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Melanin-based skin pigmentation and stress in Atlantic salmon (*Salmon salar*).

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https://www.nmbu.no/en/faculty/biovit

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Norwegian University of Life Sciences

Abstract

Anybody who has studied, fished for, handled, or consumed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) will have noticed that the skin of these fishes is typically dappled with black spots. In recent times, the spots have gained much interest within many study fields, especially stress and animal welfare. Stress is known to have detrimental effects upon growth, reproduction, food conversion efficiency and disease susceptibility, making discoveries of new stress indicators utmost important. Until now, the most utilized method to measure the level of stress has been to analyse the cortisol content in blood plasma. However, this is a time-consuming process and difficult to do in field, and consequently, finding an external indicator of the internal state is of international interest.

Previous studies have found a negative correlation between the number of ectoparasitic adult female salmon lice (*Lepeophtheirus salmonis*) and the number of spots in Atlantic salmon, in which the highly spotted individuals were found to have less adult female salmon lice than the less spotted individuals. However, these studies utilized a semi-quantitative method, in which spots are counted by eye and then subjectively adjudged depending on size. Consequently, development of automatic image analysis for the recognition and quantification of melanised areas of individual fish would be of great benefit in further research. Therefore, we wanted to investigate if the same correlation between the number salmon lice and the number of spots would be evident when utilizing automatic image analysis. Interestingly, in preliminary studies establishing methodology to quantify spots on the operculum, we noticed that some of the spots had faded and that the frequency of faded spots seemed to increase throughout the sampling session. These observations were made in fish chronically disturbed by ongoing sampling activities, e.g. crowding and netting, the same activities as during delousing procedures. Hence, it would seem that the spots do not only reflect individual stress coping ability, they also convey information about recent exposure to stressful experiences. Thorough research in established literature indicates that the sudden fading phenomenon might be stress-induced. Furthermore, as the fading was visible by the naked eye, our finding might be utilized as a new stress-indicator. This would give rise to a much easier method for detecting stress, as opposed to the blood plasma sampling. However, the fading and cortisol content were not correlated, indicating that other mechanisms are directly involved in fading. The thorough literature review finds that the activation of MCH-Rs and/or norepinephrine-receptors is the main factor in the fading process. In addition, from the results of the spot analysis, we did not find any lateralisation of spots, which in turn means that further spot analysis will only have to be based upon one of the sides. However, as time was plentiful (approximately five hours) during sampling, we cannot conclude which specific molecular process was the main fading agent. Consequently, more research regarding the molecular control of stress-induced fading is crucial in order to establish fading of spots as a marker of stress and welfare in fish aquaculture.

Contents

1. Introduction

1.1. Melanin-based skin pigmentation as an indicator of stress and parasite resistance in salmon aquaculture

The general definition of aquaculture is farming of aquatic organisms, including fish, crustaceans, molluscs and aquatic plants. The aquaculture industry in Norway produces mainly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), in which the Norwegian production of Atlantic salmon exceeded 60 billion NOK in 2017 [1], making Norway one of the largest producers of Atlantic salmon in the world. However, there are many issues involved in the production, such as escapees, diseases, and parasites, which in total not only influence the profit, but also the industry's reputation. Therefore, the industry has used a lot of resources upon solving such issues, focussing on distinct challenges to fish welfare and production biology related to stress and disease in stocked fish. Especially, infections by ectoparasitic salmon louse (*Lepeophtheirus salmonis*). The salmon louse is a natural ectoparasite of salmonids in the ocean, and its survival is dependent on the density of hosts. A normal sized open sea cage production site in Norway utilizes between six to eight sea cages, in which each sea cage contains approximately 200 000 Atlantic salmon, which makes for a perfect habitat to the salmon louse. Salmon louse is known to cause stress and decreased survival in Atlantic salmon, and as a consequence, every producer in Norway is obliged by law to monitor the number of salmon lice and report to the Norwegian Food Safety Authority. The density of adult female lice with egg strings must at any given moment be lower than 0,5 per fish. If the number exceeds this upper limit, necessary measures must be initiated. Normally, this involves delousing of fish, in which a purse seine is lowered into the sea cage with the purpose of concentrating the fish, enabling easy access for further handling. However, such crowding of fish is known to induce repetitive stress responses [2, 3]. Stress in general have detrimental effects within many aspects of fish welfare, including growth, reproduction, food conversion efficiency and disease susceptibility [3-5]. Considering the issues with salmon louse in total, the yearly cost for the industry exceeds billions of NOK, which makes any improvement of utmost interest.

Anybody who has studied, fished for, handled, or consumed salmon or trout will have noticed that the skin of these fishes is typically dappled with black spots. The spots are formed by aggregations of pigment producing cells, chromatophores, containing the black pigment eumelanin. In terrestrial animals and insects, melanin-based pigmentation is important for social signalling thermoregulation, camouflage and protection against physical damage, infectious agents, UV-light, free radicals, and toxicants [6-10]. Functions of this pigment in fishes are most likely just as diverse but have not been studied to nearly the same degree. Previous studies have shown that variable numbers of eumelanin spots on salmonid fish can provide

essential information about each animal's physiology and behavioural patterns. Within strains of Atlantic salmon and rainbow trout, more spotted individuals have a reduced cortisol response to stress, a more rapid recovery of feed intake after disturbance or transfer to novel environments [11, 12]. Notably, Kittelsen et al. (2012) [13] reported that the number of adult female lice differed significantly between less spotted individuals and highly spotted individuals: The less spotted individuals had more adult female lice than the highly spotted individuals.

In previous studies, there was no observed correlation between pigmentation and the occurrence of lice at early developmental stages [13, 14], suggesting that host factors specifically modify parasite development, but not infection rate. As the salmon louse is responsible for such tremendous economic loss, it would be of great interest to further investigate the causal mechanisms behind this association between melanin-based dermal pigmentation patterns and parasite resistance. Precise quantification of melanisation in salmon is required in this context. However, previous studies have utilized a semi-quantitative method, in which spots are counted by eye and then subjectively adjudged depending on size. Development of automatic image analysis for the recognition and quantification of melanised areas of individual fish is therefore defined as an objective in this thesis.

In this context, it should be noted that spot patterns in salmonid fish have lately come in focus also as possible means for individual identification [15, 16]. Non-invasive identification of individuals in groups of fish is often essential in scientific studies, particularly when focussing on biological traits and their variability through time. Individual traceability throughout the production process and the subsequent value chain, without the need for physical markers or tagging, is also of essential interest for quality monitoring in the aquaculture industry. The papers by Kittelsen et al. (2009 [11], 2012 [13]) preceded a recent series of studies on into the gene-environment interactions controlling skin pigmentation and correlated traits in salmonids (e.g. Colihueque et al. (2018) [17]; Jørgensen et al. (2018) [18]; Rodríguez et al. (2019) [19]). Various methods for digital image analysis for objective quantification of melanin-based pigmentation have been evaluated in these studies. Very recently, Jørgensen et al. (2018) [18] developed a macro for spot analysis in the image analysis program "ImageJ", and we were granted permission to utilize this macro. This allowed the investigation of further potential confounding factors in the analysis of body pigmentation, such as the possible occurrence of bilateral (right-left) asymmetry, and whether pigmentation on various body parts are correlated. For instance, the number of spots on the lateral side is much higher than the number of spots on the operculum, and therefore, any correlation between the two would enable a much faster method for spot analysis in Atlantic salmon. In addition, as opposed to the lateral side, the operculum does not have prominent fish scales. Fish scales will in some instances hide pigment particles, which in turn might have implications on subjective differentiation between spots and shadows between fish scales [20]. Therefore, if a correlation between the number of spots on the lateral side and the operculum exists, this finding will provide a much better area for spot analysis.

Finally, this thesis addresses a novel phenomenon, which may be of potentially high significance both regarding the possible use of melanin-based pigmentation as an indicator of stress and parasite resistance, and with regard to individual recognition. Interestingly, in preliminary studies establishing methodology to quantify spots on the operculum, we noticed that some of the spots had faded and that the fading seemed to increase throughout the sampling session (see figure 1 for an example). These observations were made in fish chronically disturbed by ongoing sampling activities, e.g. crowding and netting, the same activities as during delousing procedures. Hence, it would seem that the spots do not only reflect individual stress coping ability, they also convey information about recent exposure to stressful experiences. Thorough research in established literature indicates that the sudden fading phenomenon might be stress-induced. Hence, the study design was modified to also describe the time course of fading response and investigate whether an additional acute stress test (typically used to describe individual variation in stress responsiveness, cf. Kittelsen et al. (2009) [11]) would induce further fading. The physiological mechanisms involved in individually specific pigmentation patterns and, potentially, the fading response are further described below. Thesis goals are summarised at the end of this introduction.

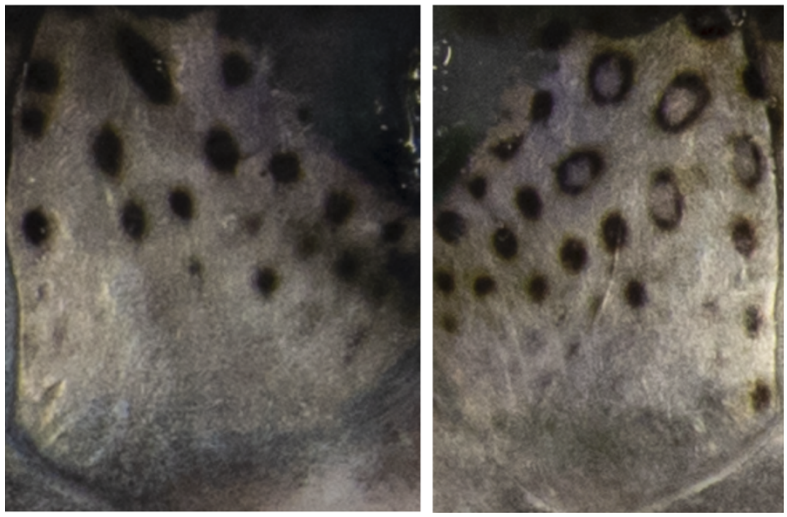

Figure 1 – The fading of the spots on the operculum. The picture to the left shows the operculum of the first control fish from our third sampling session, whilst the picture to the right shows the operculum of fish number 17 from the same sampling session. The control fish did not have any faded spots, whilst fish number 17 have many faded spots, a phenomenon that only seemed to increase throughout our third sampling session.

1.2. Colouration reflects the individual

Both colouration and pigmentation have diverged into fantastic varieties: From the striking orange of the common clownfish (*Amphiprion ocellaris*) to the camouflage of the Western diamondback rattlesnake (*Crotalus atrox*). Colouration serve many functions, as exemplified with guppies (*Poecilia reticulata*), which can change their colouration in accordance to the predation pressure [21], as well as display individual social status within a specific population, in which great tits (*Parus major*) with the widest black stripe on the chest have the highest social status [22]. Other studies have shown that melanin-based colours and pigment patterns reflect individual differences in survival rates [23-26], aggression [27, 28], dominance [29, 30], and parasite resistance [31, 32].

Colours and pigment patterns are produced by different cell types in the skin. Mammals, including humans, have only one type of cell involved in pigmentation, the melanocytes, whilst lower vertebrates, as poikilotherms and invertebrates, have six cell types, termed chromatophores [6, 33-36]. Chromatophores are specialized cells involved in a continuous synthesis and storage of pigments and light-reflecting structures, enabling a species to change its appearance in relation to environmental impact through morphological or physiological colour change [33]. Morphological colour changes manifest over days or months, depending on the distribution, density and morphology of the chromatophores in the integument's three-dimensional organization [20, 37]. Such morphological colour change can occur during a species' transition between two pre-adapted phenotypes specific to a given species' life-stages [20], which can be seen in many rivers during early spring when brown Atlantic salmon (*Salmo salar*) parr develops into silvery smolts through smoltification. The physiological colour change on the other hand, can occur within minutes or hours [33]. By altering the motility of the pigment vesicles within a species' cell, as well as the reflective structures, the colour change can be acute and transient [38]. Physiological colour change is divided into primary and secondary, in which the primary colour change is due to direct environmental effect, such as light, whilst the secondary colour change is controlled by neuroendocrine mechanisms [20, 39].

1.2.1. Chromatophores and their specific pigments

The six types of chromatophores found in poikilotherms and invertebrates are melanophores, xanthophores, erythrophores, leucophores, iridophores, and cyanophores [33]. The melanophores is produce brown and black pigment synthesis, the xanthophores synthesize ocher and yellow pigments, and the erythrophores produce red pigmentation. The leucophores produce pigments of a whitish colour, and iridophores are responsible for a metallic or iridescent appearance, which makes for a light-reflective function [39]. Iridophores is found to be important in the formation of stripes in zebrafish (*Danio rerio*) [40]. Cyanophores have been found to produce blue pigments [41]. The origin of the teleost chromatophores are pigment cells precursors

developed from neural crest cells [42, 43], formed during embryogenesis [20]. These precursor cells, termed chromatoblasts, migrate and mature into the different chromatophores, and will thereafter settle in the integument. Combining these chromatophores enables a species to develop fantastic colouration [33, 39], as can be seen in tropical waters. In general, the amount of pigmentation within Atlantic salmon varies greatly between individuals [18, 44], indicating individual differences in the amount and activity of the different chromatophores. Within the melanophores, all pigment synthesis occurs within one cell organelle, the melanosome. The melanophores are often involved in melanophore-iridophore complexes, in which melanophores normally surround the iridophores in dendritic extensions hiding the iridophores, which in turn hinders the iridophores' reflective function [20]. Therefore, the colour seen by the naked eye is due to the black eumelanin-containing organelle, the melanosome, within melanophores. The complexes are located both on top of and underneath fish scales, as well as between fish scales, on the lateral side [45, 46]. Such location presumably hides many of the chromatophores, especially when located underneath scales. However, the operculum consists of several bony plates instead of scales, and therefore, we assume that all chromatophores are visible by the naked eye. This makes the operculum of Atlantic salmon a perfect area for research.

1.3. Stress and pigment synthesis

1.3.1. Stress and stress-coping styles

Stress is defined by Chrousos (1998) [47] as any state of threatened homeostasis that can be re-established by physiological and behavioural adaptive responses. Should however the adaptive responses not be able to re-establish homeostasis, a healthy state is not achieved, in which the ultimate outcome is pathology. Normally, the concentration of circulating corticosteroids within resting or unstressed fish is 30-40ng/ml, but should ideally not exceed 5ng/ml [2]. However, individuals react different both behaviourally and physiologically when exposed to stressors, and the correlation between the two is termed stress-coping styles [48-54]. Within a population of a given species, the stress-coping styles are specific for certain individual groups, and studies have found two such groups within strains of rainbow trout: A proactive low-responsive (LR) group and a reactive high responsive (HR) group [52, 55-59]. However, it must be pinpointed that such dividing of groups is only a fish model of a conserved phenomenon. Stress-induced cortisol response is much lower for HR-fish than for LR-fish [56, 60]. In addition to cortisol production, stress induces production of catecholamines [48, 61]. Previous studies have found a negative correlation between the cortisol response and the catecholamine response [49], in which HR-fish with the low cortisol response showed a high catecholamine response, whilst LR-fish with the high cortisol response showed a low catecholamine response.

1.3.2. The melanocortin system

1.3.2.1. Proopiomelanocortin (POMC)

The vertebrate pituitary is the main producer of proopiomelanocortin (POMC) [62]. Depending on the site of proteolytic cleavage by prohormone convertase 1 (PC_1), different hormones are generated: When expressed in corticotrophs in the anterior pituitary, the cleavage generates adrenocorticotropic hormone (ACTH) whilst cleavage within melanotrophs generates melanophore-stimulating hormones (MSH) [62-67], in which α-MSH is best studied.

1.3.2.2. Melanocortins and their role in colouration

The melanocortin system regulates pigment synthesis within chromatophores and cortisol synthesis within corticosteroidogenic cells under the influence of the hypothalamus-pituitary-interrenal axis and the hypothalamus-pituitary-adrenal axis, respectively [11, 68]. The melanocortins have normally five G_s-protein-coupled melanocortin receptors, MC_1-MC_5, in which MC_1 is associated with colouration in melanophores, and MC_2 is associated with cortisol synthesis in corticosteroidogenic cells, not only in fish, but also in mammals [62, 69-74]. ACTH is specific for MC_2, while α-MSH is specific for the other receptors. When α-MSH and ACTH bind to their specific melanocortin receptors, adenylyl cyclase degrades adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP). In melanophores, this process is continuous, in which the intracellular increase of cAMP stimulates melanosome dispersion into dendritic projections [33]. In corticosteroidogenic cells on the other hand, the activation of MC_2 is stress-induced. If exposed to stress, hypothalamic neurons synthetize corticotrophin-releasing hormone (CRF) [75], which in turn initiates the proteolytical cleavage of POMC into ACTH. In addition, functional expression of MC_2 is dependent on receptor trafficking by the accessory protein melanocortin-2-receptor accessory protein (MRAP) [76]. The intracellular increase of cAMP stimulates cholesterol production, which is thereafter degraded into cortisol through several enzymatic steps. Cortisol is thereafter released into the blood.

The melanocortin signalling is regulated from not only endogenous agonists, but also competitive antagonists [62]. Interestingly, an increase of blood plasma cortisol increases expression of the α-MSH-antagonist agouti-signalling protein (ASP) in the skin of the salmonid rainbow trout [12, 77, 78]. When ASP binds to the MC_1 receptor, the production of cAMP is hindered, which in turn reduces the melanosome dispersion. Therefore, activation of MC_2 ultimately hinders melanosome dispersion.

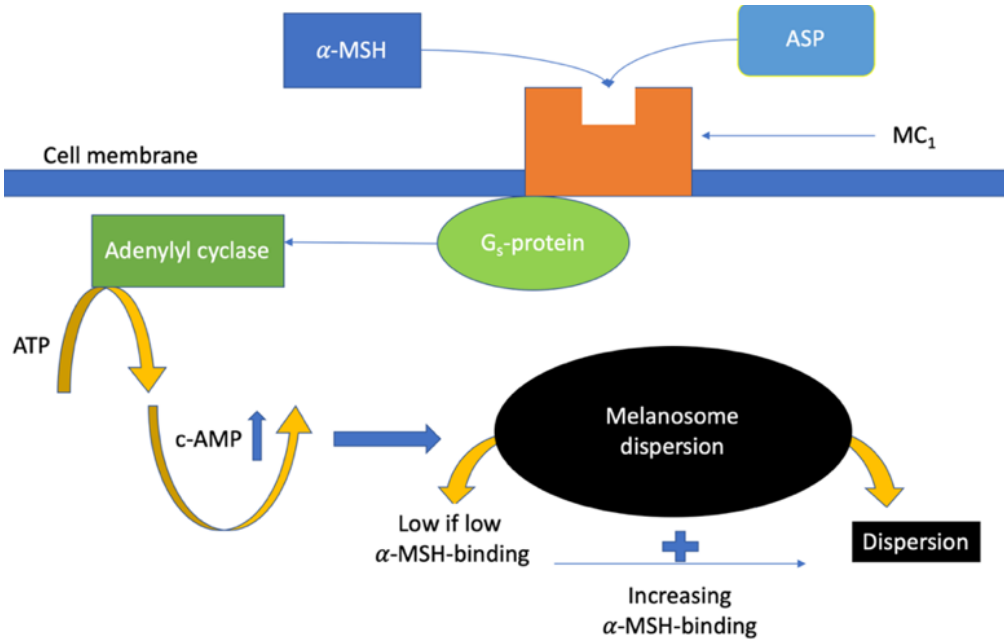

Figure 2 – The molecular processes involved in melanosome dispersion within melanophores. ASP and α-MSH compete with each other in being the first to bind to MC_1. Normally, α-MSH continuously binds to the G_s-protein-coupled MC_1, which activates adenylyl cyclase, resulting in increased intracellular level of cAMP. The amount of cAMP is positively correlated to the degree of dispersion. However, if ASP is first to bind to MC_1, the activation the G_s-protein is hindered. which in turn hinders melanosome dispersion. As a consequence, the degree of melanosome dispersion decreases.

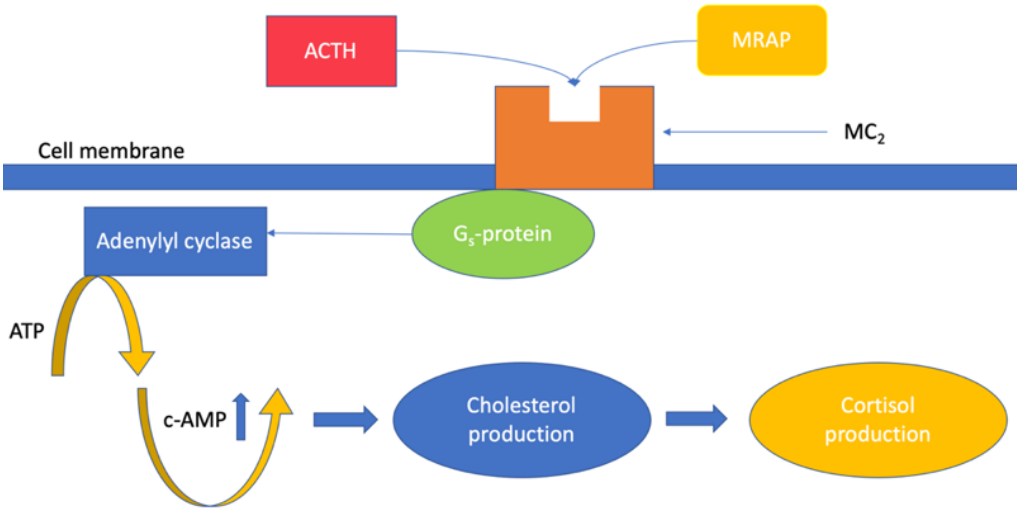

Figure 3 – The molecular processes involved in cortisol production within corticosteroidogenic cells. MRAP transports ACTH to MC_2 by receptor trafficking, which initiates adenylyl cyclase. Adenylyl cyclase generates cAMP, which in turn stimulates cholesterol production. Cholesterol is thereafter degraded to cortisol in several enzymatic steps.

1.3.3. Melanin-concentrating hormone (MCH)

Melanin-concentrating hormone (MCH) and α-MSH have opposing effect, in which activation of MCH-receptors results in melanosome aggregation [79-82]. MCH is also important in mechanisms regarding feeding and energy metabolism [83-85]. The synthesis of MCH is initiated by repeated stress [86]: If exposed to repeated stress, hypothalamus produce the precursor hormone of MCH, proMCH, which is secreted from the neurohypophysis as MCH [87-89]. None of the melanocortin receptors (MC_1-MC_5) can bind MCH, making MCH specific for MCH-receptors [68]. The number of MCH-receptors varies between species, but the G_i-protein-coupled MCH-R1 is associated with colouration. [89-91]. When MCH binds to MCH-R1, adenylyl cyclase is inhibited, and as a consequence, the production of cAMP ceases [92-96]. Therefore, instead of being dendritically dispersed around the iridophores, the melanosomes aggregate, resulting in that only the reflective function of the iridophores can be seen with naked eye.

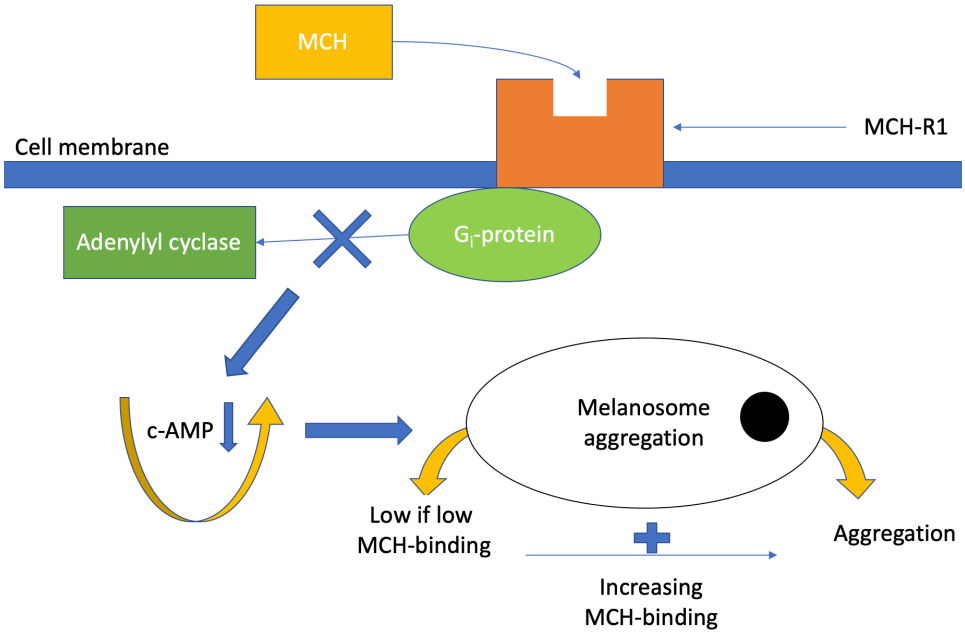

Figure 4 – The molecular processes involved in aggregation of melanosomes. MCH binds to the G_i-protein-coupled MCH-R1, which inhibits adenylyl cyclase. Thereby, the production of cAMP ceases, resulting in melanosome aggregation. The ultimate result is that only the iridophores are visible by the naked eye, which makes the skin pale.

1.3.4. Activation of MCH-R1 and MC_1 have opposing effects

In summary, the main difference between dispersion and aggregation is the effect upon adenylyl cyclase, in which the activation of MCH-R1 inhibits adenylyl cyclase, whilst the activation of MC_1 stimulates adenylyl cyclase. When MCH binds to the G_i-protein-coupled MCH-R1, cAMP-synthesis is inhibited, which in turn ceases the melanosome dispersion, resulting in aggregation of the melanosome. On the other hand, when α-MSH binds to G_s-protein-coupled MC_1, cAMP-synthesis is stimulated, which in turn disperse the melanosome. In

addition, the stress induced activation of MC_2, which ultimately results in the production of α-MSH-antagonist ASP, might have a profound effect on the total body melanisation.

1.3.5. Norepinephrine

Norepinephrine is known to aggregate melanosomes [33]. When exposed to stress, noradrenaline coupled to ATP is released into the circulation. The melanophores have receptors for both noradrenalin and ATP in the membrane. The circulating noradrenaline binds to a G_i-protein-coupled receptor, which inhibits adenylyl cyclase, ultimately aggregating the melanosome. The circulating ATP on the other hand, is degraded to adenosine, which in turn binds to g G_s-protein-coupled adenosine receptor. The binding stimulates cAMP-synthesis, and ultimately melanosome dispersion.

However, Wilkes et al. (1984) [97] reported that norepinephrine had a much lower aggregation effect than MCH at equimolar concentrations (approximately 10% within 30 minutes vs. 35% within 30 minutes). In addition, the aggregation effect of noradrenaline ceased much faster than MCH-induced aggregation.

1.4. Aim of the thesis

As our novel observation gives rise to intriguing studies within fish welfare, establishing a firm theoretical basis of the molecular mechanisms involved in fading of spots, notably within Atlantic salmon, was one of our aims for this thesis. Furthermore, as our finding has never before been reported in alive fish during a sampling session, developing a method for analysing the degree of fading was another aim. In addition, in the context of spot analysis in general, previous studies have analysed both the right and left side of fish. This might be unnecessary if the spottiness of fish is symmetric, i.e. that the spottiness is not lateralised, which in turn means that only one of the sides has to be analysed within spot analysis. This would supply further spot analysis projects a much faster method.

2. Material and methods

2.1. Study Material

2.1.1. Field work at Austevoll

We were granted permission to sample Atlantic salmon in the saltwater rearing phase from ongoing studies at the Norwegian Institute of Marine Research's (IMR) research facility at Austevoll, Norway.

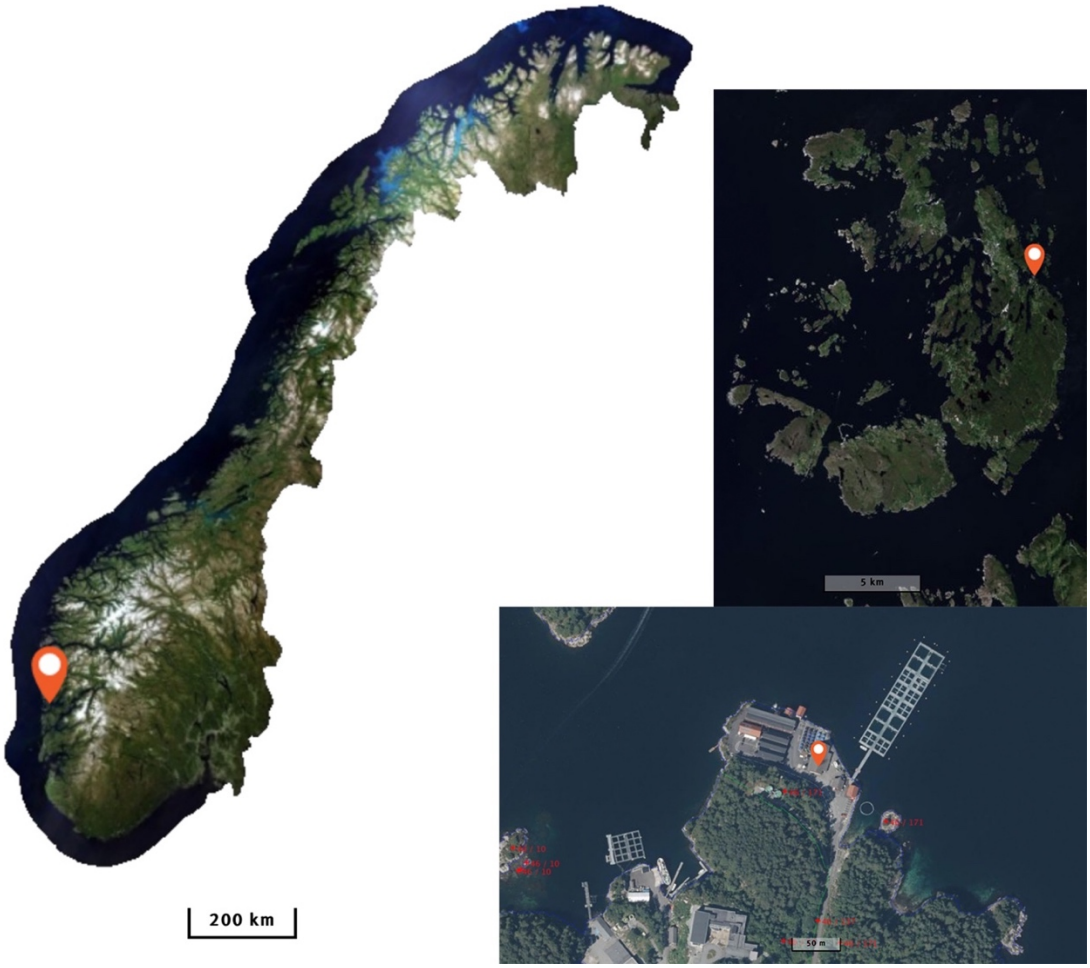

Figure 5 – The location of the sampling site, IMR, collected from Norgeskart [98].

After a period of methods development, sampling of Atlantic salmon from sea cages took place 23th of October 2018. The water temperature was 11,7°C at 0,5m depth and 12,8°C at 20m, and all fish were sampled from the same sea cage. Following a typical routine for sampling of fish in aquaculture studies, at the start of the sampling session, a purse seine was submerged into the sea cage. After being submerged, the purse seine was pulled together, capturing a representative sample of fish, and lifted to the surface. This enabled access to fish for capture by netting, and is a standard procedure for handling, capture or other treatment of fish from aquaculture. The

fish were kept confined in the purse seine during the whole sample period from 11AM to 17PM and hand netted subsequently during the day. In total, the sampling involved hand netting individual fish, transport of fish to the land-based research setup (approx. 1min), size measurements (length and weight), stress test (for 18 out of 27 individuals), blood plasma sampling, and photoshoot in the end. Fish were either assigned to a control group or acute stress testing. The controls were instantly killed, using a high dose of MS222 (0,5g/l). The fish to be stress-tested were transported alive in a wheel barrow containing a 50l box filled with saltwater to our research setup after hand netting. No anaesthesia was added and fish were kept in the boxes for approximately 20 minutes, mimicking acute stress testing previously used to assess individual variation in stress responsiveness in salmon [11]. When the stress test ended, the fish were instantly killed, using a high dose of MS222 (0,5g/l). For both directly sampled controls and stress tested fish, length and weight were measured before blood plasma was sampled with a 1ml syringe containing a tiny amount (1-2mg) of EDTA. The centrifuged samples were stored in an ice box for <2h, transferred to dry ice, and later analysed for plasma cortisol. Thereafter, individual fish were photographed with Canon EOS 60D with Sigma DC 17-50 1:2:8 EX HSM lens, utilizing manual camera settings. The sampling method for the controls, from hand netting to finished photographing, took four minutes on average. For the stress-tested fish, the sampling method took 33 minutes on average, including the stress test.

Figure 6 - Images of the research setup. The top image illustrates our research station in general, as well as the large black confinements utilized for the stress test. The bottom left image illustrates the centrifuge utilized for centrifuging of the blood plasma samples, whilst the bottom right image illustrates the working bench including tools for length and weight measurements and blood sampling. The centrifuged blood plasma samples were stored in the white ice box.

2.2. Data collection

2.2.1. Cameras and the photoshoot

All images from the sampling session were shot by one dedicated photographer, utilizing a Canon EOS 60D with Sigma DC 17-50 1:2:8 EX HSM lens. All images were saved in both JPEG-files and RAW-file format. Manual camera setting was used, and the white balance was set in the camera after a test photoshoot with a white balance card (Lastolite, LL LR1250). Thereafter, a reference image was chosen for the camera to create a white balance profile, in which the settings stayed the same throughout the sampling. In addition, the camera was rigged on a tripod, which made for high standardization of the images. Extra light was not applied at the photoshoot. All images were shot in a white box with a ruler placed next to the fish, which was utilized for calibration during the following image analysis.

Figure 7 – The photoshoot setup during the third sampling session. A tripod was utilized in order to standardize the images. The white box made all the details of the fish much more visible.

Figure 8 – An example of the result after photoshoot.

2.3. Image processing

2.3.1. Lateralisation and body-operculum correlations

All RAW-format images were analysed using Photoshop and ImageJ. The first step of the analysis was to line up the pictures using photoshop, in which the lateral line was utilized as the reference point.

Figure 9 – A before/after-scenario after lining up the pictures in Photoshop.

Thereafter, ImageJ's ability to calibrate images to a ruler was utilized, which enabled measuring of the region of interest (ROI). Selecting ROI for further analysis was done according to Jørgensen et al. (2018) [18]: They argued that in order to study a representative area of the fish for spottiness, ROI should extend from the end of the gill cover to the start of the dorsal fin with the lateral line in the middle. Further analysis was executed in ImageJ, due to the program's ability to utilize macros. I was granted permission to utilize the macro developed by Jørgensen et al. (2018) [18], but unfortunately, ImageJ was not able to process the same macro on our images. Nevertheless, the images were cropped in accordance to the ROI utilized by Jørgensen et al. (2018) [18]. Thereafter, the crop was measured by ImageJ's measuring function, which gave us the size of the crop in cm^2, and finally saved as TIFF-files.

Figure 10 – A before/after-scenario of the cropping process of ROI. The cropped ROI was done in accordance to Jørgensen et al. (2018) [18].

Thereafter, the images were transported back to Photoshop, in which spots were counted by hand utilizing Photoshop's painting function to mark each spot. Finally, the number of spots/cm^2 was found in Excel by dividing the number of spots on the size of the ROI.

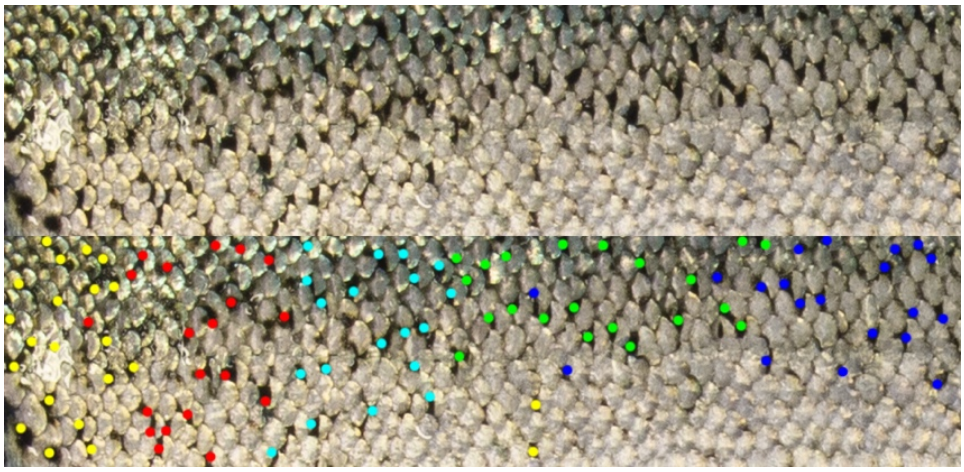

Figure 11 – An example of the cropped ROI and the spot count. 20 spots were counted at a time with a specific colour, which made it much easier to sum the total amount of spots in each ROI.

The ROI on the operculum followed the operculum's shape. However, the shape of the operculum varied greatly between individuals, and therefore, we restricted the ROI to the cheek of the fish. The shape of the cheek was the same for all individuals, enabling high degree of standardization. After lining the images with reference to the lateral line, the next step in the processing of the operculum ROI was to utilize ImageJ's ability to calibrate images to a ruler. The operculum ROI was drawn by hand in ImageJ by plotting dots along the cheek of the fish. Thereafter, the area was cropped, measured by utilizing ImageJ's measuring function, and saved as TIFF-files.

Figure 12 – The operculum ROI.. The shape of the operculum varied greatly between individuals. Therefore, we restricted the operculum ROI to the cheek of the fish. Nevertheless, the cheek ROI is emphasized as the operculum ROI. The dotted line-function in ImageJ was utilized to mark ROI.

The images were thereafter transported back to Photoshop, in which spots were counted by hand utilizing Photoshop's painting function to mark each spot. Finally, the number of spots/cm^2 was found in Excel by dividing the number of spots on the size of the ROI.

2.3.2. Processing of the faded spots

RAW-images were utilized for the processing of the faded spots.

Figure 13 – An illustration of the selected operculum ROI. The image to the left illustrates the location of the operculum ROI. The image to the right illustrates the cropped operculum ROI. The dotted line-function in ImageJ was utilized to mark ROI.

After cropping, all spots were marked and measured by hand in ImageJ, utilizing ImageJ's "freehand"-function. The visible black pigment of the none-faded spots was marked and measured. Only the visible black pigment of the faded spots was marked and measured, and a estimated original size of the faded spots was marked and measured, based on the outline of the pigment ring encircling faded areas (see figure 14). Thereafter, the degree of fading (as % of estimated total area) was calculated in Excel by comparing the total pigmented areal to the total estimated original pigmented areal. Fading was only observed in the comparatively large spots on the operculum ROI.

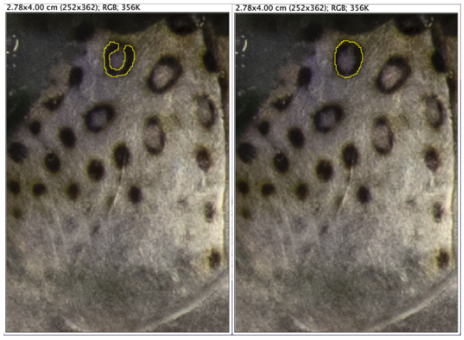

Figure 14 – The hand-drawn markings of the spots. The picture to the left shows the size of a faded spot, whilst the picture to the right shows the most probable original size of spot if not faded.

In total, the procedure described above yielded the following variables: Number of spots/cm^2 in left- and right-side ROI, estimated original pigmented area in left and right ROI (fraction of pigmented area to total ROI area, %), % faded area of estimated original area, and pigmented are after fading. As no bilateral asymmetry was indicated (see the results section), the average of right and left side measures was used to analyse relationships between variables and treatment effects on melanisation.

2.4. Cortisol analysis

“DetectX Cortisol Enzyme Immunoassay kit” was utilized for cortisol analysis of our blood plasma samples. This kit is designed to measure the total cortisol content in extracted plasma samples.

2.5. Statistical analysis

Possible bilateral asymmetry and effects of treatment (direct sampling vs. additional acute stress) on melanisation was analysed by mixed model ANOVA and linear regression between right and left side melanisation indexes, with runs test to check for deviation from linearity. Determining the relationship between operculum and body melanisation was also done by linear regression. Data from control and stress-tested individuals were pooled for the regression analysis, as no effect of additional stress on any melanisation index was indicated. Effect of additional stress testing after seining on plasma cortisol was analysed by t-Test between control and stress-tested individuals. Finally, effect of time during sampling on the fading response was determined by linear regression.

3. Results

3.1. Spot asymmetry

The first question to be addressed in this thesis was whether there are any bilateral (right-left) asymmetry in dermal melanisation in farmed Atlantic salmon. On average, the various melanisation indexes were almost completely similar on the left and right side of the body in the tested population (see table 1).

Table 1 – Comparison of right vs. left side melanisation indexes in farmed Atlantic salmon. Values are average and S.E.M. Controls (n=9), additional acute stress-test (n=18).

Body: Spots/cm^2 in ROI		
	Right	*Left*
Controls	**3,16±0,22**	**3,17±0,21**
Stress-tested	**3,32±0,21**	**3,35±0,24**
ANOVA outcome: Lateralisation, p=0,94. Effect of stress, p=0,48. Interaction, p=0,97.		
Operculum: Estimated original pigmented area before fading (% of ROI)		
	Right	*Left*
Controls	**7,99±0,45**	**7,40±0,57**
Stress-tested	**8,82±0,49**	**8,61±0,61**
ANOVA outcome: Lateralisation, p=0,56. Effect of stress, p=0,10. Interaction, p=0,76.		
Operculum: Estimated faded area (% of pigmented area in ROI)		
	Right	*Left*
Controls	**5,21±1,69**	**4,78±1,65**
Stress-tested	**7,79±2,05**	**7,98±2,16**
ANOVA outcome: Literalisation, p=0,99. Effect of stress, p=0,21. Interaction, p=0,89.		
Operculum: Pigmented area after fading (% of ROI)		
	Right	*Left*
Controls	**6,70±0,54**	**6,97±0,49**
Stress-tested	**7,57±0,59**	**7,69±0,53**
ANOVA outcome: Literalisation, p=0,55. Effect of stress, p=0,23. Interaction, p=0,80.		

ANOVA revealed no difference between right and left side nor any effect of acute stress test on any parameter. Hence, based on the lack of an effect of additional acute stress, and no confirmed deviation from linearity (Runs test, p>0,4 in all cases), the relationship between right and left side melanisation indexes could be visualised with a single regression line for control and stress-tested individuals (figure 15).

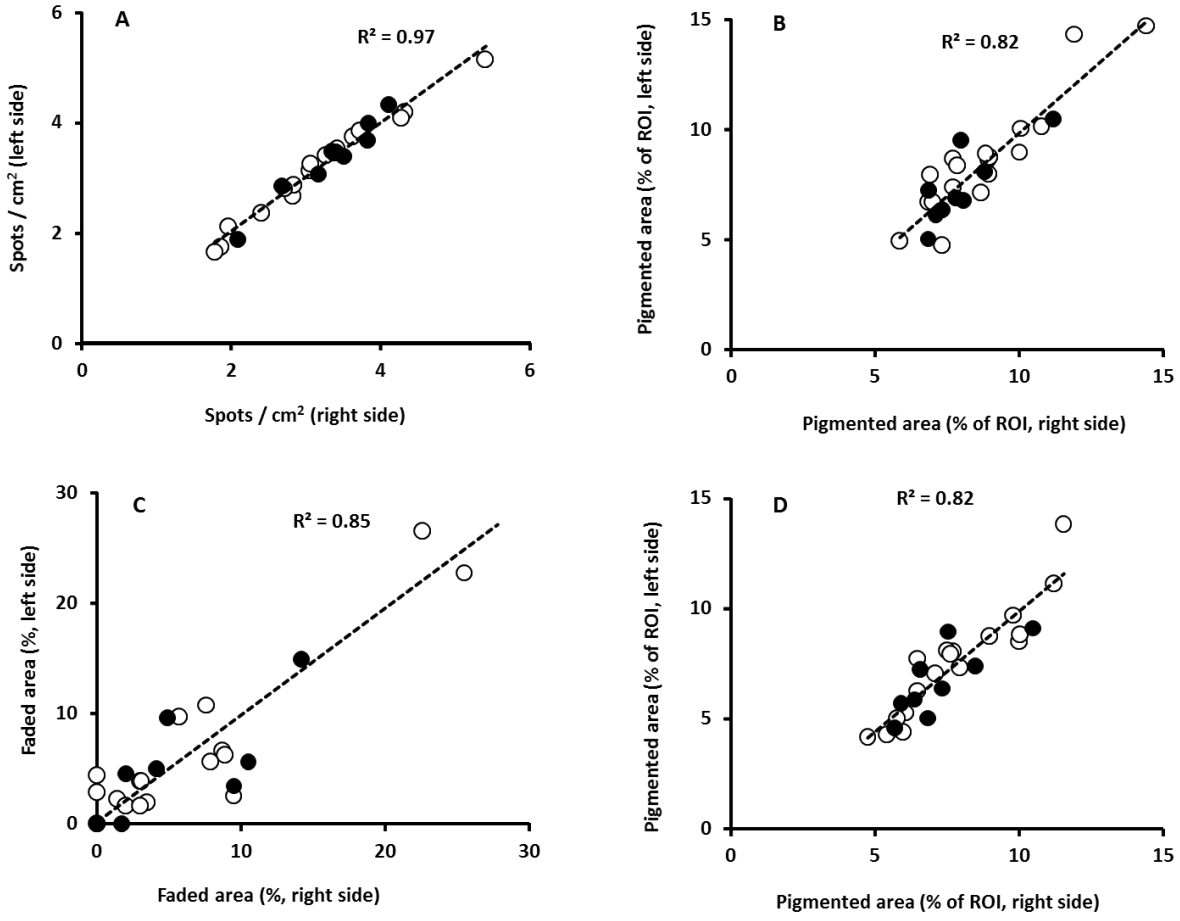

Figure 15 – Lack of bilateral asymmetry, as indicated by linear regression between right and left side melanisation indexes in farmed Atlantic salmon. Based on the lack of an effect of additional acute stress (see above), and no confirmed deviation from linearity (Runs test, p>0,4 in all cases), a single regression line for control (filled circles, n=9) and stress-tested individuals (open circles, n=18) is shown. A: Spot numbers in body side region of interest. B: Estimated original melanised area (before fading) in operculum region of interest. C: Estimated faded are (% of original pigmented area) in operculum region of interest. D: Melanised area after fading in operculum region of interest.

3.2. Association between operculum and body melanisation

Regarding the question whether operculum melanisation is representative for the overall body, I chose to simply look at the strength of the association between individually variable spot counts in the body side ROI and % pigmented areas of the operculum ROI. For this task, the average of registrations from both body sides was used. Results of linear regression is shown in figure 16.

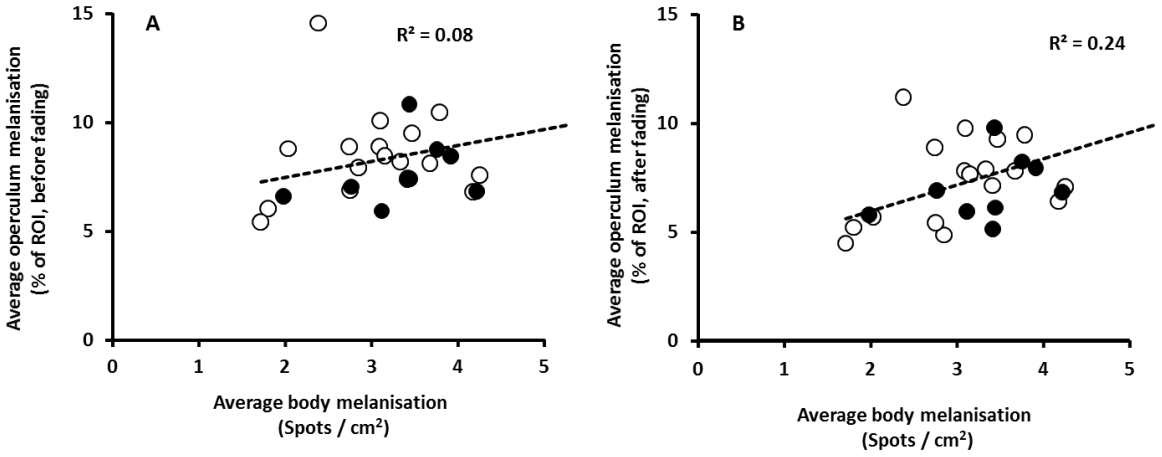

Figure 16 – Relationship between body side and operculum melanisation, as indicated by linear regression between average (right + left side) spot counts in body ROI and estimated average % pigmented area in operculum ROI before (A) and after fading (B). A single regression line for control (filled circles, n=9) and stress-tested individuals (open circles, n=18) is shown.

As can be seen, the strength of the relationship between body and operculum melanisation index was much lower than that between right and left sides within each ROI (> 80% correlation vs. 8% and 24% correlation). Of note, body melanisation was indexed by number of melanised spots, while in the case of the operculum, both spot numbers and their size were combined into % pigmented area. Different physiological mechanisms may regulate spot formation and size, so for comparison, I also assessed the relationship between body and operculum spot counts, which turned out to be somewhat stronger (see figure 17, below).

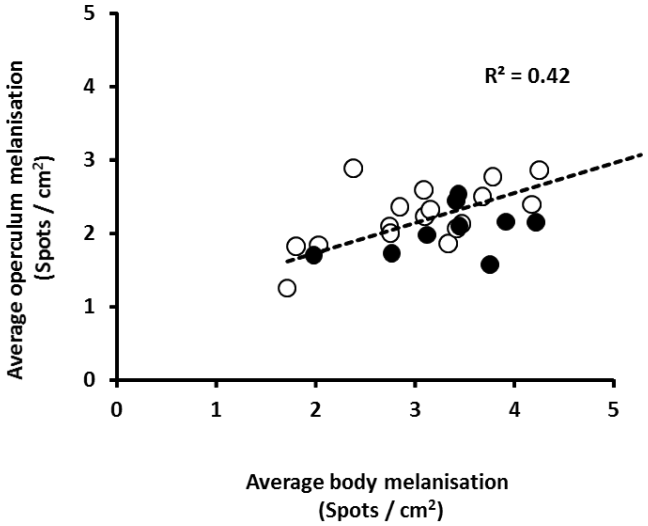

Figure 17 – Relationship between body side and operculum melanisation, as indicated by linear regression between average (left + right side) spot counts in body and operculum ROI. A single regression line for control (filled circles, n=9) and stress-tested individuals (open circles, n=18) is shown.

3.3. The stress-response and fading

A novel observation made during the work with this thesis was that a variable degree of fading of melanised spots on the operculum was seen in a majority of the observed fish. Despite a growing interest in melanisation as an indicator of stress and robustness, this is a phenomenon that to my knowledge has not yet previously been reported in Atlantic salmon. Potentially, given the many links between neuroendocrine regulation of stress responses and pigment synthesis and distribution (see introduction and discussion for details), the phenomenon may be related to the stress experienced by the test-fish during ongoing sampling activities, both long lasting confinement in the purse seine and the additional acute stress-test. Hence, it is worthwhile to look closer at the individual variable degree of fading in both stress-tested and fish sampled directly form the net pen. Of note, in terms of circulating plasma cortisol, both the fish sampled directly from the net pen and the stress-tested fish showed values high above what has been reported for completely unstressed salmon (less than 5ng/ml, see introduction for references). Nevertheless, acutely stressed fish also showed a significant elevation in circulating cortisol compared to directly sampled controls (t-test p=0,004, figure 18, below).

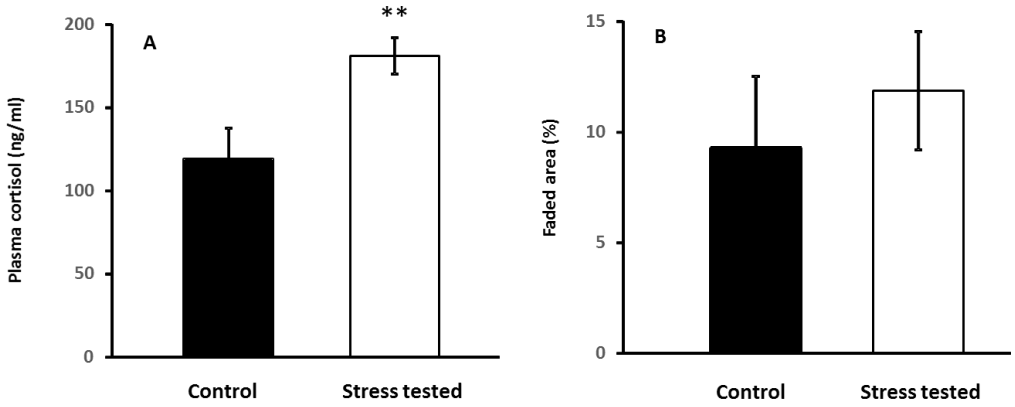

*Figure 18 – A: Circulating plasma cortisol (average ± S.E.M.) in salmon sampled directly from the purse seine ("controls") and fish subjected to an additional acute 20min confinement test (** = p<0,01). B: Fading response expressed as % faded area of estimated originally pigmented area in operculum ROI (average ± S.E.M.) (n.s).*

There was however no such effect of additional stress on average fading response (t-test, p=0,56), but variation around the mean was high for this variable (e.g. S.E.M. >30% of group mean in the "control" group). Testing the tentative hypothesis that this variability was related to the duration of ongoing sampling activity, which mostly relates to time spent in the purse seine, was done by linear regression.

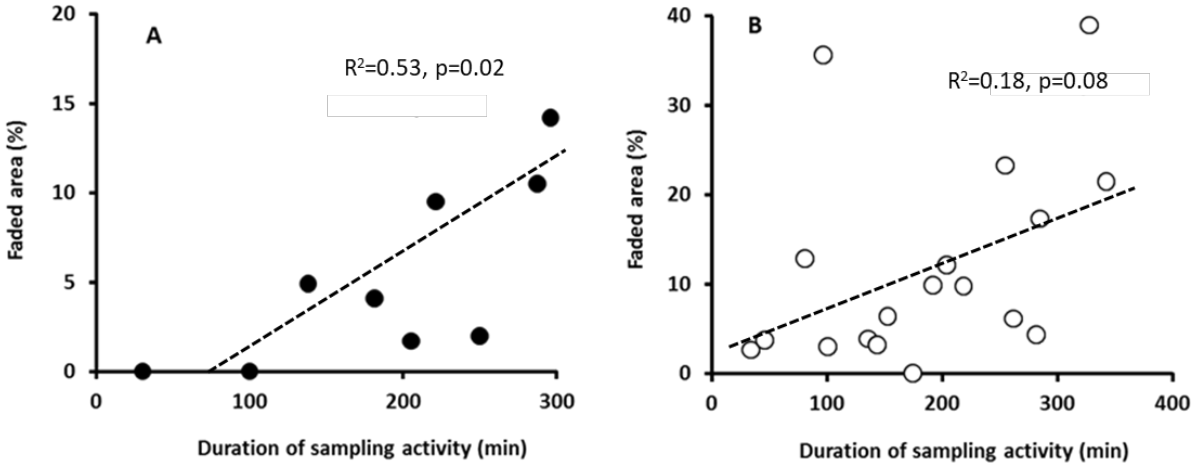

Figure 19 – Relationship between duration of sampling activity and the fading response expressed as % faded area of estimated originally pigmented area in operculum ROI in control fish sampled directly from purse seine (A) and fish subjected to additional acute stress testing (B). Linear regression R^2- and p-values are shown.

As indicated in figure 19, there was only a significant effect of time in the control group. It would appear that this effect is more variable in fish subjected to an additional acute stressor superimposed on the effect of ongoing sampling from seined fish. Regarding putative physiological causative mechanisms underlying fading, within the current data set I was only able to test whether individual variation in the fading response may be explained by plasma cortisol, but there was no correlation between cortisol and faded area in any group (r=44, p=0,23 and r=0,009, p=0,97 in control and stress-tested group, respectively).

4. Discussion

This thesis has revealed likely that fading of melanin-based skin spots in Atlantic salmon occurs during ongoing sampling activities. Furthermore, a method for digital analysis of faded and non-faded spots has been developed, in order to reveal possible lateralisation and association between melanisation of different body regions. The literature review indicates that our observed fading is stress-induced. From the results, it was evident that both the fading and cortisol content increased throughout the sampling session. However, these responses were not correlated, indicating that other processes than cortisol production had direct impact on fading. The thorough literature review finds that the fading is most likely due to activation of MCH-Rs and/or the transport of norepinephrine to the pigment producing cells. In addition, no lateralisation was found, enabling further spot analysis projects to only focus upon one of the sides.

4.1. Material and Methods

4.1.1. Study material and general implications

We were granted permission to sample fish at IMR's research facility on Austevoll, Norway, in which the fish we sampled were originally from other experiments. Therefore, the fish we utilized might have been exposed to unknown stressors before sampling, which might have implications upon our results, especially the cortisol response to stress and related responses. Nevertheless, to what degree such potential stressors affects the general stress level in our sampled fish is not known. While awaiting the outcome of further studies, it must be assumed that the observed fading phenomenon is likely to be present in salmonid fish in general. Underlying molecular mechanisms and evolutionary-ecological implications should be subject to further studies.

4.1.2. Data collection

In total, four sampling session took place at IMR's research facility on Austevoll, Norway. The first sampling session took place the 4th and 5th of June 2018. Before this sampling session, we had decided to utilize ImageJ for image analysis. However, we had little experience with ImageJ, especially regarding how the images had to be shot to enable processing of the images with macros. In addition, time was limited during the sampling, in which we didn't have the time to test images in ImageJ at the start of the sampling. As a consequence, we had to choose how to shoot the images purely on intuition, in which we decided to tilt the individuals in order to enlighten the spots upon the individuals' back. This tilting however, changed the proportions of the fish, meaning that the ROI we were to utilize became out of proportions, which ultimately made several whole samplings unusable for image analysis in ImageJ.

Figure 20 – Fish number 13 from the first sampling session. We didn't have the time to test images whilst sampling, and as a consequence, we had to shoot the images purely on intuition, in which we decided to tilt the individuals in order to enlighten the spots upon the individuals' back. This tilting changed the proportions of the fish, which made the whole sampling unusable for image analysis in ImageJ.

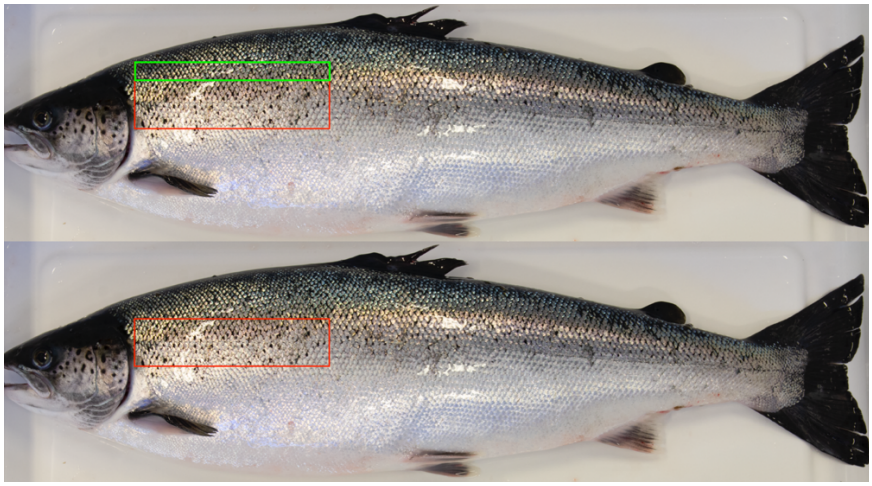

Figure 21 – The tilting-made wrong proportions. The red box on the bottom image is the proposed ROI by Jørgensen et al. (2018) [18]. The green box in the top image displays how our tilting of the fish would affect the size of ROI, in which the green box is the added areal to the ROI.

In addition to the wrong proportions of ROI, our fish were approximately 200 grams on average, whilst the fish utilized by Jørgensen et al. (2018) [18] were at least 1kg on average. During conversations regarding the use of their macro, they argued that the spottiness was not fully developed until the fish had reached a certain size, approximately 1kg. Therefore, the combination of having wrong proportions for ROI and that the fish were way too small, we decided to exclude the first sampling.

The second sampling session took place the 25th of July 2018. Before this sampling session, I had been working with Jørgensen et al. (2018) [18] in order to solve the problems with ImageJ we encountered during the processing of images from the first sampling. However, the fish were still too small, which also made this dataset unusable.

The fourth sampling session was also excluded from this thesis due to bad quality images shot by a handheld camera. ImageJ was not able to process the images with the macro from

Jørgensen et al. (2018) [18]. We were certain that as long as the spots on the lateral side were visible in the images, the handheld camera would be adequate for processing of images in ImageJ. However, ImageJ was not able to process any images, which in turn resulted in that we had to count the spots by hand. Therefore, the quality of the images became utmost important, especially during the differentiation between spots and shadows between fish scales. Nevertheless, whilst doing the manual spot analysis, it quickly became obvious that the quality of the images shot by the handheld camera was of a much poorer quality than first expected, resulting in that the manual spot counting was a very time-consuming process and gave rise to potential artefacts. As a consequence, we decided to exclude these results from this thesis.

4.1.3. Image processing

Jørgensen et al. (2018) [18] developed a macro for spot analysis in ImageJ, and we got to utilize their macro for our images. However, ImageJ was not able to analyse any images. Therefore, I contacted programmers of ImageJ in order to find a solution for enabling proper processing. In short, they said that light settings during a photoshoot is the main determinant for a fully functional processing of images in ImageJ, and when they considered our case, they concluded that our light settings were too dark, which made it impossible for ImageJ to distinguish between spots and shadows between fish scales. As a consequence, all processing of images in ImageJ had to be done manually. A manual interpretation of spots is a subjective spot analysis, which might produce artefacts. Nevertheless, as the spot analysis is based upon one subjective interpretation of each spot, the level of standardization is high.

The programmers' conclusion regarding light condition would have been fairly simple to improve during the photoshoot, but as time was limited, we were not able to test images in ImageJ whilst sampling. Therefore, if we are to do spot analysis in later projects, more time will be invested in testing the macro from Jørgensen et al. (2018) [18] at the start of the sampling session to ensure that our images are analysable.

4.2. Results

4.2.1. The Correlation in Number of Spots

For the spot analysis, we wanted to use the macro from Jørgensen et al. (2018) [18]. The number of spots on the lateral side from the sampling session were on average 91,6/ROI with an average size of 28,53cm^2. However, fish scales can hide melanophores, which in turn can make the counting process difficult, especially the differentiation between spots and shadows in-between fish scales (see figure 11). This problem had most probably been avoided if ImageJ had been able to process the images. Nevertheless, the manual count is to be considered as valid, as any shadows mistaken as spots or spots mistaken as shadows is assumed to ultimately break even, due to the total amount of utilized fish for the analysis.

4.3. Lateralisation

There was no systematic difference between right and left sides nor any effect of acute stress on any parameter. Therefore, it would appear that for any given melanisation index, an analysis including either the left or right side, or an average thereof, would likely yield the same result. Variation around the regression line seem to be generally somewhat higher (i.e., lower R^2 values) for measures of pigmented area expressed as % of ROI in the operculum, than what is the case for counts of spot numbers on the body. As the generally much smaller body spots was not amenable for calculations of % pigmented area, it is hard to determine whether this reflects a general trend for more asymmetry on the operculum, or whether the method for assessing % pigmented area is inherently less precise than spot counts.

In addition, the operculum ROI utilized for this thesis had to be limited to the cheek of the fish due to gill cover deformations (see methods for details). Such deformations are utilized to distinguish between wild Atlantic salmon and farmed Atlantic salmon [44], in which the deformations are most often defined as shortening of gill cover. Therefore, it is assumed that wild Atlantic salmon is a better model animal for spot analysis on operculum, since the whole operculum can be set as ROI.

4.4. Association between operculum and body melanisation

The relationship between body and operculum melanisation index was much lower than that between right and left sides within each ROI (>80% correlation between sides vs. 8% correlation before fading and 24% correlation after fading). In addition, the correlation between average operculum and body melanisation before fading was much lower than the correlation after fading. How come that the correlation after fading was higher than before fading, which should have been the true melanisation? This might be due to the method, as the only exact data basis for this thesis is the melanisation "after fading". Therefore, we had to propose the original size of the faded spots (see figure 23). As a consequence, in order to find degree of fading, the method required proposing the size of spots before sampling induced fading. This method is not preferred, as subjective measurements will easily produce artefacts. Therefore, new projects are urged to photograph the operculum of fish at the start of spot analysis experiments, in order to get the exact size of unfaded spots. Thereby, a much more precise before/after-scenario is obtainable, which would give rise to much more detailed and precise data.

In addition to the large differences in the average number of spots/cm^2 between wild Atlantic salmon and farmed Atlantic salmon reported by Jørgensen et al. (2018) [18], they also found that the spots were a lot smaller and more randomly scattered in farmed fish. Furthermore, the spots on the lateral are generally masked by silvery scales, which hides, either in part or totally, pigment particles and even small spots. This masking of spots made it impossible to give a valid subjective interpretation of the true body melanisation, as opposed to the spots on the operculum with no prominent scales. Consequently, we assume that if ImageJ had been able to process our images, the true body melanisation would have been obtainable. However, we also assume that the same observed fading, as the one on the operculum, is initiated by the same mechanisms on the body as well. If so, the fading of spots on the operculum should correlated to a potential fading on the body. Therefore, further studies are urged to determine if fading also occur on the body, and finally, which melanisation index is dependent on which physiological mechanism.

4.5. The stress response and fading – potential mechanisms

Our novel observation of spots fading on the operculum of Atlantic salmon during an ongoing sampling, indicates that the sampling itself induced the fading. The sampling involved lowering a purse seine into a sea cage in order to capture a representative amount of fish, before crowding them to enable easy access for hand netting. Both crowding and hand netting of fish is known to cause a stress-response [3], and as the fish were kept in the same position for a longer period of time, it's assumed that the stress-response became repetitive. From the results, all fish had a much higher level of circulating cortisol than under normal conditions, supporting the assumption.

Through a thorough research in available literature, the main regulation of visual pigmentation is found to be the activation of G_s-protein coupled receptors and G_i-protein coupled receptors in the chromatophores cell wall, in which the synthesis of cAMP is either stimulated or inhibited. Under normal conditions, only the G_s-protein coupled receptors are continuously activated, leading to a continuous stimulation of cAMP-synthesis, which in turn maintains the melanosome dispersion. However, should the G_i-protein coupled receptors be activated, the cAMP-synthesis will be inhibited, which in turn ceases melanosome dispersion, resulting in melanosome aggregation. The G_s-protein coupled receptor MC_1 in melanophores is the only receptor responsible for the melanosome dispersion, which is activated by α-MSH. However, a stress-induced activation of the G_s-protein coupled receptor MC_2 in corticosteroidogenic cells will ultimately produce the antagonist ASP. ASP competes with α-MSH in binding to MC_1, and should the antagonist ASP bind to MC_1, the continuous synthesis of cAMP is hindered, which in turn ceases the melanosome dispersion. Furthermore, the repetitive stress-response will also synthesise MCH, the main antagonist of melanosome dispersion. Activation of the G_i-protein coupled MCH-R will inhibit cAMP-synthesis, inducing melanosome aggregation. The same scenario is seen for norepinephrine, in which norepinephrine binds to a G_i-protein coupled receptor, which inhibits cAMP-synthesis. However, the aggregation of the melanosome due to norepinephrine was found to be an indirect mechanism, as norepinephrine is mainly involved in the transport of ATP to the cell (see introduction for details). Therefore, based upon the literature, the direct fading response can be restricted the mechanisms involved in activation of MC_1 and MCH-R.

Khan et al. (2016) [12] proposed a model in which pigmentation in salmonids might be influenced and regulated by MRAP-binding affinity to two different variants of MC_1, namely MC_1-176Met and MC_1-176Leu. They found that the 176Met-variant had a much better binding affinity to MRAP than the 176Leu-variant, and that only the 176Met-variant was evident within reactive HR-fish, whilst only the MC_1-176Leu-variant was evident in proactive LR-fish. This would in turn greatly influence the total amount of MRAP, in which the reactive HR-fish have

much less circulating MRAP than the proactive LR-fish. Therefore, as the activation of MC_2 in corticosteroidogenic cells is dependent on available MRAP, they argued that during a stress-response, much less MC_2 would be activated within reactive fish than in proactive fish, which ultimately results in less cortisol synthesis. Consequently, less cortisol will be processed into ASP, the antagonist of α-MSH within the activation of MC_1. Thereby, more MC_1 will be activated, leading to more pigmentation. As the proactive LR-fish have more circulating MRAP, more MC_2 will be activated, which in turn results in more cortisol synthesis. Thereby, more cortisol would be processed into ASP, which ultimately will reduce the activation of MC_1. Consequently, LR-fish will have less pigmentation than HR-fish. This might help to explain the general differences in body and operculum melanisation. However, previous studies have found a negative correlation between cortisol response and catecholamine response, in which the reactive fish, with a low cortisol response, will have an opposing high catecholamine response, whilst the proactive fish, with a high cortisol response, will have an opposing low catecholamine response. During a stress-response, both the synthesis and transport of catecholamines to the circulatory system is known to be highly efficient. Consequently, the catecholamine transport of ATP to the cells, which involves binding to a G_i-protein coupled complex, should be visible as fading within a fairly short period of time (approximately 30 minutes, see introduction for details). This assumption is partly supported from the results, as no correlation between fading and cortisol was evident (R=0,44, p=0,23 and R=0,009, p=0,97 in control and stress-tested groups, respectively), indicating that other mechanisms are involved. Therefore, we assume that HR-fish will show a fading response faster than LR-fish, due to the secondary effect from ATP-transport by norepinephrine. However, we were not able to sample catecholamine content from our sample, and consequently, no conclusions can be made. Consequently, further research is urged to determine if the stress-induced fading is under steroidogenic, catecholaminergic, or neurohormonal control. This might give a conclusion to the assumption.

Considering the results, only the degree of fading within the control group was highly correlated to the sampling duration (r=0,73), while the degree of fading within the stress-tested group was somewhat more moderately correlated to the sampling duration (r=0,42) (see figure 19). Furthermore, the linear regression did only find a significant correlation within the control (p=0,027 for controls, p=0,083 for stress-tested). This finding indicates that additional stress will remove the effect of time, meaning the fading is more dependent on individual response to the stress-test, rather than time. Based upon the literature, we find that fading in general is due to the activation of either MCH-R or norepinephrine-receptors. However, there was no available literature that focused upon the time from a stress-response to MCH-synthesis. Therefore, as time was plentiful (approximately five hours) during sampling, we cannot outlook any of the molecular processes, nor make any conclusion of which specific molecular process was the main fading agent. Consequently, more research regarding which molecular processes

control the stress-induced fading is crucial in order to establish fading of spots as a marker of stress and welfare in fish aquaculture.

Furthermore, our finding might have implications for the fairly new identification method, in which the spots are utilized as ID. Assuming that the method of sampling the ID is stressful, stress-induced fading will inflict the differentiation of individuals within a population, which in turn makes for large biases. Therefore, other ID-measures are currently preferable.

4.6. Conclusions

Based upon the literature review together with the results which concluded that the fading increased significantly throughout the sampling, we argue that the observed fading of spots was stress-induced. The observations of fading were made in fish chronically disturbed by ongoing sampling activities, e.g. crowding and netting, the same activities as during delousing procedures. Hence, it would seem that the spots do not only reflect individual stress coping ability, they also convey information about recent exposure to stressful experiences. Furthermore, as the fading was visible by the naked eye, our finding might be utilized as a new stress-indicator. This would give rise to a much easier method for detecting stress, as opposed to blood plasma sampling, which is time-consuming and requires methods difficult in field. In addition, the fading and cortisol content did not correlate, indicating that other molecular mechanisms than cortisol production are directly involved in fading. Through the thorough literature review, we argue that the fading was due to an activation of MCH-Rs and/or norepinephrine-receptors.

We did not find any lateralisation of spots, which in turn means that further spot analysis will only have to be based upon one of the sides. However, further research is urged to utilize the average of the individuals, as fading might inflict the result.

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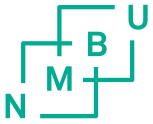

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