Examining the impact of cleaner fish on pigmentation of salmon lice (*Lepeophtheirus salmonis*) in commercial aquaculture cages.

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Abstract

Salmon lice (*Lepeoptheirus salmonis*) are one of the biggest challenges to sustainable salmonid aquaculture. The species display high evolutionary potential, which is evident by its development of resistance to numerous chemical compounds used for delousing. In response to this, salmon farms now use non-chemical delousing methods to minimize the damage done by salmon lice, including heavy reliance on cleaner fish. Anecdotal reports from farmers and fish health personnel in areas where cleaner fish are used have suggested that salmon lice are becoming less pigmented. This experiment investigated changes in the pigmentation of salmon lice in relation to the use of cleaner fish, louse stage and sex, temperature, preferred salmon swimming depth, daylength, and salinity. Salmon lice were sampled from snorkel cages on a commercial salmon farm where three cages were stocked with farmed lumpfish and ballan wrasse, and three cages were without cleaner fish. Water temperature, salinity, and depth were recorded using a conductivity, temperature, and depth (CTD) recorder. Pigmentation was measured via photographic analysis of individual lice.

The analyses showed that using cleaner fish stabilized dMGV throughout the year while lice dMGV varied between each sampling in control cages. This stabilization was also evident during environmental changes. Male lice have a higher dMGV than females independent of environment, but there is no specific change in dMGV between the mobile life stages. Overall, present findings suggest that the pigmentation of the salmon louse is not controlled by a single factor, but instead by several factors working together. Using cleaner fish throughout a single production cycle did not reduce average louse pigmentation compared to control cages, but their presence did have a stabilizing effect.

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1 Introduction

1.1 Norwegian salmon farming

With the continuous growth of the human population, the world's food demand also increases. Worldwide, aquaculture has expanded rapidly during the past decades and is currently the dominant method of seafood production (Lindland et al., 2019). The Norwegian salmon industry has been steadily growing since its start in the seventies, and as of 2019, the total production of Atlantic salmon was 1.36 million tonnes with a total value of 68 billion NOK (Statistics Norway 2020). In Norway, aquaculture consists mainly of Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss*), and brown trout (*Salmo trutta*) farming, with salmon production accounting for 94.5 percent of production (Lindland et al., 2019).

With more fish, however, come more challenges. One of the biggest challenges to the industry is ectoparasitic salmon lice (*Lepeoptheirus salmonis*) (Bui et al., 2020; Torrissen et al., 2013). Salmon lice are a multi-faceted problem. Directly, be it wild or farmed salmon, lice injure their host by feeding on its skin, blood, and mucus (Wootten et al., 1982). Indirectly, regulations that aim to curb the growth of the louse population force farmers to pursue potentially risky and expensive management strategies. In 2013, Norwegian authorities implemented increasingly stricter regulations to resolve the challenge of sea lice-induced mortality in wild salmonids (Larsen & Vormedal, 2021). As a result, all salmon farms are required to keep lice levels below 0.5 adult female lice per fish most of the year, with requirements to keep it below 0.2 during spring migration. In 2017 a traffic light system was created with thirteen production areas along the coast where lice infestation is evaluated. These areas are assigned a green, yellow, or red "traffic light" depending on the impact the area has on wild salmon. Salmon farmers are required to count lice every week when the water temperature is over 4°C and fortnightly when the temperature is below 4°C (Larsen & Vormedal, 2021). Despite these efforts, the population of salmon lice has consistently grown (Guarracino et al., 2018).

1.2 *Lepeophtheirus salmonis* (Salmon Lice)

Salmon lice have the greatest economic impact of all parasites affecting aquaculture, and they also heavily affect wild salmonid populations (Overton et al., 2019b). This marine copepod is prevalent in the North Atlantic Ocean and North Pacific Ocean where Atlantic salmon and other salmonid species are found (Skern-Mauritzen et al., 2014). The parasitic stages of lice feed on mucus, skin, and blood of salmonid fish (Wootten et al., 1982). In high concentration, salmon

lice can cause great damage to the host, eventually killing them (Wagner et al., 2008). With the increasing presence of salmon farms, the population of salmon lice has grown (Guarracino et al., 2018). Wild Atlantic salmon are targets for salmon lice at the start of their migration toward the open sea and when they return to the river to spawn (Torrissen et al., 2013).

Salmon lice cost the industry a lot of money as a result of loss of fish and, treatment expense. In 2014 the cost of sea lice was estimated to be around 3 billion NOK (Iversen et al., 2015). As the salmon industry continues to grow, the cost salmon lice inflict on the industry will likely also increase. In wild salmon populations, the mean abundance of salmon lice ranges from 6 to 33 per fish, with 0 to 17 of them being adult females (Torrissen et al., 2013).

Figure 1: Photograph of the different development stages of salmon louse *from Pre-adult I to adult stages, with both sexes represented for each stage. From left to right: Pre-adult I male, pre-adult I female, pre-adult II male, pre-adult II female, adult male, and adult female.*

1.2.1 Life cycle

Salmon lice have a direct life cycle, which consists of eight life stages, two free-living and six on a single host. There are two free-living naupliar dispersal stages, one infective copepodite stage, and five parasitic stages consisting of two chalimus stages, two pre-adult stages, and one adult stage (Hamre et al., 2013, Figure 1).

The naupliar stages (Nauplius I and II) are planktonic for 5-15 days after hatching when they molt into infective free-living copepodites (Costello, 2006). This planktonic stage is between 0.4 and 0.7 mm in length and moves with the water current while feeding only on the energy reserves they hatch with (Boxaspen, 2006).

As with the nauplius stage, the copepodite stage is also planktonic and drifts with the water current until it finds a host (Boxaspen & Næss, 2000). Copepodites respond visually to the hosts' shadows and flashes of their scales, and they respond to vibration caused by the movement of their potential host and follow them, using chemoreceptors to determine the suitability of the host (Costello, 2006; Komisarczuk et al., 2017).

After finding a potential host, the copepodite molts into a chalimus that attaches itself to the host with newly developed frontal filaments (Gonzalez-Alanis et al., 2001). On average, the chalimus phase for males lasts 12 days while for females it lasts 14 days before they molt into preadult I. Sexual dimorphism develops during the pre-adult I molt, when males become visually distinct from females.

During the pre-adult stages, the lice become mobile and can move freely over the fish (Costello, 2006).

After the final molt, salmon lice become sexually mature adults. The male lice are around 5 mm in length while females are up to 10 mm in length, approximately double in size (Hamre et al., 2009). Mature females produce a pair of egg strings which may contain up to 1000 eggs per string during each reproductive period (Brooker et al., 2018). Up to 11 pair of egg strings may be produced by a single female (Heuch et al., 2000).

1.2.2 Temperature and salinity

The growth and development of lice are dependent on water temperature. Higher temperature causes rapid development in all lice stages except copepodite (Groner et al., 2014), while lower temperatures slow metabolism and growth. For example, female lice used 72 days to mature at 6°C, but only 13 days at 21°C (Hamre et al., 2019). As a consequence of the delayed growth, the lifespan of the louse also increases at lower temperatures and total female egg production increases (Costello, 2006). Warmer water temperatures mean fewer eggs produced, but due to the short generation time the population can still increase exponentially.

Sea lice are dependent on seawater and do not tolerate freshwater exposure over prolonged periods. While full freshwater causes mortality across stages, early louse stages are more susceptible to hyposaline water than mature stages (Sievers et al., 2019). Early stages (copepodites) have a high mortality rate of 96-100% after 1-h freshwater exposure, whereas later stages could survive up to 8 days (Wright et al., 2016). The lower limit of salinity is dependent on the stage in which the sea lice are currently in, with adult females surviving a salinity of 12.5 ppt without a host for under 8 hours (Ljungfeldt et al., 2017). The lower limit

of optimal salinity is 16 ppt a 14-15°C for adult females (Berger, 1970). Lower salinity may also alter behavioral patterns, attachment rates, and the development of free-living copepodites (Groner et al., 2016). Wild salmonids exhibit strong preferences toward areas with lower salinity when infested with salmon louse (Gjelland et al., 2014). Brown trout have also been observed to prematurely return to brackish and freshwater as a response to salmon louse infestation (Serra-Llinares et al., 2020).

1.2.3 Evolution and selection

Salmon lice have an outstanding capacity to evolve, which is one of the primary reasons they are so difficult to combat. Numerous factors influence the rate of resistance evolution, including intensity and frequency of selection, the population genetics and life history, and genetic mechanisms of resistance (McEwan et al., 2015). Salmon lice have a short generation time, especially in water temperature (Johnson & Albright, 1991), which increases the possibility of new traits appearing quickly (Ljungfeldt et al., 2017). Further, the species is highly abundant and display genetic variation in several key traits, including salinity and thermal tolerance (Ljungfeldt et al., 2017).

With the high amount of fish farms, farmed hosts vastly outnumber wild hosts (Dempster et al., 2021), and as a result natural refugia are insufficient to reduce the selective pressure on salmon lice (McEwan et al., 2015). Thus, while gene flow may counteract local selective forces, when multiple farms apply the same treatments and therefore selection, this leads to strong population-wide selection (Hamre et al., 2021). Salmon farming also selects for a shorter generation time as the parasite fitness is maximized with early maturation and high fecundity, even if it damages the host (Dempster et al., 2021). Consequently, farming conditions favor rapid reproductive cycles as there is an abundance of mates, high host availability, and a need to reproduce before the farmer delouses or harvests the salmon (Dempster et al., 2021).

Usage of chemical de-lousing was the leading de-lousing method from the 1980s to 2015 (Jensen et al., 2020). As a result, salmon lice evolved resistance and/or reduced sensitivity to 4 out of 5 chemical therapeutants (Fjørtoft et al., 2020). The case of emamectin benzoate is a clear example demonstrating the evolutionary capacity of salmon lice; resistance appeared in a single farming region and then, due to strong selection and extensive use of chemicals, dispersed throughout the North Atlantic within just 8 years (Besnier et al., 2014; Ljungfeldt et al., 2014). Further, despite a decline in the use of chemical treatments in recent years (Fjørtoft et al., 2020), resistant strains still persist in regions where no chemotherapeutants are used.

Following the shift to other delousing methods from chemical treatment, there is the possibility that salmon lice may adapt similarly to these new methods as well. Non-medicinal treatments consist of freshwater, mechanical removal, thermal delousing, and the use of cleaner fish (Jensen et al., 2020). Freshwater treatment consists of exposing salmon to freshwater for a few hours to remove lice (Coates et al., 2021). Already, the potential for increased tolerance to low salinities has been observed (Ljungfeldt et al., 2017), and because wild salmonids swim into freshwater to remove lice and regain ionic balance (Groner et al., 2019), there is serious concern that salmon lice may develop tolerance to low salinities. Mechanical delousing consists of pumping salmon through automated systems where lice are physically removed using jets of pressurized water, turbulence and/or brushes (Coates et al., 2021). Although no resistance to mechanical delousing has been observed to date, as the widespread use of mechanical delousing continues it is possible that lice could develop stronger attachment ability, reducing the effectiveness of the treatment. Similarly, thermal delousing detaches lice by briefly bathing salmon in warm water (up to 36°C) (Coates et al., 2021). Similar to freshwater tolerance, host attachment during thermal delousing also has a heritable basis (Coates et al., 2021), and as such the thermal tolerance of lice may increase with strong selective pressure (Coates et al., 2021).

1.2.4 Pigmentation

Pigmentation in salmon lice is what gives them their coloration. In copepods, pigment cells synthesize carotenoids and mycosporine-like amino acids (MAA) to either function as a sunscreen or as scavengers of photo-produced radicals (Hansson et al., 2007). MAA is a water-soluble molecule found in many cyanobacteria and eukaryotic microorganisms, as well as aquatic life forms (Oren & Gunde-Cimerman, 2007). These molecules absorb UV radiation between 310 and 365 nm and act as sunscreen to protect against harmful levels of UV radiation (Oren & Gunde-Cimerman, 2007). The pigments from the pigment cells may distribute widely over the body surface giving the lice a dark appearance, or the pigments can be concentrated in the pigment cells, leaving large areas of the louse transparent (Hamre et al., 2021).

Broadly, in free-living crustaceans pigmentation is often highly plastic and changes in response to UV exposure and predator cues (Scoville & Pfrender, 2010). In salmon lice specifically, there is evidence for both genetic and environmental determination of pigmentation (Hamre et al. 2021). Degree of pigmentation consistently differed between strains regardless of environment, but also within strains lice were consistently lighter when reared indoors compared to individuals reared outdoors (Hamre et al., 2021). Further, louse placement on the fish is also a factor when it comes to pigmentation, where lice found on the dorsal side of the fish, which is

most exposed to sunlight, were significantly darker than lice found on the ventral side of the fish (Hamre et al. 2021). However, in *Daphnia*, another free-living crustacean, pigment production has been shown to be energetically costly and slow growth (Scoville & Pfrender, 2010). As a result, when *Daphnia* were removed from UV radiation they rapidly reduced pigmentation by 40% (Hansson et al., 2007). Further, in populations with predators, *Daphnia* had reduced pigmentation as the predators preyed upon the darker individuals, and remained pale across varied UV conditions (Scoville & Pfrender, 2010). Similarly, in some free-living copepod species, lighter pigmented individuals are preyed on less by predators than darker individuals of the same species (Scoville & Pfrender, 2010).

Thus, the cost of damage from UV radiation and the possibility to be seen by predators is likely a key trade-off for salmon lice, and it may be advantageous for lice to reduce pigment production when UV exposure is low. Further, this trade-off will occur differently between lice on wild and farmed salmon populations where selective pressures are different (Scoville & Pfrender, 2010).

1.3 Cleaner fish

The use of cleaner fish as a continuous louse control technique was developed in the late 1980s (Torrissen et al., 2013), and their use rapidly increased in Norway with the phase-out of chemical treatments (Overton et al., 2019a). The cleaner fish used in Norwegian aquaculture are opportunistic feeders, meaning they feed on what is available (Imsland et al., 2015), unlike obligate cleaner fish who primarily feed by cleaning other fish species (Vaughan et al., 2017). Cleaner fish are less expensive and less stressful for the salmon than other delousing methods and are generally more acceptable to the public than chemotherapeutants (Overton et al., 2020). With the widespread resistance to chemotherapeutants and the fact that non-chemical delousing strategies are stressful and elevate salmon mortality rates post-treatment, cleaner fish became a keystone control method in the fight against salmon lice. The lack of antagonistic behavior between Atlantic salmon and cleaner fish also helped spur investment (Imsland et al., 2018).

The cleaner fish species mainly used in Norwegian aquaculture are ballan wrasse (*Labrus bergylta*), corkwing wrasse (*Symphodus melops*), rock cook (*Centrolabus exoladus*), goldsinny wrasse (*Ctenolabrus rupestris*), cuckoo wrasse (*Labrus mixtus*), and lumpfish (*Cyclopterus lumpus*) (Philis et al., 2021). Lumpfish and ballan wrasse are mostly farmed for their use in aquaculture while the other species are only wild-caught.

Due to water temperature, there are limitations to the usage of cleaner fish with each species tolerating different temperature ranges. Lumpfish tolerate lower temperatures better than wrasse, and as such lumpfish is the only species used north of Trøndelag, while there is a mix of all used south of Trøndelag (Philis et al., 2021). Typically, wrasse are best deployed in spring/summer while lumpfish are best deployed in autumn/winter (Brooker et al., 2018b). Lumpfish and Atlantic salmon share feeding ground in the wild, which may explain the non-antagonistic behavior of this species when reared together in salmon sea pens (Imsland et al., 2016).

1.3.1 *Labrus bergylta* (Ballan wrasse)

Ballan wrasse are the largest wrasse species in Norway. It is a large fish in the *Labrus* genus with a relatively deep body and a big head. Ballan wrasse are the fastest-growing wrasse and can get up to 60 cm in length and live up to 29 years (Blanco Gonzalez & de Boer, 2017). It is also the largest and hardiest of the wrasse species, giving it the highest value in the industry (Skiftesvik et al., 2013). However, Ballan wrasse are also the least abundant of the wrasse species in the wild. Farms that produce Ballan wrasse for use in aquaculture have thus been created to relieve the pressure on wild populations. Wrasse are demersal fish living in shallow coastal rocky reefs and kelp beds at depths of 20-30 meters, and commonly shelter overnight in rock crevices (Leclercq et al., 2018). Ballan wrasse are a temperate species found in the northeast Atlantic from Trondheim, Norway, in the north to Morocco in the south. In sea cages, ballan wrasse were observed to rarely be above the halocline and thermocline, usually staying deeper in the cages (Geitung et al., 2020).

When used as cleaner fish in sea cages strict biofouling control is important as these alternative food sources can preclude delousing (Deady et al., 1995). Ballan wrasse prefer to feed on large adult lice but will also consume smaller mobile lice (Leclercq et al., 2014). Ballan wrasse use eyesight to find their prey, which is thought to limit their ability to spot and eat smaller louse stages (Blanco Gonzalez & de Boer, 2017). This could also apply if the pigmentation makes it difficult to see the louse on the salmon.

1.3.2 *Cyclopterus lumpus* (Lumpfish)

Lumpfish is a bony fish belonging to the order Scorpaeniformes, family Cyclopteridae, and is the only species in the genus *Cyclopterus.* It is considered a sub-Arctic species and is found on both sides of the North Atlantic, and is commonly found along the coastlines of Iceland, Norway, the United Kingdom, and the East coast of North America (Pountney et al., 2020). Lumpfish are generally solitary and spend most of their adult life in the open sea (Bañón et al.,

2008). Lumpfish are often found in association with floating seaweed (Ingolfsson & Kristjansson, 2002). Lumpfish are twice as long as they are deep, and the body is compressed both anteriorly and posteriorly (Davenport, 1985). One of their more distinctive features are their pectoral fins which have formed into a suction disc that used to latch onto rocks, seaweed, or other smooth surfaces to rest (Davenport, 1985). This suction disc constitutes around 20% of their body length. Lumpfish do not have a swim bladder (Powell et al., 2018).

The diet of lumpfish consists mainly of planktonic organisms living near the surface or mid waters, but sometimes they feed on benthic species, especially organisms which dwell on weed (Davenport, 1985). Like wrasse, they use eyesight to locate their prey (Paradis et al., 2019). Gut contents of adults across different studies show different types of organisms such as crustaceans, ctenophores, polychaetes, seagrass, insects, small fish, and fish eggs (Davenport, 1985). In juvenile lumpfish, cannibalism has been observed from gut contents in individuals as small as 11 mm (Ingolfsson & Kristjansson, 2002). In sea cages, lumpfish have been observed to be highly opportunistic and seem to switch their choice of food depending on availability within their environment (Imsland et al., 2015). For the majority of their daytime cycle, lumpfish are either foraging for food or resting among floating seaweed (Imsland et al., 2014). Lumpfish will feed at temperatures as low as 4°C making it an ideal species during winter months or in areas where the water temperatures are low (Powell et al., 2018).

1.4 Aims of the study

Salmon lice are an ongoing problem for salmon aquaculture. With the inherent challenges and problems involved with other methods of combating lice, cleaner fish use has dramatically increased (Brooker et al., 2018). As cleaner fish are thought to be dependent on eyesight to locate prey, their widespread and intense usage may be exerting selective pressure on the pigmentation of lice. No previous studies have examined the effect of cleaner fish predation on salmon louse pigmentation, but previous work has demonstrated that louse pigmentation is both genetically and environmentally influenced (Hamre et al. 2021). Given the high evolutionary capacity of salmon lice, some fear that lice may adapt to become less vulnerable to predation. One potential adaptive direction is altered appearance such that the lice become more difficult for the cleaner fish to visually detect.

The primary aim of this thesis is to determine if the presence of cleaner fish in marine net cages alters louse pigmentation, with a secondary aim to examine the influence of other factors

including season, environmental conditions, life stage, and sex on louse pigmentation. To that end, the following hypotheses were formed:

$H0_a$ = There is no difference in pigmentation, as measured by dMGV, between cleaner fish and control treatment groups.

$H1_a$ = Lice are more transparent (have lower dMGV) in cages stocked with cleaner fish compared to controls.

$H0_b$ = Temperature does not affect louse pigmentation, as measured by dMGV.

$H1_b$ = Louse pigmentation increases (higher dMGV) with increasing temperature.

$H0_c$ = Depth of optimal temperature for salmon does not affect louse pigmentation, as measured by dMGV.

$H1_c$ = dMGV varies with the optimal depth of the salmon, with deeper waters having lighter pigmented lice (lower dMGV).

$H0_d$ = Daylength does not affect louse pigmentation, as measured by dMGV.

$H1_d$ = Daylength affects the pigmentation of the salmon louse with longer days causing them to become darker pigmented (higher dMGV).

$H0_e$ = Salinity does not affect louse pigmentation, as measured by dMGV.

$H1_e$ = Louse pigmentation increases (higher dMGV) with higher mean salinity.

$H0_f$ = Life-stage does not affect louse pigmentation, as measured by dMGV.

$H1_f$ = Pigmentation differs among the three lice life-stages measured, with adults being the darkest (highest dMGV) and pre-adult I being lightest (lowest dMGV).

$H0_g$ = Sex does not affect louse pigmentation, as measured by dMGV.

$H1_g$ = Degree of pigmentation differs between males and females, with females being lighter pigmented (lower dMGV).

2 Materials & Methods

2.1 Sampling

All lice were collected from a commercial salmon farm located at Fosså (59.269 N, 6.143 E) in Boknafjorden, Hjelmeland municipality (Figure 2). Throughout this study the farm consisted of six 200 m circumference polar circle cages equipped with 20 m deep snorkels (90 m circumference), two aeration devices positioned at 22 m depth (Midt-Norsk ringen, NorseAqua, Norway), submerged feed distribution beginning at 18 m (SubFeeder, AKVA group) and two submerged lights at 18 m (150W/1200W Aurora SubLED Combi light, AKVA group). In addition, three cages were stocked with a combination of farmed Ballan wrasse and Lumpfish throughout production, added when seasonally appropriate (Table 1), while three remained control cages with no cleaner fish. To maximize cleaner fish welfare and performance, cages with cleaner fish were also supplied with plastic 'kelp' style hides and species-specific cleaner fish feed. Cage 1, 4, and 5 contained cleaner fish while cage 2, 3, and 6 were control cages and did not contain cleaner fish. During the last two samplings, the cleaner fish cages and control cages were interchanged so that cleaner fish were in cages 2, 3, and 6 and cages 1, 4, and 5 were without cleaner fish. A schematic of the fish farm with each numbered cage and the snorkel cage used can be seen in figure 3.

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Table 1: Species of cleaner fish used with stocking date and number of cleaner fish stocked in each cage.

		Lumpfish	
	Cage 1	Cage 4	Cage 5
Week 46 2020	10697	10521	10470
Week 8 2021	9792	9805	9817
		Ballan wrasse	
Week 21 2021	10654	9876	9935

Figure 2: Location and overview of the Fosså fish farm.

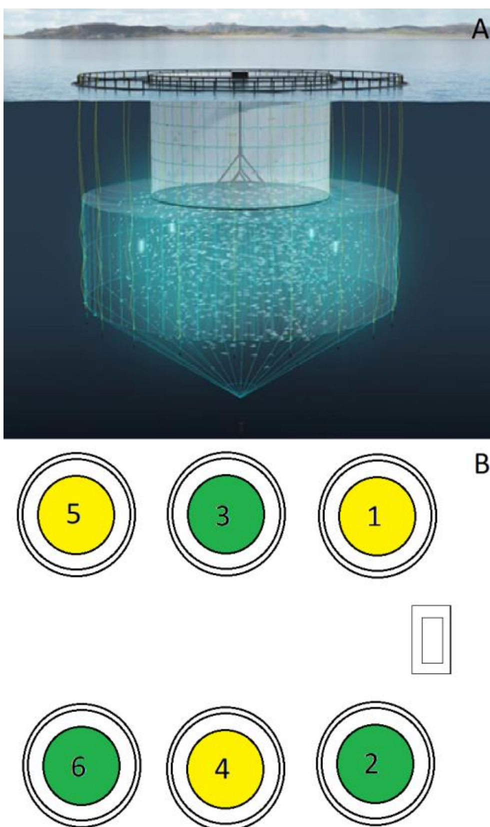

Figure 3: A) Schematic diagram of a snorkel cage as used on the Fosså farm. (Snorkel schematics photo: Mowi ASA). B) Cage layout of the Fosså salmon farm. Each cage is numbered according to how they are numbered at the site. Cages 1, 4, and 5 contained cleaner fish (yellow) while cages 2, 3, and 6 were without cleaner fish (green). This was swapped for the last two samplings.

Salmon were collected from cages using a ‘jumpnet’. The jumpnet is a 5 x 5 x 5 m rectangular net with small buoys around the upper perimeter to hold the top of the net flush with the water’s surface. Because each individual salmon jumps, on average, at least once per day (pers. comm., Frode Oppedal), jumpnets allow for the passive capture of salmon without the need for crowding or feed restriction. After having the net out for around 60 min the fish were collected and placed individually in buckets where they were given an overdose of Finquel MS-222 (Tricaine Methanesulfonate).

At each site visit, the goal was to sample 30 fish from each cage. However, due to difficulties capturing fish during some visits, the number of fish sampled per cage ranged from 20-30. In one instance, we were not able to gather any fish from one cage. Samplings were performed throughout one year from January to December 2021 for a total of thirteen samplings., Generally, there was a period of 2-3 weeks between each sampling, but during the summer there was a gap of 2 months between samplings due to covid restrictions.

For each fish, every louse was counted, and life stage recorded. The three early life stages, copepodite, chalimus I, and chalimus II, were not included in this trial because they are physically attached to the fish and are too small for consumption by cleaner fish. As such, the stages which are included in these data are pre-adult I, pre-adult II, and adults of both the male and female sex. After counting, all mobile lice were collected and placed in a seawater-filled petri dish for photographic examination.

Temperature and salinity were recorded at a central reference location down to a depth of 30 m using a conductivity, temperature and depth (CTD) recorder (SD204, saiv.no). Daylength here was calculated as the time between sunrise and sunset, excluding twilight, and was determined using online data (timeanddate.com).

2.2 Photography

After all lice were counted and collected from an entire cage the lice were prepared for photographing. To do this, lice were removed from the petri dish and placed on tissue paper to remove excess water which could distort the image. After drying, each louse was placed on a 240 lumen LED lightbox (Wafer 1, daylightcompany.com) to ensure even lighting from below. Several lice were then arranged according to stage, close together but not overlapping, next to a scale. An Olympus Tough TG-6 camera atop an opaque, black polyvinyl chloride box was placed over the scale and lice, such that all light is from the LED lightbox. In this way, the lighting conditions and camera position of all photographs were standardized and consistent

between samplings, regardless of ambient conditions. The exposure setting varied from -0.3 to +1.3 until it was set permanently set at +1.3 in August.

2.3 Measuring pigmentation

Photographic analyses were done in the image analysis software ImageJ which can be downloaded for free (https://imagej.nih.gov/ij/download.html). To calibrate the size of the image, a 1 cm scale was included in each photo. Because all photos were stored as .jpg files which compress brightness information to complement human vision, each image required linearization before analysis. Images were linearized by photographing six grey standards ranging from 1 to 99% reflectance (https://www.xrite.com/) and modelling the linearization curve using the mica toolbox plugin (https://www.empiricalimaging.com/download/micatoolbox/). The resultant linear model was then used to generate a linear normalized version of each photograph.

After linearization, quantitative measurement of pigmentation was obtained by measuring the amount of light passing through each louse in a representative, fixed-size circular area on the cephalothorax (Figure 4). Because size varies with louse life stage, specific diameters for the measurement area were chosen for each life stage: 50 pixels for adult females, 35 pixels for adult males and pre-adult II females, 25 pixels for pre-adult II males and pre-adult I females, and 20 pixels for pre-adult I males and *Caligus elongatus* (Figure 4). An example of pigmentation types can be seen in figure 5. A second circular area of the same size was measured next to each louse to provide a measurement of background lighting. To assess pigmentation the average grey value of every pixel within the measurement area was calculated (mean grey value-MGV). To standardize for possible differences within and between each image, the MGV of each louse was subtracted from the MGV of the paired background area, giving a difference in MGV for each individual louse compared to the background (dMGV). Less pigmented lice are more transparent and have lower dMGV values, while more pigmented lice absorb light and have higher dMGV values. These measurements were done on 4698 lice, where 2423 were adults, 1494 were pre-adult II, 667 were pre-adult I, and 114 were *C. elongatus*. *C. elongatus* were originally included to examine how they differ in pigmentation from *L. salmonis*, but because of their small number and variability between samplings, they were not included in the final analyses.

Figure 4: All three stages of L. salmonis with both sex showing the area where MGV was measured on the louse with the corresponding size of that area.

Figure 5: Two differently pigmented adult L. salmonis males with different dMGV. The male to the left has a dMGV of 40.78 and the one to the right has a dMGV of 25.56.

2.4 Statistical analyses

All data analyses were performed using R version 4.1.2 (R Core Team, 2021). Packages used were lattice (Sarkar, 2008), ggplot2 (Wickham, 2016), plotrix (Lemon, 2006), car (Fox & Weisberg, 2019), FSA (Ogle et al., 2022), and lme4 (Bates et al., 2015).

For all potential response and predictor variables, dotplots were used to check for outlying observations. No outliers were detected. Unless otherwise specified, summary data presented are mean ± SD.

A Levene's test was done to check for homogeneity of variance for the fixed effects chosen for this study by using the car package in R. This test shows whether the variance for a variable with two or more groups is equal or not. In biological studies where variation is expected to be high, it has been suggested that the assumption of homogeneity of variance can be accepted when $F \leq 20$ (Høisæter, 1989). As most tests revealed highly significant Levene's test (indicating non-homogeneity of the variances between groups) it was decided to apply non-parametric Kruskal-Wallis test (Chan & Walmsley, 1997) for all one-way combinations.

As Kruskal-Wallis test is not able to test for interactions between different parameters, a linear mixed effects models (lmer) were used to test for interaction between variables. A two-way mixed nested analysis of variance (ANOVA) was applied to check for the interaction between factors (Table 2) and their relevance to the response value dMGV. In this analysis the cages (random) were nested within the predictor (fixed) variables.

In cases of significant Kruskal-Wallis test a post-hoc test using Dunn test using the FSA package in R was performed to test for possible differences between experimental groups.

Data were visualized as boxplots to show how each variable changed through time. The boxplots used here follow the common standards. The box itself is the core of the plot and contains the 25th percentile (also called 1st quartile, Q1) representing 25 % of the data in one end and the 75th percentile (also called 3rd quartile, Q3) representing 75 % of the data in the other end. The line going through the box shows the median (also known as the 2nd quartile, Q2). Together, these are commonly known as the interquartile range (IQR). The lines on either end of the box show the minimum and maximum values, known as 'whiskers', and are calculated according to the formula: $Q1 - 1.5*IQR$ for the minimum value and $Q3 + 1.5*IQR$ for the maximum value. Outside the whiskers, outliers are presented as individual data points.

Table 2: The predictor variables used in statistical analyses

Predictor variables	Data type	Levels
Treatment	Categorical	2
Date	Categorical	7
Temperature	Continuous	5
Optimal depth	Continuous	6
Salinity	Continuous	3
Daylength	Continuous	7
Sex	Categorical	2
Stage	Categorical	3

3 Results

Initial examination of the data suggested that dMGV was higher in the earlier samplings. However, because different camera exposure settings were used in the early samplings, data were checked to examine the impact of exposure setting on measured dMGV. By treating exposure setting as a continuous variable and plotting it against dMGV, there was a clear trend that the exposure setting of the camera affected dMGV, even after linearization and background subtraction. Specifically, mean dMGV was inversely correlated with exposure setting, and decreased with increasing image brightness. Because the majority of lice were photographed on the highest exposure setting (Table 3), all data with exposure settings other than +1.3 were excluded.

C. elongatus lice were also excluded as there were too few observations. Seven sampling dates with a total of 3601 individual lice remained for analysis. The remaining sampling dates were 2021-01-05 and the period 2021-08-16 to 2021-11-22. The remaining sampling dates had a big range in the number of lice collected (Table 4). All potential outliers identified during data exploration were checked, but no issues were identified and none were excluded. Explanatory variables were chosen because of their potential relevance to dMGV and how they are a part of either the lice or a factor which may influence the lice as part of their habitat.

Table 3: Number of lice per camera exposure setting. All lice from exposure settings other than +1.3 were excluded from the final analyses.

Exposure setting	-0.3	+0.3	+0.7	+1.0	+1.3
Number of lice	368	256	335	46	3693

Table 4: Number of observed lice from each of the remaining sampling dates after the exclusion of exposure settings lower than +1.3 and C. elongatus.

Date	21-01-05	21-08-16	21-08-30	21-09-20	21-10-04	21-11-01	21-11-22
Number of lice	56	195	325	112	479	582	1852

A Kruskal-Wallis test was done for all variables used in this study as the Levene's test reported highly significant p-values, indicating that the variances between the groups were non-homogeneous. Even though the F-value for some variables were ≤ 20, Kruskal-Wallis non-

parametric test were applied in order to be consistent in statistical treatment of the experimental groups.

3.1 dMGV by treatment

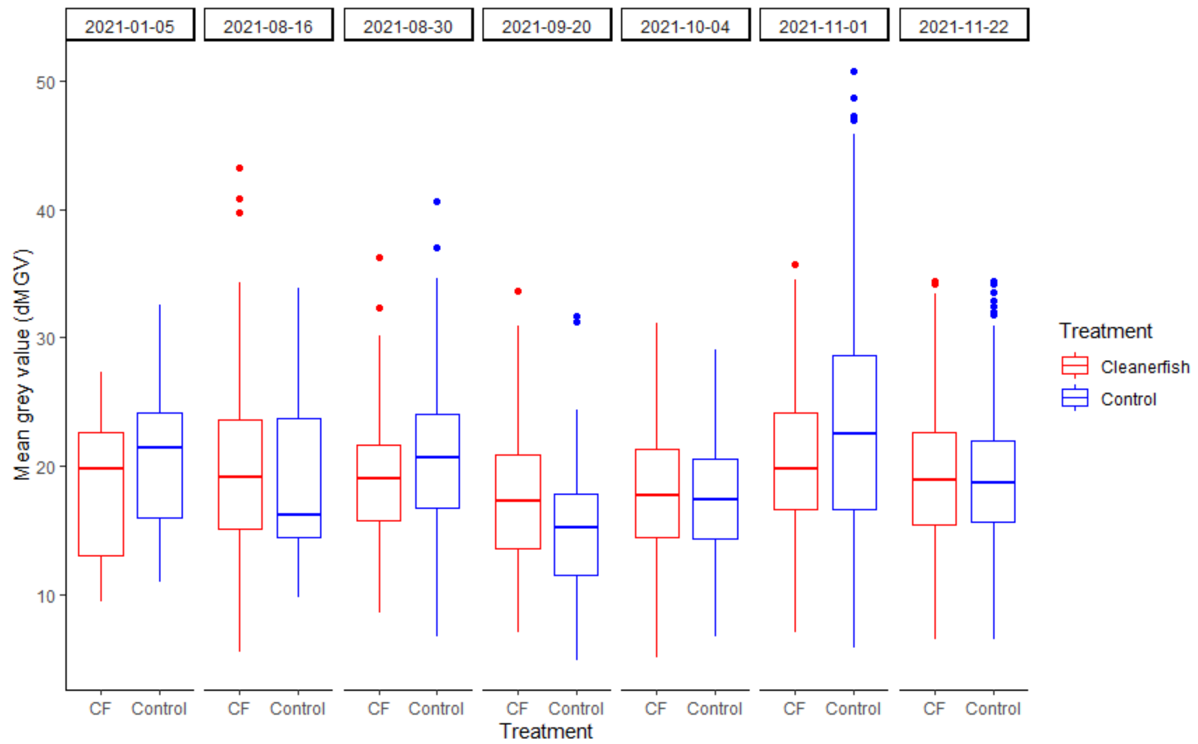

Figure 6: Boxplot showing the interaction between treatment and date. Y-axis shows mean grey value (dMGV) while the x-axis shows treatment. The treatment is compared at each date with cleaner fish cages in red and control cages in blue.

In all 7 samplings, there were only minor differences between cleaner fish (dMGV = 17.4 ± 5.6 to 20.4 ± 5.4) and control treatments (dMGV = 15.9 ± 6.2 to 23.0 ± 8.7). However, dMGV was more variable in control cages than those stocked with cleaner fish (Figure 6). There was no clear trend as the treatment with highest dMGV changed between samplings. In 3 out of 7 samplings, control cage had higher mean dMGV than cleaner fish. In 3 of them they were identical and for the last one cleaner fish cage had higher mean dMGV.

A Kruskal-Wallis test showed there was not a statistically significant difference between treatments ($H(1) = 0.020$, $p > 0.05$).

3.2 Seasonal changes of dMGV

3.2.1 Average Sea temperature

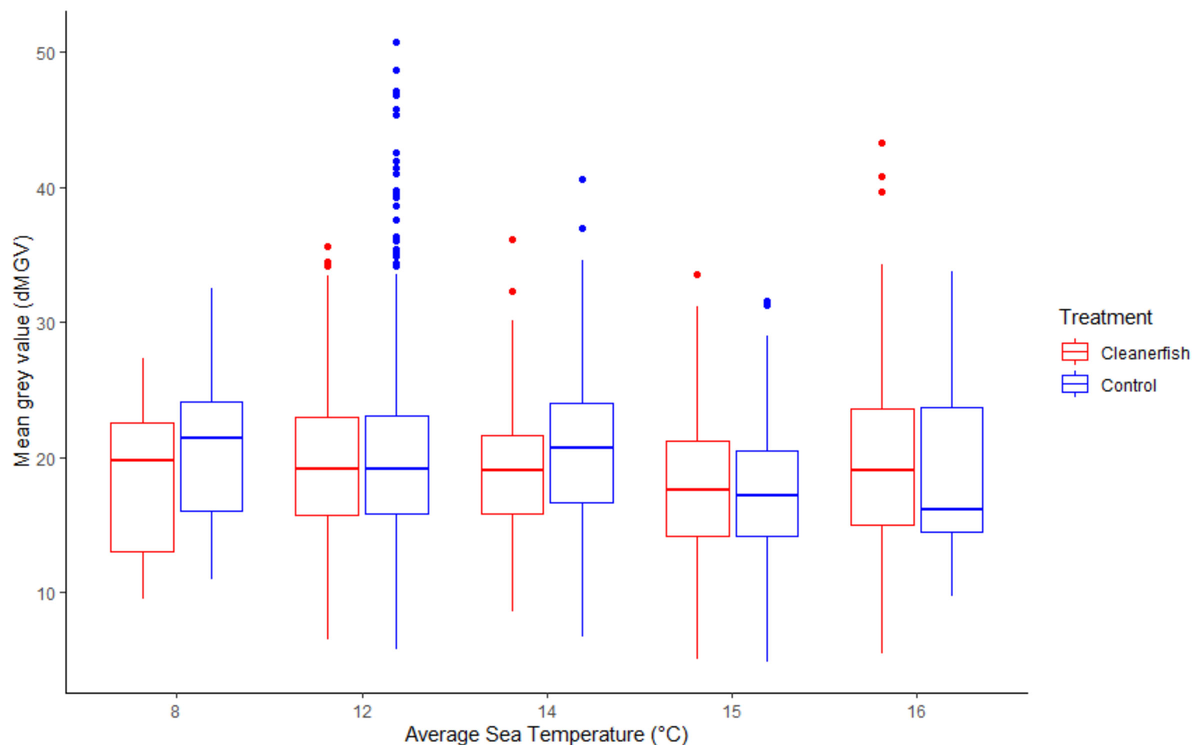

Figure 7: Boxplot showing the interaction between average sea temperature and treatment. Y-axis shows mean grey value (dMGV) while the x-axis shows the average sea temperature. The treatment is compared at each temperature with cleaner fish cages in red and control cages in blue.

The temperature from the samplings ranged from 5,3 to 17,4°C near the surface (upper 15 m) and 6,8 to 16,0°C in the deeper waters (lower 15 m). For all 5 temperatures, there were only minor differences between cleaner fish (dMGV = 17.8 ± 5.0 to 19.7 ± 6.5) and control treatments (dMGV = 17.3 ± 4.7 to 21.1 ± 6.4). However, dMGV was more variable in control cages than those stocked with cleaner fish (Figure 7). There was no clear trend as the treatment with highest dMGV changes between samplings. Control cages (dMGV = 21.10 ± 6.39) were more pigmented than cleaner fish (dMGV = 18.73 ± 5.24) at 8°C. At 14°C, control cages (dMGV = 20.52 ± 5.68) were darker than cleaner fish cages (dMGV = 19.18 ± 4.89). At temperatures at 15 and 16°C, dMGV stays about the same for both treatments. At 12°C in the control cages (dMGV = 19.89 ± 6.17).

A Kruskal-Wallis test showed there was a statistically significant difference between the temperatures (H (4) = 72.897, $p < 0.001$). Dunn's test between temperatures showed there was a difference between 4 out of 10 comparisons ($p < 0.001$) using Bonferroni correction.

A two-way ANOVA was performed to analyze the effect of the interaction between temperature and treatment on dMGV. The two-way ANOVA revealed that there was a significant interaction of temperature and treatment ($F(4, 66) = 2.593$, $p < 0.05$).

3.2.2 Optimal temperature-depth

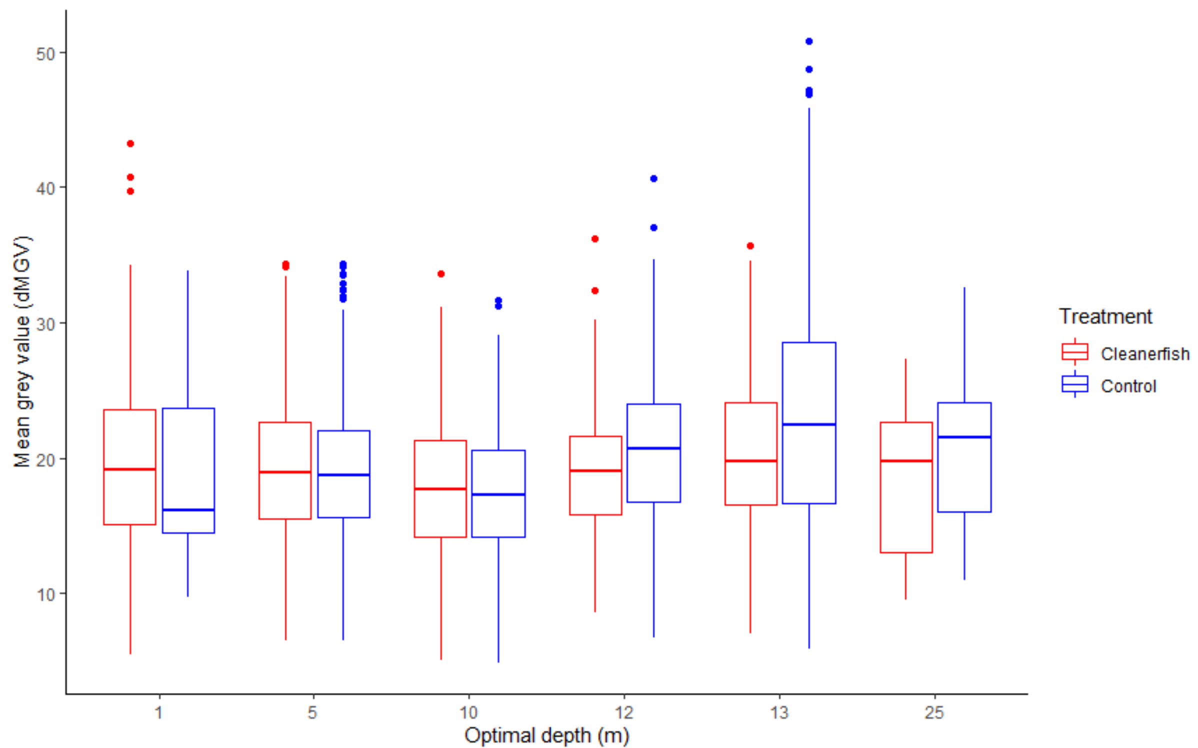

Figure 8: Boxplot showing the interaction between optimal depth and treatment. Y-axis shows mean grey value (dMGV) while the x-axis shows the optimal depth (m). The treatment is compared at each depth with cleaner fish cages in red and control cages in blue.

An optimal temperature-depth was chosen by looking at the temperature profile from the CTD and locating the shallowest depth where the temperature was closest to 14°C. The optimal depth ranged from 1 to 25 m. For all 6 depths, there were only minor differences between cleaner fish (dMGV = 17.8 ± 5.0 to 20.4 ± 5.4) and control treatments (dMGV = 17.3 ± 4.7 to 23.0 ± 8.7). However, dMGV was more variable in control cages than those stocked with cleaner fish (Figure 8). Control cages had higher dMGV than cleaner fish cages when optimal temperature was deeper than 10 m. During the first 10 m, the dMGV decreased with depth, and from 12 m it started to increase. The darkest louse being found at the preferred depth of 13 m for both control (dMGV = 23.00 ± 8.74) and cleaner fish (dMGV = 20.39 ± 5.37) cages. The least pigmented louse was found at the preferred depth of 10 m for both control (dMGV = 17.26 ± 4.71) and cleaner fish cages (dMGV = 17.76 ± 4.49).

A Kruskal-Wallis test showed there was a statistically significant difference between the optimal depth ($H (5) = 102.15$, $p < 0.001$). Dunn's test between depths showed that there was a statistically significant difference between 9 out of 15 comparisons ($p < 0.001$) using Bonferroni correction.

A two-way ANOVA was performed to analyze the effect of the interaction between optimal depth and treatment on dMGV. A two-way ANOVA revealed that there was a statistically significant interaction of optimal depth by temperature and treatment ($F (5, 132) = 7.133$, $p < 0.001$).

3.2.3 Hours of Daylight

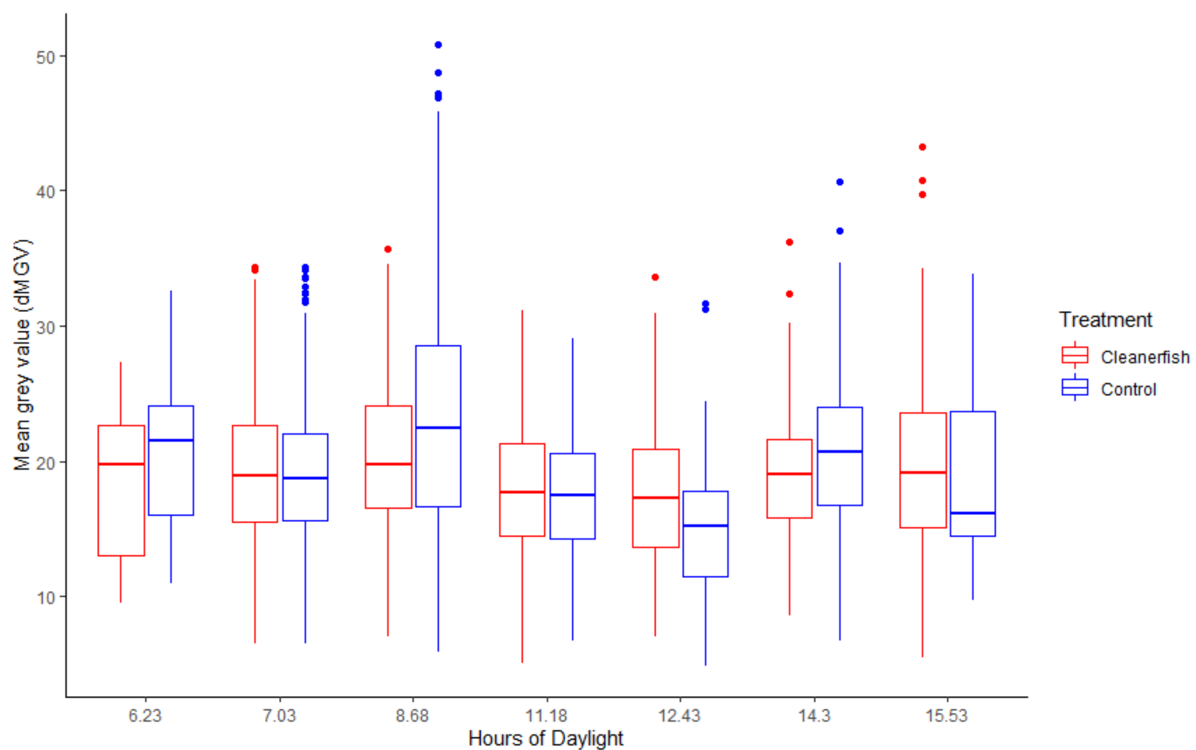

Figure 9: Boxplot showing the interaction between hours of dayligh and treatment. Y-axis shows mean grey value (dMGV) while the x-axis shows the length of days. The treatment is compared at each daylength with cleaner fish cages in red and control cages in blue.

The longest day of the sampling dates was at 16.08.21 and lasted 15.53 hours, and the shortest day was on 05.01.21 and lasted 6.23 hours. In all 7 samplings regarding daylength, there were only minor differences between cleaner fish (dMGV = 17.4 ± 5.6 to 20.4 ± 5.4) and control treatments (dMGV = 15.9 ± 6.2 to 23.0 ± 8.7). However, dMGV was more variable in control cages than those stocked with cleaner fish (Figure 9). There was no clear trend as the treatment with highest dMGV changes between samplings. The day with the highest measured dMGV had a daylength of 8.68 hours for both cleaner fish (dMGV = 20.39 ± 5.37) and control (dMGV = 23.00 ± 8.74) treatment. The lowest measured dMGV had a daylength of 12.43 hours for both

control (dMGV = 15.92 ± 6.22) and cleaner fish (dMGV = 17.44 ± 5.58) (Figure 9). In cages with cleaner fish, the dMGV is stable throughout the whole sampling period with a small decrease at 11.18 hours (dMGV = 17.89 ± 4.7).

A Kruskal-Wallis test showed there was a statistically significant difference between the daylength ($H(6) = 121.65$, $p < 0.001$). Dunn's test between daylength showed there was a statistically significant difference between 15 out of 21 comparisons ($p < 0.001$) using Bonferroni correction.

A two-way ANOVA was performed to analyze the effect of the interaction between daylight hours and treatment on dMGV. A two-way ANOVA revealed that there was a statistically significant interaction of daylight hours and treatment ($F(6, 65) = 6.294$, $p < 0.001$).

3.2.4 Salinity

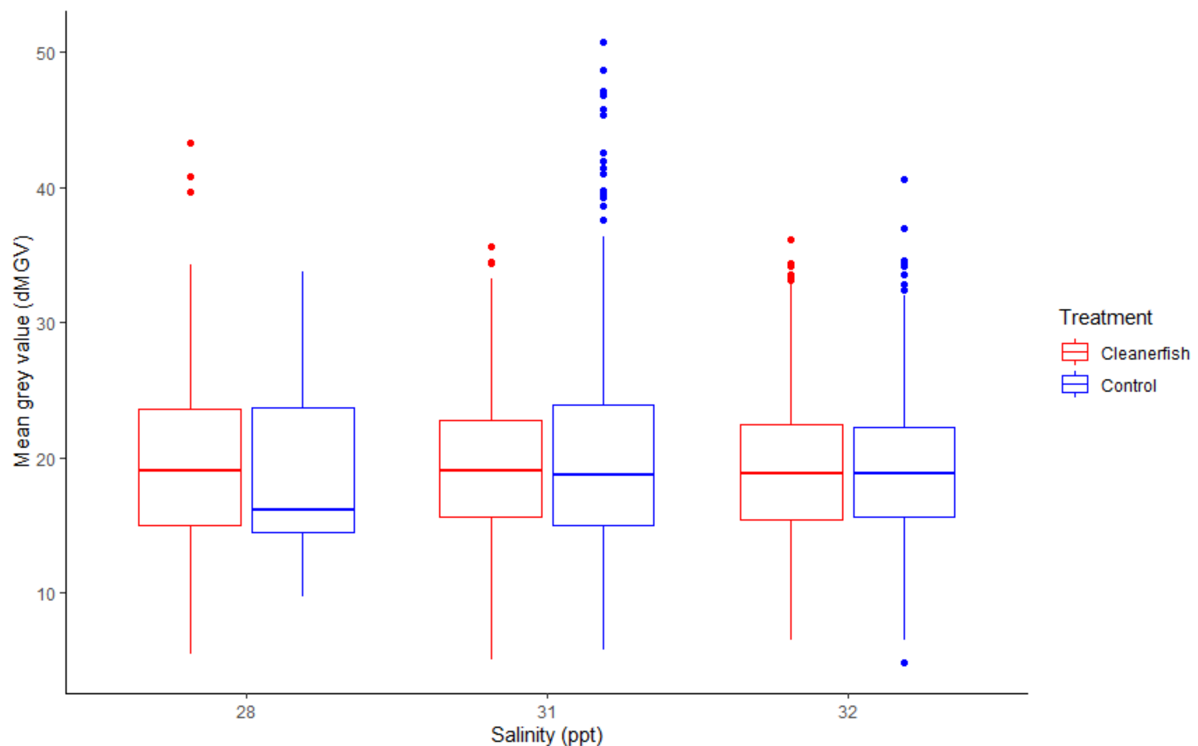

Figure 10: Boxplot showing the interaction between salinity and treatment. Y-axis shows mean grey value (dMGV) while the x-axis shows the salinity (ppt). The treatment is compared at each point of salinity with cleaner fish cages in red and control cages in blue

Salinity ranged between 20,6 to 31,9 ppt on the surface and 30,1 to 34,9 ppt in the deeper water levels. For all 3 salinities, there were only minor differences between cleaner fish (dMGV = 19.1 ± 5.1 to 19.7 ± 6.5) and control treatments (dMGV = 19.0 ± 6.6 to 20.2 ± 7.4). dMGV in both cleaner fish and control cages was similar for all salinity level (Figure 10). There was no clear trend as the treatment with highest dMGV changes between samplings. Control cages had

less pigmented lice at 28 ppt (dMGV = 19.02 ± 6.58) while cleaner fish cages had less pigmented lice at 32 ppt (dMGV = 19.13 ± 5.08).

A Kruskal-Willis test showed there was not a statistically significant difference between the salinity ($H(2) = 2.58$, $p > 0.05$).

A two-way ANOVA was performed to analyze the effect of the interaction between salinity and treatment on dMGV. A two-way ANOVA revealed that there was not a statistically significant interaction of daylight hours and treatment ($F(2, 3499) = 1.72$, $p > 0.05$).

Looking at the interaction between the seasonal changes and treatment, there was found a significant interaction. Cages with cleaner fish had a more stable change in dMGV than control cages had, which changes between each value of the predictor.

3.3 dMGV by stage and sex

3.3.1 dMGV by Stage

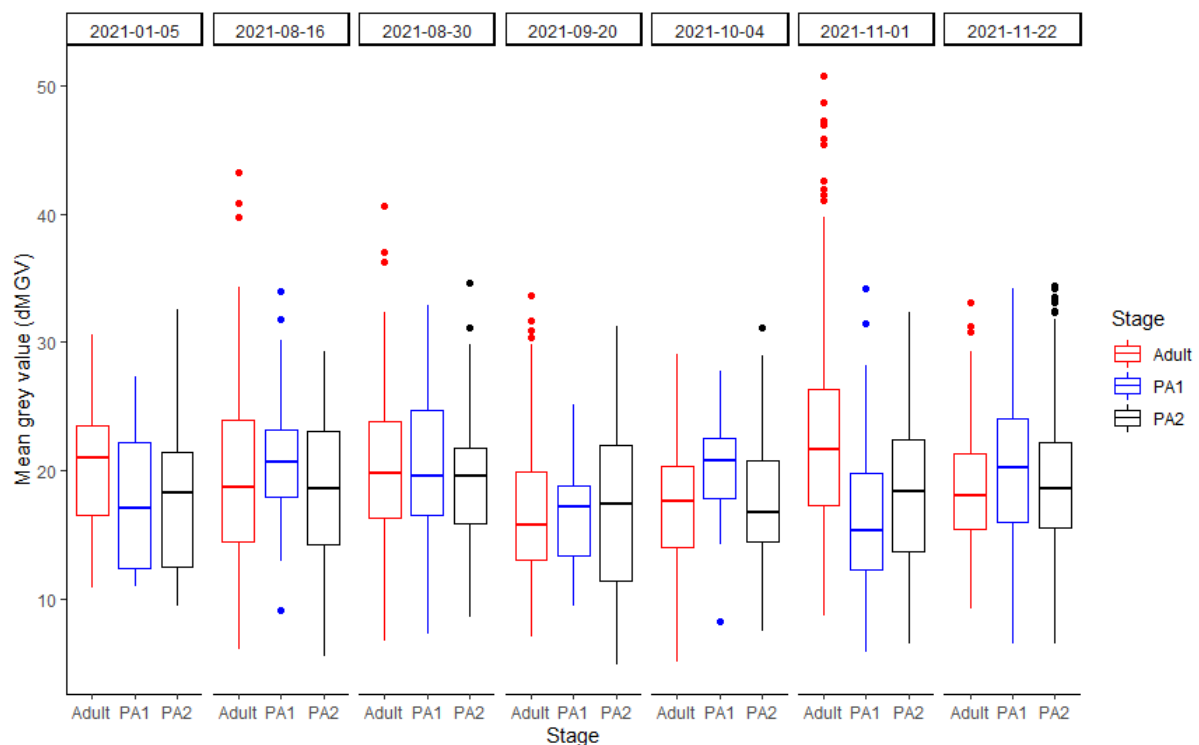

Figure 9: Boxplot showing the interaction between louse life-stage and date. Y-axis shows mean grey value (dMGV) while the x-axis shows the life-stage. The stages are compared at each date with adult in red, pre-adult I in blue, and pre-adult II in black.

2423 adult, 667 pre-adult I, and 1494 pre-adult II were all recorded individually, and each stage include both males and females. Pre-adult I, pre-adult II, and adult had a notably different dMGV throughout the sampling period. In all 7 samplings, there were small differences between the adult (dMGV = 17.3 ± 6.0 to 22.4 ± 7.1), pre-adult II (dMGV = 16.6 ± 6.2 to 19.3

± 4.8) and pre-adult I stage (dMGV = 16.6 ± 4.3 to 20.8 ± 5.6). However, dMGV was more variable for adult and pre-adult I than pre-adult II (Figure 11). There was no clear trend as the stage with highest dMGV changes between samplings.

A Kruskal-Wallis test showed there was a statistically significant difference between the stages (H (2) = 17.49, $p < 0.001$). Dunn's test between stages showed there was a statistically significant difference between adult and pre-adult II ($p < 0.05$), and pre-adult I and pre-adult II ($p < 0.001$) using Bonferroni correction.

A two-way ANOVA was performed to analyze the effect of the interaction between louse stage and date on dMGV. A two-way ANOVA revealed that there was a statistically significant interaction of louse stage and date ($F (12, 3578) = 6.41$, $p < 0.001$).

3.3.2 dMGV by Sex

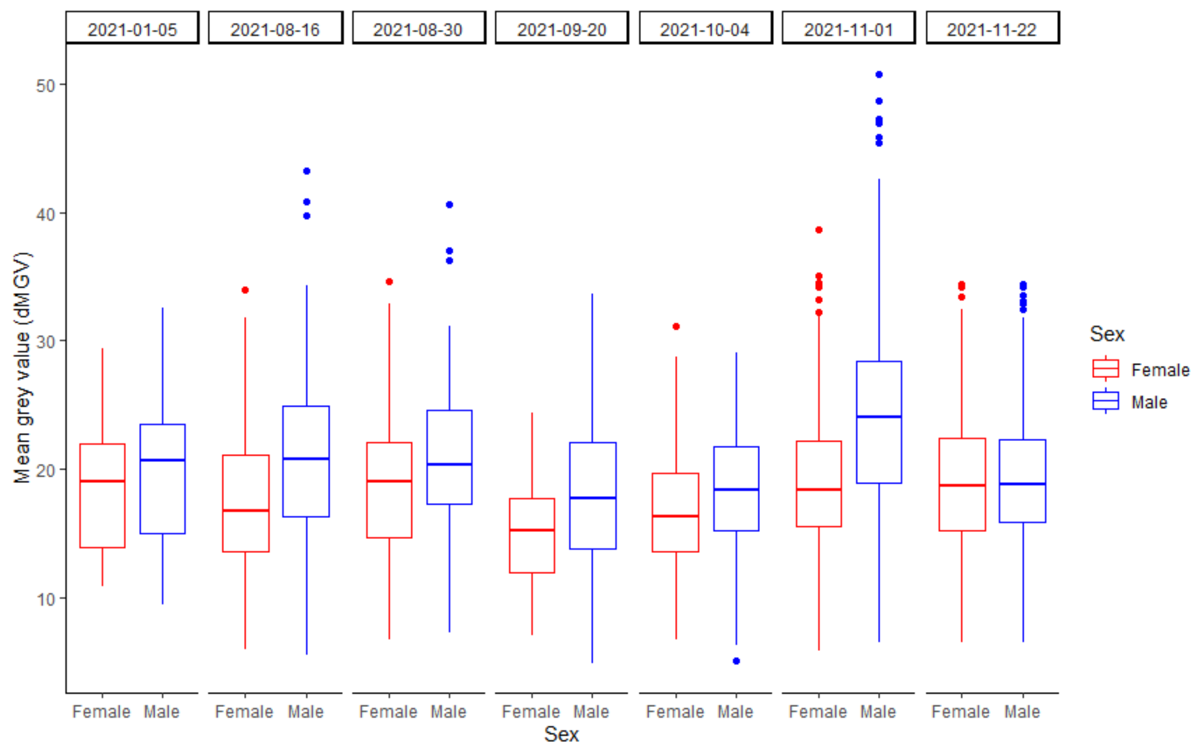

Figure 10: Boxplot showing the interaction between sex and date. Y-axis shows mean grey value (dMGV) while the x-axis shows the sex. The treatment is compared at each date with females in red and males in blue.

1845 males and 1756 females were recorded for all three stages. In all 7 samplings, there were clear differences between females (dMGV = 14.9 ± 4.3 to 19.1 ± 5.5) and males (dMGV = 18.1 ± 6.1 to 24.1 ± 7.9). dMGV varied about the same for both females and males (Figure 12). There was a clear trend as males were darker than females for most of the samplings except the last one. Males was darkest at the sampling at 2021-11-01 (dMGV = 24.09 ± 7.88), which was

where the males were darkest pigmented. Both female (dMGV = 14.93 ± 4.32) and male (dMGV = 18.11 ± 6.14) lice were lightest pigmented at the sampling during 2021-09-20.

A Kruskal-Wallis test showed there was a statistically significant difference between the sex ($H(1) = 64.35$, $p < 0.001$).

A two-way ANOVA was performed to analyze the effect of the interaction between sex and date on dMGV. A two-way ANOVA revealed that there was a statistically significant interaction of sex and date ($F(6, 3583) = 17.9$, $p < 0.001$).

4 Discussion

Little is known about pigmentation in salmon lice. Unvalidated reports suggest that in regions where cleaner fish are frequently used lice appear less pigmented, and concern exists that as a result lice may be less visible to cleaner fish (Soltveit, 2018). However, no previous scientific studies have examined this hypothesis. In the one study which looked at factors affecting pigmentation in salmon lice, Hamre et al. (2021) found evidence for both genetic and environmental control of pigmentation, demonstrating the potential for both plastic and adaptive responses to selection. This study took the next logical step and examines, (a) how pigmentation varies among lice on salmon in sea cages throughout a 12-month period, and (b) if the selective pressure from cleaner fish affects louse pigmentation

4.1 Treatment

Using cleaner fish did not result in less pigmented lice, as stated in hypothesis $H0_a$. However, even though there was no significant difference in mean degree of pigmentation between the two treatments, cleaner fish cages did have more stable pigmentation than control cages (Figure 6). These results are in contrast to previous work, where (a) *Daphnia* were observed to become less pigmented when under selective pressure from predators, even in high UV environments (Scoville & Pfrender, 2010), and (b) unvalidated reports that lice become more transparent as a result of cleaner fish use.

One possible explanation for the lack of change in average lice pigmentation is that there may have been insufficient selection pressure exerted by the cleaner fish, for which there could be several reasons. First, eyesight may not be the only sense used by cleaner fish to detect prey. Lumpfish use olfaction to detect potential predators (Staven et al., 2021), and may also be used for foraging. If olfaction is used by cleaner fish for foraging, this would reduce the possibility for selection on pigmentation by cleaner fish.

Second, both species of cleaner fish used in this experiment are opportunistic feeders (Brooker et al., 2018). Although previous research has shown that at 8% density cleaner fish can reduce the number of salmon lice found within a cage to equal or lower than previously recorded counts (Imsland et al., 2018), lumpfish also eat crustaceans, salmon feed, and hydrozoans when used in salmon cages (Imsland et al., 2015). According to Imsland et al. (2015), only 33 - 38% of lumpfish had ingested sea lice after 77 days in salmon cages. Therefore, even if cleaner fish are entirely reliant on eyesight to locate prey, selective pressure on louse pigmentation could still be weak if cleaner fish are primarily feeding on alternative food sources. For example, although

both ballan wrasse and lumpfish have been observed to eat salmon lice, the swimming speed of Atlantic salmon is higher than that of both cleaner fish species and may be a reason why they do not eat enough lice to change pigmentation (Hvas et al., 2021). In addition, the cages used in this study were snorkel cages, which may also affect cleaner fish performance. Snorkel cages work by uncoupling salmon from salmon louse larvae while providing access to surface air (Geitung et al., 2019). Ballan wrasse is found to be at deeper, warmer, and more saline water than lumpfish which is found at shallower, cooler, and more brackish water (Geitung et al., 2020). Ballan wrasse spends most of their day at 15 m or deeper (Leclercq et al., 2018). Lumpfish spends most of their day at 10 m or above and used hides extensively (Leclercq et al., 2018). Different depth distribution for salmon and cleaner fish leads to less interaction between them, and fewer lice feeding (Gentry et al., 2020).

In 3 out of 7 sampling events, mean dMGV was higher in the control cages than those stocked with cleaner fish. Only one of the samplings had cleaner fish cages with higher mean dMGV. This may indicate that, although selection strength was not strong enough to reduce the mean dMGV between treatments, the dark phenotype of the lice was removed by the cleaner fish.

4.2 Temperature

Lice pigmentation varied with temperature, but with no apparent pattern. At most temperatures measured pigmentation did not differ between treatments, except for 8°C and 14°C where control cages were darker pigmented than cages with cleaner fish (Figure 7). The differences in pigmentation through different temperature supports the alternative hypothesis $H1_b$.

Temperature may have both direct and indirect effects on lice pigmentation. The water temperature's primary function for salmon lice is to dictate the growth rate of the lice with lower temperatures making their metabolism slow down and higher temperature speeds it up (Hamre et al., 2019). As lice body size is correlated with pigmentation, with larger individuals being darker (Hamre et al., 2021), and higher temperatures make them grow faster (Samsing et al., 2016), a co-selection may occur for size and color (Coates et al., 2021).

Temperature affects the behavior of salmon following their depth preference. Temperature is a key environmental factor influencing salmon swimming depth and density. The optimal temperature for growth of post-smolt Atlantic salmon is 13-16C (Handeland et al., 2008), they will follow the temperature and either go deeper or closer to the surface depending on where the temperature closely resembles their preference. Seasonal changes in the vertical distribution

have concurred with temperature shifts, suggesting that salmon prefer the highest available temperature or avoid colder temperatures (Oppedal et al., 2001).

In this trial salmon may then have been swimming closer to the surface, thereby exposing lice to more UV radiation than at other temperatures. Because salmon avoid thermal extremes it is possible that salmon was swimming closer to the surface during this period, exposing lice to more UV radiation. At higher temperatures, cleaner fish become more active giving them more opportunities to eat salmon louse. This gives the possibility for louse dMGV to become lower with increased activity. For both treatments, the pigmentation stays at a stable level as there was little variation between temperatures. Even if the temperature does not affect pigmentation directly, it may affect the lice by changing their position on the host or changing the preferred depth of the host. At 16°C both treatments are at their highest dMGV (Table 7.

4.3 Optimal depth

The depth of the preferred temperature of the salmon, here termed 'optimal depth', can vary with fish conditions, but was here defined as the nearest temperature to 14°C (Handeland et al., 2008). As with temperature, treatments with cleaner fish have a stable pigmentation with minor differences while control cages have more variation in the measured dMGV. Lice are attached to salmon, which means that when fish swim close to the surface, lice are more exposed to UV radiation than when they swim deeper. Despite this, in this trial lice were found to be lighter pigmented when optimal depth was within the upper 10 m, and darker pigmented when the optimal depth was deeper than 10 m. The darkest lice were found at a depth of 13 m. The differences in pigmentation at different depths supports the alternative hypothesis $H1_c$.

A possible reason why lice at the upper layer were lighter pigmented than in deeper layers may be because the salmon lice may have not been at this depth prior to sampling. Even if it were the optimal depth for the salmon based on water temperature, salmon moves freely through the water column and may be at a depth other than the optimal depth. Salmon usually avoid water warmer than 18°C and colder than 11°C (Johansson et al., 2009). All salmon collected for analysis were collected at the surface as it is required for them to jump into the jumpnets for collecting. This means it is unknown if they stayed at the depth and/or for how long they have stayed this depth. Also, because the preferred temperature is measured on the sampling date, it does not mean that the optimal temperature was at this depth the days between the samplings. The temperature may change from day to day and may also change throughout the day. Vertical mixing can completely change the temperature profile of the water in the matter of hours, and

this happens frequently at the farm location during autumn. This way, the temperature may change quickly over short periods throughout the depth.

4.4 Daylight hours

Daylight is defined as the period between sunrise and sunset and was chosen because this period is when UV radiation is strongest. There was no consistent increase in dMGV with increasing hours of daylight for either treatment. However, there was a decrease in dMGV in control cages as daylength increased from 8.7 hours to 12.4 hours (Figure 9). dMGV started to increase again for the last couple of days, increasing with the increase in daylength. The change in dMGV supports the alternative hypothesis $H1_d$. Between the two treatments, lice in cleaner fish cages had a more stable dMGV than lice in control cages for all values of daylength measured. A trade-off between UV radiation and being preyed upon causes the lice to have a pigmentation to avoid both, causing a stable pigmentation with little variation. As with *Daphnia*, who remained pale across UV because of predators (Scoville & Pfrender, 2010), a similar result of cleaner fish selection may affect salmon lice to have a stable pigmentation (Ehrenreich & Pfennig, 2016). However, control cages have potentially more room for phenotypic plasticity as they do not have any selection pressure caused by cleaner fish. This may also explain the larger variation in dMGV.

It has been shown that lice pigmentation is strongly influenced by environmental conditions, most likely light (Hamre et al., 2021). From observations between outdoor and indoor reared lice, the outdoor reared lice are found to be darker pigmented. Pigmentation is costly and slows growth for other free-living crustaceans species such as *Daphnia* (Scoville & Pfrender, 2010), and this may be the case for salmon lice as well. As this experiment was done on a salmon farm where the conditions are different from controlled environments, other factors may contribute to the pattern where dMGV do not show any consistent trend with increasing daylength.

The absorption of UV radiation is dependent on how clear the water is, but at depths of 50-70 m, there is still ~10% of the surface UVA when the sun is at its highest point (Lee et al., 2013). The penetration of UV radiation is dependent on the dissolved and particulate as well as concentration of phytoplankton (Häder, 1997). The preferred depth of the salmon may change with the increasing daylength with the changes in the water temperature as their preferred temperature is at 14°C (Handeland et al., 2008). This may cause the salmon to swim deeper in the water as it may be too warm during the summer period, giving less UV radiation than the day length would give if the salmon stayed close to the surface.

4.5 Salinity

There was no overall difference in pigmentation between the different treatments in relation to salinity. Both treatments had the same dMGV for all measured salinities of 28, 31 and 32 ppt so that the null hypothesis for salinity. $H0_e$, is not rejected.

Salinity does not necessarily affect how pigmented a louse may be on its own, but it may change the depth or location of the lice, causing other factors to play a role with salinity in terms of pigmentation. Copepodites actively avoid lower salinity waters (Crosbie et al., 2019), as salinity as high as 29 ppt limits the growth of copepodites (Powell et al., 2015). The salinity is more linked to survival as lice do not tolerate lower salinity, with different lice stages tolerating different levels of salinity (Andrews & Horsberg, 2020). The individuals used in this study, tolerated salinity around the values in the experiment, but it could limit future generations. This in turn may limit which individual survives and may change how the pigmentation changes in the population. The salmon lice life stages studied in this experiment have a higher tolerance to lower salinity than copepodites (Andrews & Horsberg, 2020). As the lower limit of optimal salinity of adult females is 16 ppt at 14-15°C (Ljungfeldt et al., 2017), it is believed that the lower limit of pre-adult and pre-adult II may be lower than 28 ppt which was the lowest average ppt in this study. The fact that adults have a higher tolerance for lower salinity than the earlier stages may be overlooked by aiming freshwater treatments toward the other stages (Andrews & Horsberg, 2020).

4.6 Stage

dMGV for the different stages changed from each sampling with pre-adult II being the most stable of the three stages used in this experiment. As adult and pre-adult I had more changes in dMGV than pre-adult II, pre-adult II had the most stable dMGV through the sampling period. There was no clear trend in dMGV for either of the stages from each sampling date. Adults and pre-adult I alternated in being the most pigmented stage during the samplings when they were not equal in pigmentation. As there were different dMGV by stage the null hypothesis for lice stage, $H0_f$, is rejected in favor of the alternative hypothesis $H1_f$. The size differences may play a role in the pigmentation differences between the stages as larger lice are darker (Hamre et al., 2021). This explains why adults were darker pigmented in several of the samplings, but it does not explain why pre-adult I was darker during other samplings. There were, however, differences in pigmentation between the stages, and salmon lice may possibly change their pigmentation during molting, making them either lighter or darker pigmented.

The placement of the lice on the host may be a reason for the differences in dMGV between them. A study by Bui et al. (2020) shows where the different stages of salmon lice prefer to be on their host. However, this study does not distinguish between pre-adult I and pre-adult II. Pre-adults is mainly found on the dorsal part of the host (57%) which is also where adult males are primarily found (Bui et al., 2020). In contrast adult females were primarily found in the head region of the fish (Bui et al., 2020). As these stages are primarily found on the dorsal side of the fish, they are all bound to get similar amount of sunlight which may help to explain the small differences in dMGV between lice stages seen in this study.

4.7 Sex

Possible differences in lice pigmentation between males and females was investigated. Male salmon lice were on average darker than females throughout the samplings except for the last sampling where they were equally pigmented, and this supports the alternative hypothesis $H1_g$. This difference between sexes accounts for all life stages recorded in this experiment. In line with present findings Hamre et al. (2021) found that male lice were darker than females.

As male lice are smaller than females, approximately half the size of a female (Hamre et al., 2009), which may make them less likely to be seen, and eaten, by cleaner fish. The size differences may also affect how the pigmentation cells are spread in their body. The size difference is strongly correlated to pigmentation where larger individuals are darker pigmented than smaller individuals (Hamre et al., 2021). This is opposite to findings of this study where the smaller-sized males were found to be darker than the larger-sized females.

Another point to take into consideration is the placement of the different sexes on the host. Adult males are mostly found on the dorsal part of the fish where the skin is darker than the rest of the fish (Bui et al., 2020). Most of the adult females are found on the head of the fish (Bui et al., 2020), and depending on the placement, may be found on either a darker or lighter spot. The study by Bui et al. (2020) did not separate the sexes at the pre-adult stages. As males are mostly found on the dorsal side of the fish causes them to be more exposed to UV radiation than the females who are found more on the head of the fish. As pigmentation may help camouflage the lice, the pigmentation may reflect the colorization of their placement on the host. The placement on the host, whether it was on the ventral side or dorsal side, affected the pigmentation of the louse (Hamre et al., 2021). Individuals found on the ventral side of the host were lighter pigmented than those found on the dorsal side for both sexes (Hamre et al., 2021).

4.8 Present findings in a larger context

Present results show how different factors are affecting the pigmentation of salmon lice. Cleaner fish stabilized salmon lice pigmentation (measured as dMGV) for all environmental changes investigated in this study, while there was more variation in salmon lice pigmentation in the control cages.

Two or more environmental factors may together affect the pigmentation of salmon lice by either affecting the lice behavior or affecting how the host behaves. These variables may also work in antagonistic ways where they negatively affect the other, causing conditions that might have increased pigmentation to affect it less than it could have. Hamre et al. (2021) found that environmental conditions strongly influence the pigmentation of salmon lice which is similar to what was found in the present study. In this study cleaner fish stabilized the salmon lice pigmentation across different temperatures, depths and salinities. In contrast, the control cages had more variation between pigmentation values for these environmental factors.

Salmon lice pigmentation may change as the factors are influencing each other by altering lice behavior and growth. These changes cause the pigmentation to vary with the stage and sex of the lice. Sex is the only variable where a clear trend was found in the present study, where salmon lice males were darker pigmented than females.

5 Conclusion

Little variation in salmon lice pigmentation (measured as dMGV) was found between the different treatments. No clear seasonal effect in salmon lice pigmentation was found.

Presence of cleaner fish in salmon cages stabilized the salmon lice pigmentation compared to sea cages without cleaner fish present. Presence of cleaner fish did not lead to more transparent salmon lice.

Control cages had higher dMGV than cages stocked with cleaner fish at 8 and 14°C. Both treatments have the same dMGV in the top 10 m of the sea pens. At depths greater than 10 m, the salmon lice in control cages were darker than lice in cages stocked with cleaner fish. The darkest lice were found at 13 m depth. Salmon lice did not have a specific increase in dMGV with increasing daylength for both treatments. Larger variation in dMGV was seen for salmon lice in the control cages compared to the cages stocked with cleaner fish dMGV was stable for all values of salinity for both treatments indicating little effect of salinity on salmon lice pigmentation.

All three salmon lice stages showed variation in dMGV through the sampling period. Pre-adult I was more different than the other two stages through the samplings. With the exception of the final sampling, salmon lice males were found to be darker than female lice.

5.1 Future research

For future research on lice pigmentation at commercial fish farms it should be aiming at exploring more specifically how cleaner fish affect the pigmentation of salmon lice. The samplings should be done at more regular intervals than was possible in this study. The samplings should also increase to include more fish farms to see if the location of the farms may affect the salmon lice pigmentation. Environmental effects may be different depending on location. The farms should be spread along the Norwegian coast where the environment may differ to see if the same effects affect the lice pigmentation. Future research should focus on how the pigmentation cells found in salmon lice differ from each other and how they change to make the lice appear darker or lighter. Also, comparing salmon lice to *Caligus elongatus* would be interesting to see if pigmentation of these species are affected differently.

A multiple regression analysis would allow for a more integrated perspective on how each variable influence pigmentation could provide greater insight. Such an analysis would be able to account for antagonistic relationships between multiple variables, and therefore increased ability to detect patterns which may have been masked in the current analysis.

More research on how cleaner fish find food, and what sensory system they use to find it, will increase our understanding on how they can find lice.

Hopefully, this experiment gives inspiration and guidance for further research in this field.

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Appendix A – Limitations of the study

As salinity was measured as a mean salinity over the 30 m may be the reason no specific result is shown. A different measurement may have given better results as they may have looked at other parts of the spread of salinity. Using a mean as measurement causes outliers to affect the mean more against the result and may give a false result regarding the other values. Other measurements could be more informative than the one used here. A possibility is to look at the median salinity to see what the salinity the median is through the depth.

The range between each point, 28 to 32 ppt, may be too narrow to cause any specific differences in pigmentation between each gradient of salinity, or have any effect on the louse used in this experiment. The value of salinity is the average for the whole cage, all 30 m, and as such does not fully explain the salinity for all the water layers. However, as it is the Atlantic salmon swim through the water masses and thus decides what salinity layer the attached lice is staying at. They may drop off hosts at lower salinities and move towards potential hosts at higher salinity.

As the results did not reveal much difference between the treatments, a possibility is that the delousing used at farm could change the pigmentation adaptation of the lice. Commercially used delousing methods do not discriminate on how pigmented the lice is so there is limited possibility for adaptation of lice towards being lighter.

Homogeneity of variances was tested using the standard Levene's test. Usage of parametric (GLM) test assumes that the variances between experimental groups are equal. If that is not the case alternative tests should be considered. In biological studies where variation is expected to be high, it has been suggested that the assumption of homogeneity of variance can be accepted when $F \leq 20$ (Høisæter, 1989). However, most of the Levene's test in this study revealed $F > 20$. So further one-way testing was conducted using a non-parametric Kruskal-Wallis test. For sake of consistency the Kruskal-Wallis test was applied for all one-way testing.

There are certain assumptions in the Kruskal-Wallis test (Chan & Walmsley, 1997).

- It is assumed that the observations in the data set are independent of each other.
- It is assumed that the distribution of the population should not be necessarily normal and the variances should not be necessarily equal.
- It is assumed that the observations must be drawn from the population by the process of random sampling.

All these assumptions were fulfilled in the present study allowing for the use, and interpretation of, Kruskal-Wallis for the present data set

Appendix B – Alternative figures

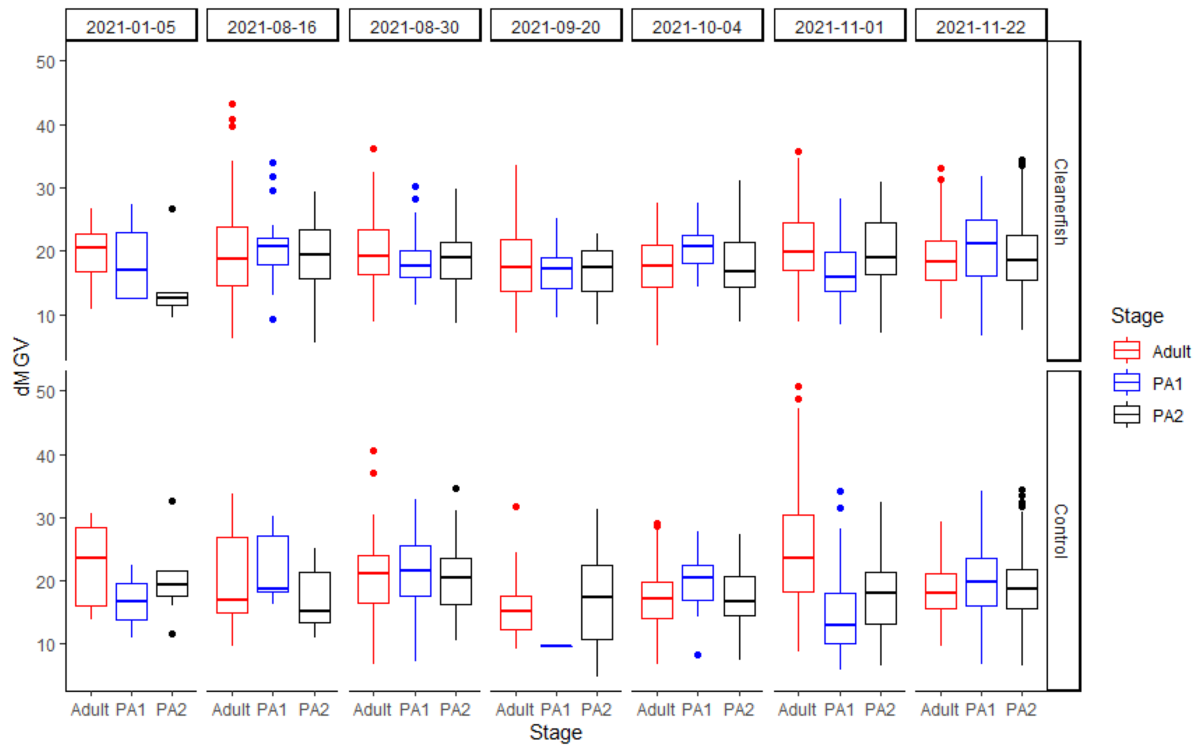

Figure B.1: Alternative figure for the different stages distributed on each treatment. This show the average dMGV by each stage for both treatments.

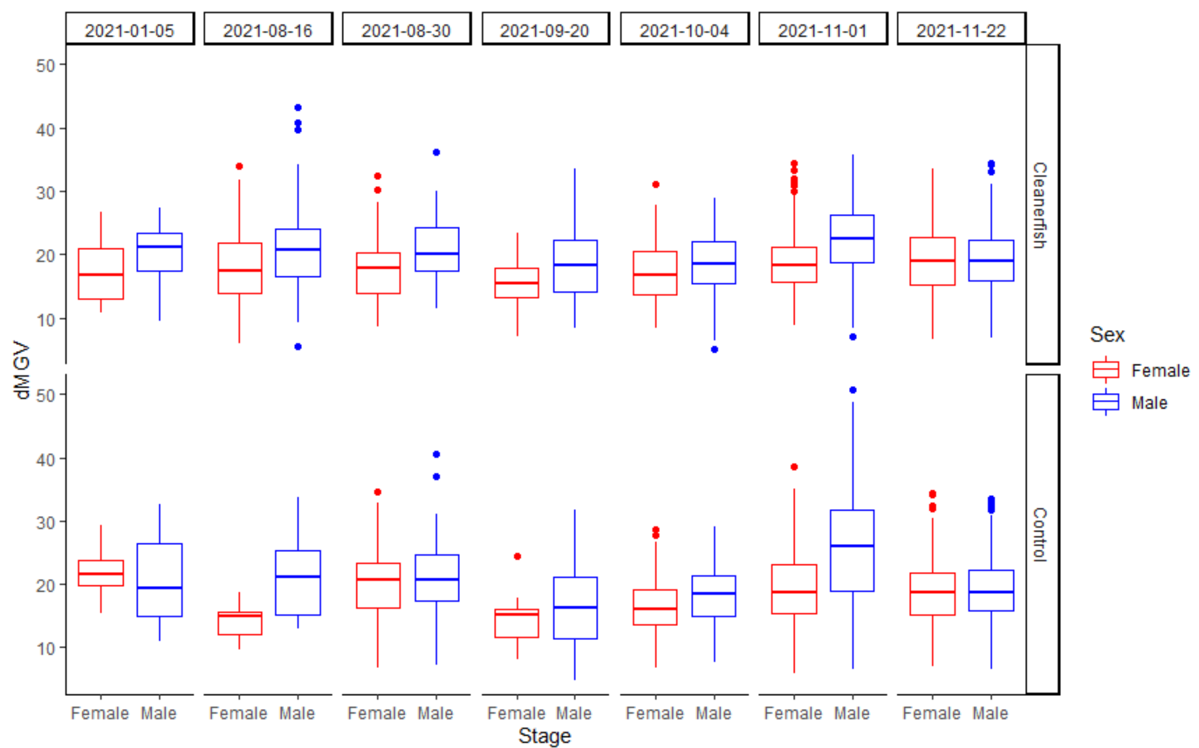

Figure B.2: Alternative figure showing the difference in dMGV between the sexes for each treatment.

Appendix C – statistics

r-code used in the study

```
#Import the SWIM data

Fish <- read.table(file = "CAC18-SWIM-Lice(1).txt",header = TRUE,dec = ",")

dim(Fish)

names(Fish)

str(Fish)


#Import the Lice data

Lice <- read.table(file = "CAC18-dmgv.txt",header = TRUE,dec = ".")

dim(Lice)

names(Lice)

str(Lice)


#Import the environmental data

env <- read.table(file = "CAC18-CTD.txt",header = TRUE,dec = ".")

dim(env)

names(env)

str(env)


#Import the daylight hours data

day <- read.table(file = "CAC18-Day.txt",header = TRUE,dec = ",")

dim(day)

names(day)

str(day)


#combine Lice + Environmental dataframes

d <- merge(Lice, env, by="Date")

write.csv(d, file = "CAC18LiceEnv.csv")
```

```
#combine LiceEnv + Fish dataframe
d1 <- merge(d, Fish, by=c("Date", "Treatment"))
write.csv(d1, file = "CAC18LiceEnvFish.csv")

#combine LiceEnvFish + day dataframe
d1 <- merge(d1, day, by="Date")
write.csv(d1, file = "CAC18LiceEnvFishDay.csv")

head(d1)
str(d1)
names(d1)

# Remove rows without dMGV data
d1 <- d1[complete.cases(d1$dMGV),]

# Remove columns with extraneous data
d1$Background <- NULL
d1$Lice <- NULL
d1$Hour <- NULL
d1$Minutes <- NULL
d1$MinProp <- NULL
d1$Sunrise <- NULL
d1$Sunset <- NULL

str(d1)

#Load packages
library(lattice)
library(ggplot2)
library(plotrix)
library(lme4)
```

```
library(car)
library(FSA)

# Housekeeping - make sure all categorical covariates are coded as factors
d1$Sex <- factor(d1$Sex)
d1$Stage <- factor(d1$Stage)
d1$Cage <- factor(d1$Cage)
d1$Treatment <- factor(d1$Treatment)
d1$DaylightHours <- factor(d1$DaylightHours)
d1$Temp <- factor(d1$Temp)
d1$Salinity <- factor(d1$Salinity)
d1$OptimalTempDepth <- factor(d1$OptimalTempDepth)

# Day in year
d1$Day365 <- strftime(d1$Date, format = "%j")
d1$Day365 <- as.numeric(d1$Day365)

# Check data formatting
str(d1)
str

# Subset to only data with exposure setting +1.3
d2 <- subset(d1, d1$fExposure == 5)
write.csv(d2, file = "CAC18LiceEnvFishDayExp.csv")
str(d2)
head(d2)

#Data exploration
#A Outliers in Y / Outliers in X
#B Collinearity X
#C Relationships Y vs X
```

```
#D Spatial/temporal aspects of sampling design (spatial not a problem, temporal we'll have to deal with)

#E Interactions (is the quality of the data good enough to include them?)

#F Zero inflation Y (not a problem here)

#G Are categorical covariates balanced?


# X. Basics


tapply(d1$dMGV, d1$Treatment, mean)

tapply(d1$dMGV, d1$Treatment, std.error)

boxplot(dMGV ~ Treatment, data = d1)


tapply(d1$dMGV, d1$Sex, mean)

tapply(d1$dMGV, d1$Sex, std.error)

boxplot(dMGV ~ Sex, data = d1)


tapply(d1$dMGV, d1$Stage, mean)

tapply(d1$dMGV, d1$Stage, std.error)

boxplot(dMGV ~ Stage, data = d1)


tapply(d1$dMGV, d1$Date, mean)

tapply(d1$dMGV, d1$Date, std.error)

boxplot(dMGV ~ Date, data = d1)


tapply(d1$dMGV, d1$fExposure, mean)

tapply(d1$dMGV, d1$fExposure, std.error)

boxplot(dMGV ~ fExposure, data = d1)


# A. Outliers in Y

MyVar <- c("dMGV")

Mydotplot(d1[,MyVar])
```

```
# check Outliers in dMGV
par(mfrow = c(1, 1))
plot(x = d1$Day365,
   y = d1$dMGV)
identify(x = d1$Day365,
       y = d1$dMGV)

# nothing of great concern, but let's check out the darkest individuals anyway
d1[1067, ]
d1[1092, ]
d1[1100, ]
d1[1362, ]
d1[2360, ]

# ALL look legitimate, can't see any reason to exclude.
# May have to remove if they skew the results because don't
# have enough data within that high range to support analysis.

# Outliers in X
# Treatment : Control or CF
MyVar <- c("Salinity", "Temp", "DO", "DOmg", "Exposure", "MobileLice", "Caligus", "OptimalTempDepth")
Mydotplot(d1[,MyVar])

# Check for homogenity in all covariates.
leveneTest(dMGV~Treatment, data=d3, center=mean)
leveneTest(dMGV~Temp, data=d3, center=mean)
leveneTest(dMGV~OptimalTempDepth, data=d3, center=mean)
leveneTest(dMGV~DaylightHours, data=d3, center=mean)
leveneTest(dMGV~Salinity, data=d3, center=mean)
```

```
leveneTest(dMGV~Sex, data=d3, center=mean)
leveneTest(dMGV~Stage, data=d3, center=mean)
leveneTest(dMGV~Cage, data=d3, center=mean)
# No homogenity was found. All values are unequal.

# Getting rid of Caligus
d3 <- d2 [!(d2$Stage=="Caligus"),]

# Relationships

#Plot every covariate versus Y

# Treatment by date
p <- ggplot()
p <- p + geom_boxplot(data = d3,
           aes(y = dMGV,
             x = Treatment, colour=Treatment, shape=Treatment)) +
  scale_color_manual(values=c("Cleanerfish"="red", "Control"="Blue")) +
  scale_x_discrete(labels=c("CF", "Control"))
p <- p + xlab("Treatment") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p <- p + facet_grid(.~Date)
p

# Temperature by Treatment
p <- ggplot()
p <- p + geom_boxplot(data = d3,
          aes(y = dMGV,
            x = Temp, colour=Treatment, shape=Treatment)) +
  scale_color_manual(values=c("Cleanerfish"="red", "Control"="Blue"))
```

```
p <- p + xlab("Average Sea Temperature (°C)") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p

# OptimalDepthLayer by treatment
p <- ggplot()
p <- p + geom_boxplot(data = d3,
                  aes(y = dMGV,
                     x = OptimalTempDepth, colour=Treatment, shape=Treatment)) +
  scale_color_manual(values=c("Cleanerfish"="red", "Control"="Blue"))
p <- p + xlab("Optimal Depth (m)") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p

# dMGV by daylight hours
p <- ggplot()
p <- p + geom_boxplot(data = d3,
                  aes(y = dMGV,
                     x = DaylightHours, colour=Treatment, shape=Treatment)) +
  scale_color_manual(values=c("Cleanerfish"="red", "Control"="Blue"))
p <- p + xlab("Hours of Daylight") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p

# Salinity by Treatment
p <- ggplot()
p <- p + geom_boxplot(data = d3,
                  aes(y = dMGV,
```

```
                    x = Salinity, colour=Treatment, shape=Treatment)) +
  scale_color_manual(values=c("Cleanerfish"="red", "Control"="Blue"))
p <- p + xlab("Salinity (ppt)") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p

# Stage by date
p <- ggplot()
p <- p + geom_boxplot(data = d3,
                  aes(y = dMGV,
                    x = Stage, colour=Stage, shape=Stage)) +
  scale_color_manual(values=c("Adult"="red", "PA1"="Blue", "PA2"="Black"))
p <- p + xlab("Stage") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p <- p + facet_grid(.~Date)
p

# Sex by Date
p <- ggplot()
p <- p + geom_boxplot(data = d3,
                  aes(y = dMGV,
                    x = Sex, colour=Sex, shape=Sex))  +
  scale_color_manual(values=c("Female"="red", "Male"="Blue"))
p <- p + xlab("Sex") + ylab("Mean Grey Value (dMGV)")
p <- p + theme(text = element_text(size=15))
p <- p + theme_classic()
p <- p + facet_grid(.~Date)
p
```

```
kruskal.test(dMGV ~ Treatment, data = d3)
kruskal.test(dMGV ~ Temp, data = d3)
kruskal.test(dMGV ~ OptimalTempDepth, data = d3)
kruskal.test(dMGV ~ DaylightHours, data = d3)
kruskal.test(dMGV ~ Salinity, data = d3)
kruskal.test(dMGV ~ Stage, data = d3)
kruskal.test(dMGV ~ Sex, data = d3)

dunnTest(dMGV ~ Treatment, method = "bonferroni", data = d3)
dunnTest(dMGV ~ Temp, method = "bonferroni", data = d3)
dunnTest(dMGV ~ OptimalTempDepth, method = "bonferroni", data = d3)
dunnTest(dMGV ~ DaylightHours, method = "bonferroni", data = d3)
dunnTest(dMGV ~ Salinity, method = "bonferroni", data = d3)
dunnTest(dMGV ~ Stage, method = "bonferroni", data = d3)
dunnTest(dMGV ~ Sex, method = "bonferroni", data = d3)

# lmer tests for all variables
anova(lmerTest::lmer(dMGV~Treatment*Date + (1|Cage),
            data = d3, na.action=na.exclude))

anova(lmerTest::lmer(dMGV~Temp*Treatment + (1|Cage),
              data = d3, na.action=na.exclude))

anova(lmerTest::lmer(dMGV~OptimalTempDepth*Treatment + (1|Cage),
              data = d3, na.action=na.exclude))

anova(lmerTest::lmer(dMGV~DaylightHours*Treatment + (1|Cage),
              data = d3, na.action=na.exclude))

anova(lmerTest::lmer(dMGV~Salinity*Treatment + (1|Cage),
              data = d3, na.action=na.exclude))
```

```
anova(lmerTest::lmer(dMGV~Stage*Date + (1|Cage),
                data = d3, na.action=na.exclude))

anova(lmerTest::lmer(dMGV~Sex*Date + (1|Cage),
                data = d3, na.action=na.exclude))

# Used to find specific mean dMGV and standard deviation for all variables.
agg <- aggregate(dMGV ~ Treatment*Date, data=d3, function(x) c(mean = mean(x), sd = sd(x)))
agg[order(agg$Treatment), ]

agg <- aggregate(dMGV ~ Temp*Treatment, data=d3, FUN = function(x) c(M=mean(x), SD=sd(x)))
agg[order(agg$Treatment), ]

agg <- aggregate(dMGV ~ Daylength*Treatment, data=d3, FUN = function(x) c(M=mean(x), SD=sd(x)))
agg[order(agg$Treatment), ]

agg <- aggregate(dMGV ~ OptimalTempDepth*Treatment, data=d3, FUN = function(x) c(M=mean(x),
SD=sd(x)))
agg[order(agg$Treatment), ]

agg <- aggregate(dMGV ~ Salinity*Treatment, data=d3, FUN = function(x) c(M=mean(x), SD=sd(x)))
agg[order(agg$Treatment), ]

agg <- aggregate(dMGV ~ Stage*Date, data=d3, FUN = function(x) c(M=mean(x), SD=sd(x)))
agg[order(agg$Stage), ]

agg <- aggregate(dMGV ~ Sex*Date, data=d3, FUN = function(x) c(M=mean(x), SD=sd(x)))
agg[order(agg$Sex), ]

#G. Are categorical covariates balanced?
```

table(d3$Treatment)

table(d3$Sex)

table(d1$Stage)

table(d3$Cage)

table(d3$Date)

table(d1$Exposure)

table(d3$DaylightHours)

table(d3$Temp)

table(d3$Date, d3$Treatment)

table(d3$Sex, d3$Treatment)

Collinearity

MyVar <- c("Salinity", "Temp", "DO", "DOmg", "Day365", "Exposure", "MobileLice", "Caligus", "DaylightHours", "Sex", "Stage")

Mypairs(d3[,MyVar])

Levene's test

leveneTest(dMGV~Treatment, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	1	11.467	0.000716 ***
	3599		

leveneTest(dMGV~Temp, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	4	6.1382	6.411e-05 ***
	3596		

leveneTest(dMGV~OptimalTempDepth, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	5	27.822	< 2.2e-16 ***
	3595		

leveneTest(dMGV~DaylightHours, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	6	24.165	< 2.2e-16 ***
	3594		

leveneTest(dMGV~Salinity, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	2	29.276	2.443e-13 ***
	3598		

leveneTest(dMGV~Sex, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	1	8.3062	0.003974
	3599		

leveneTest(dMGV~Stage, data=d3, center=mean)

Levene's Test for Homogeneity of Variance (center = mean)

	DF	F VALUE	PR(>F)
GROUP	2	9.5006	7.67e-05 ***
	3598		

leveneTest(dMGV~Cage, data=d3, center=mean)

	DF	F VALUE	PR(>F)
GROUP	5	11.283	8.131e-11 ***
	3595		

ANOVA & Kruskal-Wallis

Table C.1: The results from the two-way ANOVA test for the interaction between treatment and date.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Treatment	0.4	0.35	1	43.8	0.0120	0.9134
Date	5150.5	858.42	6	3532.2	29.1152	< 2.2e-16
Treatment:Date	1113.5	185.58	6	65.0	6.2944	2.959e-05

Table C.1.1: Kruskal-Wallis test for treatment.

Kruskal-Wallis	chi-squared	df	p-value
Treatment	0.020621	1	0.8858

Table C.2: The results from the two-way ANOVA test for the interaction between temperature and treatment.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Temp	2390.81	597.70	4	3563.3	19.6380	5.294e-16
Treatment	0.01	0.01	1	38.3	0.0004	0.9847
Temp:Treatment	315.73	78.93	4	65.7	2.5934	0.0443

Table C.2.1: Kruskal-Wallis test for temperature

Kruskal-Wallis	chi-squared	df	p-value
Temperature	72.897	4	5.546e-15

Table C.2.2: Post hoc test using Dunn test with Bonferroni correction for Temperature

	Comparison	Z	P.unadj	P.adj
1	12 – 14	-0.77591179	4.378011e-01	1.0000e+00
2	12 – 15	8.26781886	1.364320e-16	1.3643e-15
3	14 – 15	6.15373945	7.567697e-10	7.5677e-09
4	12 – 16	0.64853144	5.166413e-01	1.0000e+00
5	14 – 16	1.03872671	2.989319e-01	1.0000e+00
6	15 – 16	-4.00646490	6.163428e-05	6.1634e-04
7	12 – 8	-0.07561963	9.397217e-01	1.0000e+00
8	14 – 8	0.24606790	8.056297e-01	1.0000e+00

9	15 – 8	-2.78475966	5.356741e-03	5.3567e-02
10	16 – 8	-0.38577811	6.996610e-01	1.0000e+00

Table C.3: The results from the two-way ANOVA test for the interaction between optimal depth by temperature and treatment.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
OptimalTempDepth	5211.8	1042.37	5	3573.8	35.3406	< 2.2e-16
Treatment	45.5	45.45	1	114.1	1.5410	0.217
OptimalTempDepth:Treatment	1051.9	210.38	5	132.3	7.1326	6.145e-06

Table C.3.1: Kruskal-Wallis test for Optimal Depth

Kruskal-Wallis	chi-squared	df	p-value
Optimal Depth	120.15	5	< 2.2e-16

Table C.3.2: Post hoc test using Dunn test with Bonferroni correction for Optimal Depth

	Comparison	Z	P.unadj	P.adj
1	1 – 10	4.0064649	6.163428e-05	9.2451e-04
2	1 – 12	-1.0387267	2.989319e-01	1.0000e+00
3	10 – 12	-6.1537395	7.567697e-10	1.1352e-08
4	1 – 13	-3.5872428	3.341931e-04	5.0129e-03
5	10 – 13	-10.7486351	6.014504e-27	9.0218e-26
6	12 – 13	-2.9276309	3.415553e-03	5.1233e-02
7	1 – 25	-0.3857781	6.996610e-01	1.0000e+00
8	10 – 25	-2.7847597	5.356741e-03	8.0351e-02
9	12 – 25	0.2460679	8.056297e-01	1.0000e+00
10	13 – 25	1.7034429	8.848522e-02	1.0000e+00
11	1 – 5	0.3963765	6.918273e-01	1.0000e+00
12	10 – 5	-6.3718256	1.867912e-10	2.8019e-09
13	12 – 5	2.0607109	3.933063e-02	5.8996e-01
14	13 – 5	6.8741625	6.235511e-12	9.3533e-11
15	25 – 5	0.6512256	5.149008e-01	1.0000e+00

Table C.4: The results from the two-way ANOVA test for the interaction between daylight hours and treatment.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
DaylightHours	5150.5	858.42	6	3532.2	29.1152	< 2.2e-16
Treatment	0.4	0.35	1	43.8	0.0120	0.9134
DaylightHours:Treatment	1113.5	185.58	6	65.0	6.2944	2.959e-05

Table C.4.1: Kruskal-Wallis test for DaylightHours

Kruskal-Wallis	chi-squared	df	p-value
DaylightHours	121.65	6	< 2.2e-16

Table C.4.2: Post hoc test using Dunn test with Bonferroni correction for DaylightHours

	Comparison	Z	P.unadj	P.adj
1	11.18 - 12.43	1.2246185	2.207190e-01	1.0000e+00
2	11.18 - 14.3	-5.5743944	2.483925e-08	5.2162e-07
3	12.43 - 14.3	-4.8292493	1.370487e-06	2.8780e-05
4	11.18 - 15.53	-3.6083327	3.081712e-04	6.4716e-03
5	12.43 - 15.53	-3.6693999	2.431205e-04	5.1055e-03
6	14.3 - 15.53	1.0387267	2.989319e-01	1.0000e+00
7	11.18 - 6.23	-2.5845236	9.751364e-03	2.0478e-01
8	12.43 - 6.23	-3.0155563	2.565083e-03	5.3867e-02
9	14.3 - 6.23	0.2460679	8.056297e-01	1.0000e+00
10	15.53 - 6.23	-0.3857781	6.996610e-01	1.0000e+00
11	11.18 - 7.03	-5.3973859	6.761883e-08	1.4199e-06
12	12.43 - 7.03	-4.1642361	3.123967e-05	6.5603e-04
13	14.3 - 7.03	2.0607109	3.933063e-02	8.2594e-01
14	15.53 - 7.03	0.3963765	6.918273e-01	1.0000e+00
15	6.23 - 7.03	0.6512256	5.149008e-01	1.0000e+00
16	11.18 - 8.68	-9.7797917	1.374933e-22	2.8873e-21
17	12.43 - 8.68	-7.0928976	1.313326e-12	2.7580e-11
18	14.3 - 8.68	-2.9276309	3.415553e-03	7.1727e-02

19	15.53 - 8.68	-3.5872428	3.341931e-04	7.0180e-03
20	6.23 - 8.68	-1.7034429	8.848522e-02	1.0000e+00
21	7.03 - 8.68	-6.8741625	6.235511e-12	1.3095e-10

Table C.5: The results from the two-way ANOVA test for the interaction between salinity and treatment.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Salinity	362.31	181.153	2	3594.9	5.8383	0.0029
Treatment	0.01	0.015	1	3595.0	0.0005	0.9824
Salinity:Treatment	106.87	53.434	2	3499.4	1.7221	0.1788

Table C.5.1: Kruskal-Wallis test for Salinity

Kruskal-Wallis	chi-squared	df	p-value
Salinity	2.5753	2	0.2759

Table C.6: The results from the two-way ANOVA test for the interaction between stage and date.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Stage	319.08	159.54	2	3576.0	5.4617	0.0043
Date	860.43	143.40	6	3572.9	4.9093	5.219e-05
Stage:Date	2247.67	187.31	12	3578.2	6.4122	2.143e-11

Table C.6.1: Kruskal-Wallis test for Stage

Kruskal-Wallis	chi-squared	df	p-value
Stage	17.487	2	0.0001595

Table C.6.2: Post hoc test using Dunn test with Bonferroni correction for Stage

	Comparison	Z	P.unadj	P.adj
1	Adult - PA1	-1.748	8.051e-02	0.2415
2	Adult - PA2	2.944	3.239e-03	0.0097
3	PA1 - PA2	3.908	9.311e-05	0.0003

Table C.7: The results from the two-way ANOVA test for the interaction between sex and date.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)

Sex	1802.1	1802.05	1	3583.1	63.622	2.01e-15
Date	5276.7	879.45	6	3583.1	31.049	< 2.2e-16
Sex:Date	3042.1	507.01	6	3583.2	17.900	< 2.2e-16

Table C.7.1: Kruskal-Wallis test for Sex

Kruskal-Wallis	**chi-squared**	**df**	**p-value**
Sex	63.345	1	1.735e-15