After / Control Before  0.746 0.1332 Inf    1  -1.640  0.7259
 Current After / Bubbles Before  1.148 0.1521 Inf    1   1.042  0.9681
 Current After / Current Before  1.099 0.1196 Inf    1   0.871  0.9886
 Current After / Stones Before   0.923 0.1216 Inf    1  -0.606  0.9988
```

```
 Stones After / Control Before   0.620 0.1218 Inf    1  -2.433  0.2254
 Stones After / Bubbles Before   0.954 0.1352 Inf    1  -0.330  1.0000
 Stones After / Current Before   0.914 0.1277 Inf    1  -0.644  0.9982
 Stones After / Stones Before    0.768 0.0855 Inf    1  -2.375  0.2538
 Control Before / Bubbles Before 1.539 0.2750 Inf    1   2.410  0.2360
 Control Before / Current Before 1.473 0.2654 Inf    1   2.152  0.3815
 Control Before / Stones Before  1.237 0.2377 Inf    1   1.109  0.9551
 Bubbles Before / Current Before 0.958 0.1289 Inf    1  -0.321  1.0000
 Bubbles Before / Stones Before  0.804 0.1093 Inf    1  -1.603  0.7486
 Current Before / Stones Before  0.840 0.1124 Inf    1  -1.305  0.8975

P value adjustment: tukey method for comparing a family of 8 estimates
Tests are performed on the log scale
```

```
> emmeans(glmer1, list(c(pairwise ~ Treatment|Phase)), type = "response") #Compares all treatment
s with phases
$`emmeans of Treatment | Phase`
Phase = After:
 Treatment rate    SE  df asymp.LCL asymp.UCL
 Control   4.46 0.675 Inf      3.31      6.00
 Bubbles   6.28 0.688 Inf      5.06      7.78
 Current   4.03 0.498 Inf      3.16      5.14
 Stones    3.35 0.451 Inf      2.57      4.36

Phase = Before:
 Treatment rate    SE  df asymp.LCL asymp.UCL
 Control   5.40 0.794 Inf      4.05      7.21
 Bubbles   3.51 0.432 Inf      2.76      4.47
 Current   3.67 0.461 Inf      2.87      4.69
 Stones    4.37 0.561 Inf      3.39      5.62

Confidence level used: 0.95
Intervals are back-transformed from the log scale

$`pairwise differences of Treatment | Phase`
Phase = After:
 2                ratio    SE  df null z.ratio p.value
 Control / Bubbles 0.710 0.123 Inf    1  -1.971  0.1990
 Control / Current 1.106 0.201 Inf    1   0.551  0.9462
 Control / Stones  1.330 0.266 Inf    1   1.429  0.4814
 Bubbles / Current 1.557 0.187 Inf    1   3.684  0.0013
 Bubbles / Stones  1.873 0.244 Inf    1   4.816  <.0001
 Current / Stones  1.203 0.166 Inf    1   1.341  0.5364

Phase = Before:
 2                ratio    SE  df null z.ratio p.value
 Control / Bubbles 1.539 0.275 Inf    1   2.410  0.0751
 Control / Current 1.473 0.265 Inf    1   2.152  0.1370
 Control / Stones  1.237 0.238 Inf    1   1.109  0.6840
 Bubbles / Current 0.958 0.129 Inf    1  -0.321  0.9885
 Bubbles / Stones  0.804 0.109 Inf    1  -1.603  0.3767
 Current / Stones  0.840 0.112 Inf    1  -1.305  0.5600

P value adjustment: tukey method for comparing a family of 4 estimates
Tests are performed on the log scale
```

Appnedix E. R-script for cell proliferation analysis

```
> combi_glmer <- glmer(Nuclei_count ~ Treatment + Brain_region + Treatment:Brain_region + (1|Fish
ID), family = 'poisson', data = tel_nuc_combi.df)
> summary(combi_glmer)
Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
 Family: poisson  ( log )
Formula: Nuclei_count ~ Treatment + Brain_region + Treatment:Brain_region +
    (1 | FishID)
   Data: tel_nuc_combi.df

     AIC      BIC   logLik deviance df.resid
  3386.9   3431.8  -1680.4   3360.9      221

Scaled residuals:
    Min      1Q  Median      3Q     Max
-9.1853 -2.1201 -0.7849  1.8318 10.6048

Random effects:
 Groups Name        Variance Std.Dev.
 FishID (Intercept) 0.7608   0.8722
Number of obs: 234, groups:  FishID, 11

Fixed effects:
                             Estimate Std. Error z value Pr(>|z|)
(Intercept)                   2.98317    0.62043   4.808 1.52e-06 ***
TreatmentBubbles             -1.06717    0.88334  -1.208  0.22701
TreatmentCurrent             -0.47236    0.80199  -0.589  0.55587
TreatmentStones               0.80297    0.75897   1.058  0.29007
Brain_regionDM               -0.24489    0.09228  -2.654  0.00796 **
Brain_regionVV               -0.98208    0.11714  -8.384  < 2e-16 ***
TreatmentBubbles:Brain_regionDM  0.25973    0.19487   1.333  0.18260
TreatmentCurrent:Brain_regionDM  0.31514    0.09953   3.166  0.00154 **
TreatmentStones:Brain_regionDM  -0.40964    0.10400  -3.939 8.19e-05 ***
TreatmentBubbles:Brain_regionVV  0.24313    0.24420   0.996  0.31943
TreatmentCurrent:Brain_regionVV  0.73026    0.12397   5.891 3.85e-09 ***
TreatmentStones:Brain_regionVV  -0.82716    0.13897  -5.952 2.65e-09 ***
```

```
> #ANOVA
> car::Anova(combi_glmer, type = 3)
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: Nuclei_count
                         Chisq Df Pr(>Chisq)
(Intercept)            23.1189  1  1.523e-06 ***
Treatment               7.1112  3    0.06844 .
Brain_region           70.3562  2  5.276e-16 ***
Treatment:Brain_region 393.6870  6  < 2.2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
> emmeans(combi_glmer, list(pairwise ~ Brain_region))
NOTE: Results may be misleading due to involvement in interactions
$`emmeans of Brain_region`
 Brain_region emmean    SE  df asymp.LCL asymp.UCL
 DL             2.80 0.277 Inf      2.26      3.34
 DM             2.60 0.277 Inf      2.05      3.14
 VV             1.85 0.280 Inf      1.30      2.40

Results are averaged over the levels of: Treatment
Results are given on the log (not the response) scale.
Confidence level used: 0.95

$`pairwise differences of Brain_region`
 1       estimate     SE  df z.ratio p.value
 DL - DM    0.204 0.0510 Inf   3.989  0.0002
 DL - VV    0.946 0.0646 Inf  14.626  <.0001
 DM - VV    0.742 0.0654 Inf  11.350  <.0001
```

```
> emmeans(combi_glmer, list(c(pairwise ~ Treatment*Brain_region)), type = "response") #Compare al
l treatments with each other in and across all ROI
$`emmeans of Treatment, Brain_region`
 Treatment Brain_region  rate    SE  df asymp.LCL asymp.UCL
 Control   DL           19.75 12.25 Inf     5.854      66.6
 Bubbles   DL            6.79  4.27 Inf     1.981      23.3
 Current   DL           12.31  6.26 Inf     4.549      33.3
 Stones    DL           44.09 19.27 Inf    18.715     103.9
 Control   DM           15.46  9.60 Inf     4.575      52.2
 Bubbles   DM            6.90  4.33 Inf     2.011      23.6
 Current   DM           13.21  6.71 Inf     4.880      35.8
 Stones    DM           22.91 10.03 Inf     9.710      54.1
 Control   VV            7.40  4.63 Inf     2.171      25.2
 Bubbles   VV            3.24  2.08 Inf     0.923      11.4
 Current   VV            9.57  4.87 Inf     3.535      25.9
 Stones    VV            7.22  3.19 Inf     3.038      17.2

Confidence level used: 0.95
Intervals are back-transformed from the log scale

$`pairwise differences of Treatment, Brain_region`
 1                       ratio      SE  df null z.ratio p.value
 Control DL / Bubbles DL 2.907  2.5680 Inf    1   1.208  0.9885
 Control DL / Current DL 1.604  1.2862 Inf    1   0.589  1.0000
 Control DL / Stones DL  0.448  0.3400 Inf    1  -1.058  0.9963
 Control DL / Control DM 1.277  0.1179 Inf    1   2.654  0.2498
 Control DL / Bubbles DM 2.864  2.5298 Inf    1   1.191  0.9897
 Control DL / Current DM 1.495  1.1989 Inf    1   0.501  1.0000
 Control DL / Stones DM  0.862  0.6547 Inf    1  -0.195  1.0000
 Control DL / Control VV 2.670  0.3128 Inf    1   8.384  <.0001
 Control DL / Bubbles VV 6.087  5.4323 Inf    1   2.024  0.6772
 Control DL / Current VV 2.063  1.6548 Inf    1   0.903  0.9991
 Control DL / Stones VV  2.735  2.0833 Inf    1   1.321  0.9766
 Bubbles DL / Current DL 0.552  0.4460 Inf    1  -0.736  0.9999
 Bubbles DL / Stones DL  0.154  0.1180 Inf    1  -2.442  0.3773
 Bubbles DL / Control DM 0.439  0.3884 Inf    1  -0.930  0.9988
 Bubbles DL / Bubbles DM 0.985  0.1691 Inf    1  -0.086  1.0000
```

```
 Bubbles DL / Current DM  0.514  0.4157 Inf    1  -0.823  0.9996
 Bubbles DL / Stones DM   0.297  0.2272 Inf    1  -1.586  0.9144
 Bubbles DL / Control VV  0.918  0.8145 Inf    1  -0.096  1.0000
 Bubbles DL / Bubbles VV  2.094  0.4486 Inf    1   3.449  0.0280
 Bubbles DL / Current VV  0.710  0.5738 Inf    1  -0.424  1.0000
 Bubbles DL / Stones VV   0.941  0.7230 Inf    1  -0.079  1.0000
 Current DL / Stones DL   0.279  0.1872 Inf    1  -1.903  0.7580
 Current DL / Control DM  0.797  0.6393 Inf    1  -0.283  1.0000
 Current DL / Bubbles DM  1.786  1.4436 Inf    1   0.718  0.9999
 Current DL / Current DM  0.932  0.0348 Inf    1  -1.883  0.7700
 Current DL / Stones DM   0.538  0.3606 Inf    1  -0.925  0.9989
 Current DL / Control VV  1.665  1.3416 Inf    1   0.633  1.0000
 Current DL / Bubbles VV  3.795  3.1061 Inf    1   1.630  0.8984
 Current DL / Current VV  1.286  0.0522 Inf    1   6.206  <.0001
 Current DL / Stones VV   1.706  1.1484 Inf    1   0.793  0.9997
 Stones DL / Control DM   2.852  2.1662 Inf    1   1.379  0.9676
 Stones DL / Bubbles DM   6.394  4.8952 Inf    1   2.423  0.3900
 Stones DL / Current DM   3.337  2.2368 Inf    1   1.798  0.8196
 Stones DL / Stones DM    1.924  0.0923 Inf    1  13.647  <.0001
 Stones DL / Control VV   5.960  4.5478 Inf    1   2.339  0.4483
 Stones DL / Bubbles VV  13.587 10.5474 Inf    1   3.361  0.0373
 Stones DL / Current VV   4.605  3.0876 Inf    1   2.278  0.4926
 Stones DL / Stones VV    6.106  0.4565 Inf    1  24.199  <.0001
 Control DM / Bubbles DM  2.242  1.9816 Inf    1   0.914  0.9990
 Control DM / Current DM  1.170  0.9392 Inf    1   0.196  1.0000
 Control DM / Stones DM   0.675  0.5129 Inf    1  -0.517  1.0000
 Control DM / Control VV  2.090  0.2539 Inf    1   6.068  <.0001
 Control DM / Bubbles VV  4.765  4.2551 Inf    1   1.748  0.8457
 Control DM / Current VV  1.615  1.2964 Inf    1   0.597  1.0000
 Control DM / Stones VV   2.141  1.6322 Inf    1   0.999  0.9978
 Bubbles DM / Current DM  0.522  0.4219 Inf    1  -0.804  0.9997
 Bubbles DM / Stones DM   0.301  0.2306 Inf    1  -1.567  0.9209
 Bubbles DM / Control VV  0.932  0.8266 Inf    1  -0.079  1.0000
 Bubbles DM / Bubbles VV  2.125  0.4542 Inf    1   3.526  0.0215
 Bubbles DM / Current VV  0.720  0.5823 Inf    1  -0.406  1.0000
 Bubbles DM / Stones VV   0.955  0.7337 Inf    1  -0.060  1.0000
 Current DM / Stones DM   0.577  0.3868 Inf    1  -0.821  0.9996
 Current DM / Control VV  1.786  1.4392 Inf    1   0.720  0.9999
 Current DM / Bubbles VV  4.072  3.3320 Inf    1   1.716  0.8615
 Current DM / Current VV  1.380  0.0552 Inf    1   8.057  <.0001
 Current DM / Stones VV   1.830  1.2319 Inf    1   0.897  0.9992
 Stones DM / Control VV   3.097  2.3649 Inf    1   1.481  0.9461
 Stones DM / Bubbles VV   7.061  5.4846 Inf    1   2.516  0.3295
 Stones DM / Current VV   2.393  1.6059 Inf    1   1.300  0.9793
 Stones DM / Stones VV    3.173  0.2522 Inf    1  14.528  <.0001
 Control VV / Bubbles VV  2.280  2.0425 Inf    1   0.920  0.9989
 Control VV / Current VV  0.773  0.6228 Inf    1  -0.320  1.0000
 Control VV / Stones VV   1.024  0.7844 Inf    1   0.032  1.0000
 Bubbles VV / Current VV  0.339  0.2774 Inf    1  -1.322  0.9765
 Bubbles VV / Stones VV   0.449  0.3500 Inf    1  -1.027  0.9971
 Current VV / Stones VV   1.326  0.8930 Inf    1   0.419  1.0000

P value adjustment: tukey method for comparing a family of 12 estimates
Tests are performed on the log scale
```

```
> emmeans(combi_glmer, list(c(pairwise ~ Treatment|Brain_region)), type = "response") #Compare tr
eatments within each ROI
$`emmeans of Treatment | Brain_region`
Brain_region = DL:
 Treatment  rate    SE  df asymp.LCL asymp.UCL
 Control   19.75 12.25 Inf     5.854      66.6
 Bubbles    6.79  4.27 Inf     1.981      23.3
 Current   12.31  6.26 Inf     4.549      33.3
 Stones    44.09 19.27 Inf    18.715     103.9

Brain_region = DM:
 Treatment  rate    SE  df asymp.LCL asymp.UCL
 Control   15.46  9.60 Inf     4.575      52.2
 Bubbles    6.90  4.33 Inf     2.011      23.6
 Current   13.21  6.71 Inf     4.880      35.8
 Stones    22.91 10.03 Inf     9.710      54.1

Brain_region = VV:
 Treatment  rate    SE  df asymp.LCL asymp.UCL
 Control    7.40  4.63 Inf     2.171      25.2
 Bubbles    3.24  2.08 Inf     0.923      11.4
 Current    9.57  4.87 Inf     3.535      25.9
 Stones     7.22  3.19 Inf     3.038      17.2

Confidence level used: 0.95
Intervals are back-transformed from the log scale

$`pairwise differences of Treatment | Brain_region`
Brain_region = DL:
 2                ratio    SE  df null z.ratio p.value
 Control / Bubbles 2.907 2.568 Inf    1   1.208  0.6217
 Control / Current 1.604 1.286 Inf    1   0.589  0.9354
 Control / Stones  0.448 0.340 Inf    1  -1.058  0.7151
 Bubbles / Current 0.552 0.446 Inf    1  -0.736  0.8827
 Bubbles / Stones  0.154 0.118 Inf    1  -2.442  0.0694
 Current / Stones  0.279 0.187 Inf    1  -1.903  0.2269

Brain_region = DM:
 2                ratio    SE  df null z.ratio p.value
 Control / Bubbles 2.242 1.982 Inf    1   0.914  0.7976
 Control / Current 1.170 0.939 Inf    1   0.196  0.9973
 Control / Stones  0.675 0.513 Inf    1  -0.517  0.9549
 Bubbles / Current 0.522 0.422 Inf    1  -0.804  0.8524
 Bubbles / Stones  0.301 0.231 Inf    1  -1.567  0.3974
 Current / Stones  0.577 0.387 Inf    1  -0.821  0.8447

Brain_region = VV:
 2                ratio    SE  df null z.ratio p.value
 Control / Bubbles 2.280 2.043 Inf    1   0.920  0.7943
 Control / Current 0.773 0.623 Inf    1  -0.320  0.9887
 Control / Stones  1.024 0.784 Inf    1   0.032  1.0000
 Bubbles / Current 0.339 0.277 Inf    1  -1.322  0.5489
 Bubbles / Stones  0.449 0.350 Inf    1  -1.027  0.7337
 Current / Stones  1.326 0.893 Inf    1   0.419  0.9753

P value adjustment: tukey method for comparing a family of 4 estimates
Tests are performed on the log scale
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