Master's thesis

NTNU
Norwegian University of Science and Technology
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Immunological effects of acute stress and immunostimulant feed in Atlantic salmon (*Salmo salar*)

Master's thesis in Ocean Resources (MSOCEAN)

Supervisor: Rolf Erik Olsen

Co-supervisors: Kjell Inge Reitan, Atle Lillehaug & Viviane Verlhac-Trichet

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Abstract

Infectious disease is a major challenge for the aquaculture industry, and contributes to substantial losses. With the aim of limiting the burden of disease, acute stress exposure and immunostimulatory feeds hold promise in enhancing the immunocompetence of the recipient. In the present experiment, post-smolts of Atlantic salmon were fed either a control feed or a treatment feed supplemented with β-1,3/1,6-glucan, nucleotides, vitamin C and vitamin E for 53 days. Cell counts of peripheral blood samples and transcriptional analyses of head kidney tissue samples were conducted before, 23 hours after and 71 hours after acute stress exposure to assess dietary and stress-mediated effects on chosen immunological parameters.

The growth performance was unaffected by the assigned diet. The proportion of circulating immature erythrocytes was significantly higher in the treatment group, which was likely correlated with the higher haematocrit measured in the same group. None of the counted leukocyte cell types were affected by the diet. Acute stress induced a significant reduction in the proportion of circulating lymphocytes, likely due to a migration into peripheral tissues, and displayed close to a full recovery within 71 hours. Phagocytes (monocytes and neutrophil granulocytes) displayed a comparatively weak response to acute stress, with a significantly increased proportion 71 hours post stress in the treatment group only. A significant interaction between the diet and acute stress was found in the thrombocyte proportion, which displayed an increase in the control group and a decrease in the treatment group 23 hours post stress. None of the target genes (IL-1β, IFNγ, TNFα, Lys C II, IL-10, IκBα, MHC I, CD8α and hsp 70) were differentially regulated in the head kidney from any dietary effects. Acute stress induced a significant decrease in the number of transcripts for all the target genes except IL-1β. This was suspected to be a result of leukocytes being released from the head kidney as part of the stress response. The effect of acute stress on gene regulation could therefore not be determined. 71 hours post stress mirrored a gradual return of leukocytes to the head kidney.

Sammendrag

Sykdom er en stor utfordring for akvakulturnæringen, og forårsaker store tap. Med et mål om å redusere belastningen av sykdom, kan utsettelse for akutt stress og fôring av immunostimulanter potensielt øke immunkapasiteten til mottakeren. I det aktuelle forsøket ble post-smolt av Atlantisk laks gitt enten et kontrollfôr eller et behandlingsfôr supplert med β-1,3/1,6-glukan, nukleotider, vitamin C og vitamin E i 53 dager. Celletellinger av perifert blod og analyser av hodenyretranskripsjon ble utført før, 23 – og 71 timer etter utsettelse for akutt stress for å vurdere effektene av diett og stress på valgte immunparametere.

Vekst var ikke påvirket av de ulike diettene. Andelen umodne erytrocytter i sirkulasjon var signifikant høyere i behandlingsgruppen. Denne observasjonen var sannsynligvis korrelert med de høyere hematokrit-verdiene som ble observert i den samme gruppen. Ingen av de telte leukocytt-populasjonene var påvirket av dietten. Akutt stress induserte en signifikant nedgang i andelen sirkulerende lymfocytter, som trolig skyldes en videre migrering til perifert vev, og viste til en nær fullverdig tilbakekomst innen 71 timer. Fagocytter (monocytter og nøytrofile granulocytter) viste til en forholdsvis svak respons på akutt stress, med kun en signifikant økning etter 71 timer i behandlingsgruppen. En signifikant interaksjon mellom diett og stress ble funnet for andelen trombocytter, som viste til en økning i kontrollgruppen og en nedgang i behandlingsgruppen 23 timer etter stress. Ingen av de valgte genene (IL-1β, IFNγ, TNFα, Lys C II, IL-10, IκBα, MHC I, CD8α og hsp 70) var regulert i hodenyra som følge av diett. Akutt stress induserte en signifikant nedgang i antall transkripter for alle de valgte genene utenom IL-1β. Det ble antatt at dette skyldtes en frigjøring av leukocytter fra hodenyra som en del av stressresponsen. Effekten av akutt stress på genregulering i hodenyra kunne derfor ikke avgjøres. 71 timer etter stress viste til en gradvis tilbakekomst av leukocytter til hodenyra.

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Introduction

The state of aquaculture

Aquaculture has currently the largest growth of any major food production sector with an annual 5.8% increase in production volume, and stood for a production of 110.2 million tonnes (of which 54.1 million tonnes constituted finfish) in 2016 (1). A continued growth in the aquaculture sector will be essential for meeting the nutritional demand of an increasing world population and reducing the amount of losses over the course of a production cycle would greatly benefit the sustainability of the industry. During 2018, 1 253 000 tons of Atlantic salmon were produced in Norway (2). During the sea-phase of the production cycle the same year, a mortality of 14.7% was recorded, in which infectious disease played a major role (2). This number does not include losses due to escapes, rejects and factors such as lice-treatment related losses or slaughtering for disease control purposes (2). The development of effective vaccines have been critical for alleviating the impact of infectious diseases. However, the development of effective vaccines against rapidly evolving RNA viruses can be extremely challenging (3). Thus, it should come as no surprise that the most influential infectious diseases currently ravaging the Norwegian shore are caused by viral agents, such as the RNA virus *Salmonid alphavirus* (SAV), being the responsible agent for the ongoing pancreas disease (PD) (4) pandemic on the western and mid-Norway shoreline (2).

The immune system

In order for any immune system to function, it needs to be able to distinguish between self and non-self cells and molecules, and then eradicate the material deemed as foreign (5). Non-self cells include extracellular pathogens, such as a bacteria, and formerly self cells supporting an uncharacteristic protein production, which indicates that the cell is either cancerous or infected by virus. Viral particles transitioning between cells can also be recognised as foreign. The immune system consists of two interacting components generating the innate and the acquired immune response (5). The innate immunity is non-specific and has a broad range of action, while the acquired immunity initiate target specific responses through antibody production and receptor recognition (5). The mammalian and the teleost immune systems share many similarities, and relevant comparisons can often be made. However, certain distinctions ought to be kept in mind. The hematopoietic bone marrow and lymph nodes are not present in fish, where the anterior kidney serves a major lymphoid role, together

with the thymus, spleen and mucosa-associated lymphoid tissues (6). The anterior part of the teleost kidney (head kidney) is comprised of, among other types of tissues, lymphoid and myeloid tissues (6). It shares many of the immunological functions of the mammalian bone marrow, including haematopoiesis (5).

Fish, like other vertebrates, possess the white blood cell lineages of granulocytes, monocytes, thrombocytes and lymphocytes (7). Which cell types that are present in different fish species and which cell type one can expect to encounter in different body compartments is often unclear. Part of the challenge is probably that there is no well-established consensus on the identification of cell types in fish and how to categorize them. The development of targeted monoclonal antibodies (mabs) for the classification of leucocyte subpopulations in fish is currently under development (8, 9). In the meantime, morphological classification of leucocytes can be employed, as in the present study. However, this methodology is not devoid some level of uncertainty.

Innate immunity

Constituents of the innate immune system include the cell types of granulocytes, monocytes, thrombocytes and certain lymphocytes as well as humoral components such as lysozyme and the complement system. Granulocytes include neutrophils, eosinophils and basophils. As their name suggests, these cells are characterized by the presence of granules in their cytoplasm, containing antimicrobial agents and enzymes (10). The site for granulopoiesis in teleost fish is in the pronephros and head kidney (10). The presence of basophils in teleosts are somewhat unclear, but neutrophils are common (5, 10). For most teleost species, eosinophils are rarely found to be present in circulation (10). The neutrophils of many teleosts, including trout, share the subdivided (lobed) nuclear morphology as can be seen in mammalian neutrophils (10). These are mobile cells, often first responders, and mediate the acute inflammatory response (10). After vasodilation, they migrate from the pronephros into circulation, and onwards to sites of inflammation or injury by chemokinesis (10). Neutrophils are potent phagocytes and harbour peroxidase systems, allowing the release of free radicals (oxidative burst), toxic for ingested material or cells in close proximity (5). Increased peripheral neutrophil numbers can indicate inflammation, but netrophilia is also a response to stress (10).

Monocytes are among the largest leucocytes. They are circulating cells that secrete pro-inflammatory cytokines and show some phagocytic capabilities (11). Monocytes are equipped with chemokine and adhesion receptors that mediate migration to tissues during infection, where they can mature to macrophages (11). Hematopoietic progenitors may also mature directly to macrophages (11). Macrophages constitute most often immobile cells with established populations situated in the kidney, spleen, liver, walls of the atrium and intestine and in the mesenteries (5). They harbour a broad range of pattern recognition receptors (PRRs), thereby making them efficient phagocytes and mediators of inflammation (11). Depending on the stimuli, fish macrophages can develop into four different activation states (12).

Microbial stimuli, detected by membrane receptors, induces the development of innate activated macrophages, which are characterized by increased phagocytic and oxidative burst activity, together with an increased production of pro-inflammatory cytokines (12). The combination of microbial stimuli and the presence of γ-interferon (IFNγ) induces the development of classically activated macrophages (12). The additional presence of tumour necrosis factor-α (TNFα) further facilitates pro-inflammatory cytokine production in these macrophages, including the interleukins (IL) IL-1, IL-6, IL-12, IL-23 and more TNFα (12). The combination of a pro-inflammatory cytokine milieu and the presence of pathogen-associated molecular patterns (PAMPs), microbial stimuli, synergistically promote phagocytosis and oxidative burst activity in macrophages (12). The presence of the cytokines IL-4 and IL-13 induces the development of alternatively activated macrophages (12). These cells continue to metabolize L-arginine, but through another metabolic pathway, thereby reducing the oxidative burst activity of neighbouring macrophages through substrate competition (12). Finally, the presence of IL-10 induces the development of regulatory macrophages, playing roles in the dampening of inflammation (12). Secondary signals such as glucocorticoids and apoptotic cells further facilitates this response (12). This framework of different macrophage activation states have to varying degrees been demonstrated in fish, and is based on work done on models of mammals (12). However, the presence of cytokine analogues in fish indicates that similar mechanisms may be present (12).

Thrombocytes are primarily involved in blood clotting, but also possess phagocytic functions (5). Nagasawa et al. (2014) characterized sophisticated phagocytic capabilities of thrombocytes in common carp and an amphibian model (*Xenopus laevis*) (13), demonstrating

its importance in the innate immunity of lower vertebrates. Comparable studies for Atlantic salmon was not found, but the thrombocyte function is presumed to be similar in this species, as Atlantic salmon would be situated between these two model organisms phylogenetically. An analogue to the mammalian natural killer (NK) cell is reported to be present in fish (5). These are lymphocytes whose function is primarily in the innate immune system, to enzymatically degrade the membranes of cells displaying cancer or pathogen infection (5).

Acquired immunity

The acquired immune system is composed of two classes of lymphocytes, B-cells and T-cells (5). B-cells initiate specific responses to extracellular pathogens by maturing to plasma cells that produce antibodies (5). Mature B-cells are produced in the head kidney (14). These cells then migrate through the circulatory system to the posterior kidney and the spleen, where upon antigen-exposure they can develop into short-lived antibody-producing plasmablasts or plasma cells (14). Long-lived plasma cells seems dependent upon successful resettlement in the head kidney, where they can propagate a sustained memory response (14). Li et al. (2006) discovered phagocytic capacities in the B-cells of rainbow trout, comparable to that of professional phagocytes, i.e. macrophages and neutrophils, demonstrating innate functionalities in these cells as well (15). T-cells initiate specific responses to intracellular pathogens through the recognition of a specific antigen presentation (5). The cytotoxic activities of T-cells in fish seems to involve the same patterns of functionality as in the mammalian system (9, 16), where the $CD8^+$ co-receptor on cytotoxic T-cells will bind to MHC-I (class I major histocompatibility complex) molecules of nucleated somatic cells (17, 18). MHC-I are membrane-bound glycoproteins that presents peptides from its intracellular protein production (18). If the antigen profile presented on the MHC-I molecule reflects cancer or the presence of intracellular pathogens, the cytotoxic T-cell will induce apoptosis in the cell (17).

Lymphoblasts, which are enlarged antigen-stimulated T-cells (19), and plasma cells are reported to be present in circulation (5, 7, 14). These appear to originate from the head kidney (5), potentially indicating further movement of mature antibody-producing cells situated in this organ. Pettersen et al. (2000) found that IgM-specific mabs bound to mononuclear cells in peripheral blood samples of Atlantic salmon, which adds additional support for the suspected presence of plasma cells in circulation (8). Zwollo et al. (2005), on the other hand, suggested

that trout blood only contain non-Ig-secreting cells and that plasma cells are not present in circulation (20). Consequently, the subject is still a matter of debate.

Cytokines

Humoral signalling molecules are essential for the function of the immune system (5). Cytokines are signalling proteins that is secreted by and elicit a response in every nucleated cell (21). According to their functions, five classes of cytokines can be classified; lymphokines, pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines and growth factors (21). Lymphokines are secreted by T-cells to regulate the immune system, pro-inflammatory cytokines elicit inflammatory responses, anti-inflammatory cytokines dampens inflammatory responses, chemokines attracts the migration of immune cells to specific tissues and growth factors promotes cell survival (21). This and the following sections is primarily based on literature involving mammalian models, but the overarching functions are presumed to be similar in teleosts, as all of the major cytokine families have been found to be present in fish and no apparent discrepancies have been characterized (22).

Before highlighting the functions of specific cytokines relevant for this paper, the regulatory mechanisms of the transcription factor nuclear factor-kappa B (NF-κB) ought to be introduced. A considerable number of cytokines is either regulated by, or regulates the activity of NF-κB (23). NF-κB usually resides in an inactive form in the cytoplasm, bound to an IκB protein (23). The activation of NF-κB is then, in most cases, initiated by the phosphorylation and release of the IκB protein, which allows NF-κB to migrate into the nucleus and regulate gene expression, facilitating inflammation (23, 24). This gene regulation also plays a crucial role during the development and survival of immune cells by facilitating the transcription of antiapoptotic factors, and also in some cases proapoptotic factors (23). These antiapoptotic factors are essential for a functional innate and acquired immune response, by assisting for example neutrophil survival during mobilization and mature B-cell longevity in propagating a memory response (23). The proapoptotic factors seems to be important in early T-cell selection (23). PRR recognition of PAMPs may also activate NF-κB (23). After a recognition of a potential infection, and a subsequent activation of NF-κB, pro-inflammatory cytokines are synthesised, such as TNFα (23). NF-κB is also seemingly involved in negative feedback loops that suppress inflammation by, among others, the downregulation of IFNγ production in NK cells (23).

IL-1β is a systemic pro-inflammatory cytokine (25) which is primarily released in response to the presence of PAMPs (26), but is also regulated by non-microbial factors, such as other cytokines (25). It is expressed and synthesised in the innate immune cells of monocytes and macrophages (26). Local effects of IL-1β has also been described in fish, where it can serve as a chemoattractant for leukocytes (22). Peritoneal injections lead to a migration of phagocytes to the area as well as increased phagocytic and lysozyme activity in macrophages (22). Locally upregulated TNFα and IL-1β expression has also been characterized in muscle tissue injected with an IL-1β-encoding expression plasmid (22). As well as serving pro-inflammatory purposes, IL-1β has also been shown to regulate its own activity by inducing the synthesis of anti-inflammatory cytokines, such as IL-10 and TGF-β1b (22). IFNγ is a cytokine secreted from T-cells and NK cells with a vast array of functions (27). IFNγ stimulates the activation of NK cells and macrophages, in addition to stimulating antigen presentation of MHC-molecules and thus indirectly affecting the activity of cytotoxic T-cells (27).

TNFα is another pro-inflammatory cytokine which can activate neutrophils and enhance macrophage and NK cell function (28). It is synthesised by macrophages in response to PAMPs, such as lipopolysaccharide (LPS) (28). Its mode of action involves an upregulation of inflammatory cytokines, such as IL-1β, IL-8, IL-17C and itself, as well as activating NF-κB (22). Some growth factor-like and chemoattractant functionalities has also been demonstrated, where TNFα promoted macrophage survival in zebrafish and likely through secondary agents, stimulated the recruitment of leucocytes to tissues (22). IL-10 is a pronounced anti-inflammatory cytokine (29, 30) synthesised by most cells of the innate and acquired immune system (31). It inhibits the production of pro-inflammatory cytokines, such as IL-1 and TNFα, and the activation of NF-κB in macrophages activated by LPS and IFNγ (24, 30). Similar inhibitory effects have been observed in monocytes and neutrophils (29). IL-10 has been shown to stimulate the development of B cells (25, 31) and $CD8^+$ (cytotoxic) T cells, while inhibiting the function of $CD4^+$ (helper) T cells (29).

The stress response

The stress response involves a range of behavioural and physiological changes suited to overcome a threat (32). Stressors are intrinsic or extrinsic stimuli that induce a stress

response, and include sudden or extreme changes in the physical environment, animal interactions, human disturbance and pollutants (32). Three phases of the stress response can be classified; the primary, secondary and tertiary stress response. The primary stress response involves the perception of a stressor and the subsequent activation of brain centers, which result in the release of the catecholamines (CAs) adrenaline and noradrenaline, and corticosteroids, the major one being cortisol in fish (32). The secondary stress response involves the effect of these hormones on the blood and tissue level (32). Long-lasting (chronic) and particularly severe stress events can elicit a tertiary stress response, which includes impairment on growth, reproductive development and immune function as well as a reduced capacity to tolerate further stress (32).

CAs are mainly produced and stored in chromaffin cells, situated in the head kidney and in the walls of the posterior cardinal vein (32, 33). The primary mechanism for CA secretion is through sympathetic nerve signals, releasing the neurotransmitter acetylcholine which upon receptor binding to chromaffin cells triggers exocytosis of catecholamine-containing granules (33). CA release is a near immediate response, where soaring levels in circulation can be detected in a matter of a few minutes (32). Normally, the half-life of circulating CAs is below 10 minutes, but in situations of chronic stress, the elevated CA levels may persist for days (32). CA effects on tissues include increased oxygen uptake and ventilation rate of the gills, accompanied by increased haemoglobin affinity to oxygen, due to a lowered pH in blood (32). Haematocrit levels rise as the spleen contracts, releasing stored erythrocytes into circulation (32). The liver is also stimulated to release stored glucose into circulation (32).

In addition to the release of CAs, the stress response involves the hypothalamic-pituitary-interrenal (HPI) axis, with the main end product being the corticosteroid cortisol (32). Briefly summarized and simplified, this endocrine pathway functions as follows. A stressor is perceived, which stimulates the release of corticotropin-releasing factor (CRF) from cells situated in the hypothalamus, mainly the preoptic area (34). Axons connecting the CRF-producing cells and the pituitary gland are present in fish, which allows localized release of CRF through synapses (34). CRF stimulates the secretion of adrenocorticotropic hormone (ACTH) in the pars distalis, whose output is regulated by dopamine (34). ACTH is then transported with the blood stream to interrenal tissue, intermingled with chromaffin cells along the cardinal veins in the head kidney, where it signals the production and release of cortisol (34). The time it takes for circulating cortisol levels to rise is longer than that of CA

release, and a spike occurs minutes to an hour after a stressor is perceived (35). Cortisol exhibit numerous functions in different tissues and organs, by acting on the ligand-dependent transcription factors of corticosteroid receptors (CR) (36). It affects the chloride cells in the gills by enhancing ion export in seawater and ion intake in freshwater (32), reverting the hydromineral imbalance caused by the CA-induced increased permeability of the gills. In the liver, cortisol interacts with glucocorticoid receptors (GR) which upregulates metabolic pathways such as gluconeogenesis (35, 37), helping to sustain the elevated energy demand. Data strongly suggest a proteolytic function of cortisol in the liver and white muscle tissue as well as peripheral and hepatic lipolytic functions (35). Reduced nutrient uptake from the intestine is also associated with heightened cortisol levels (35). Needless to say, stressful environments do not promote solid growth conditions, and rather shifts the metabolic framework towards expenditure.

Heat-shock proteins (hsp) are a highly conserved family of proteins with a variety of housekeeping functions within every cell, the most prominent function being involvement in protein folding (38). Also commonly referred to as stress proteins, they are heavily regulated by cellular stressors (38). Shifts in temperature, metabolic stress and the presence of toxic compounds upregulate their synthesis, where prevention of protein denaturation and facilitation of further protein synthesis becomes essential tasks (38). Hsp 70 has been found to be the most prominent stress-induced protein (38). Their presence also seems to protect cells from oxidative damage sustained from free radicals (38). An interaction between the physiological stress response and the cellular stress response has been documented in fish, where elevated levels of cortisol were found to supress the production of hsp 70 in heat-shock treated cells in, among others, rainbow trout (39). However, it would not seem evident that cortisol affects hsp 70 transcription under normal circumstances (40).

Endocrine tissue (chromaffin and interrenal cells) lies embedded in hematopoietic tissue in the head kidney, allowing for direct paracrine interactions between the two systems (34). Leucocytes also express receptors for CAs and corticosteroids (41). As has been described in humans (42), acute stress is related to a mobilization and enhanced immune response, while chronic stress has mainly suppressing effects on the immune response, rendering the organism more susceptible to disease. Similar trends are seen in teleost fish (43). Mediated by CAs, leucocytes, together with erythrocytes, are released into the blood stream (43). Lymphocytes and monocytes are then transported to outer tissues such as the skin, while the numbers of

circulating neutrophils continue to increase (41, 43). Evolutionarily, the migration of immune cells to the skin can be regarded as an anticipatory response to a potential injury and subsequent pathogen exposure, as stressors, such as the presence of a predator, raises the likelihood of sustaining external injuries. The early signalling hormones from the HPI axis, CRF and ACTH have been reported to affect immune function (43). CRF has been shown to promote leucocyte aggregation and increase the adhesiveness of cells, while also suppressing proliferation and NK cell activity (43). ACTH has been shown to promote TNFα and IL-6 transcription, while inhibiting IL-1β transcription (43). ACTH has also been reported to enhance respiratory burst activity in trout macrophages (43). Cortisol, on the other hand, has mainly suppressive effects on immune function, including an inhibition of leucocyte migration and cytokine release, and in induction of apoptosis (43). Specifically, the NF-κB pathway and the production of IL-12, IFN, IL-1 and TNF is reduced as a consequence of cortisol activity, while the anti-inflammatory IL-4 and IL-10 may have heightened synthesis (25, 28, 43). Glucocorticoids both upregulate IκBα protein levels and interact with receptors that inhibit the function of activated NF-κB subunits (24). Although cortisol generally promote apoptosis in leucocytes, antiapoptotic mechanisms for neutrophils has also been reported (44). As cortisol mainly functions as an immunosuppressive agent, and due to energy not being allocated to the production of antibodies, proteins and leucocytes under a sustained stress response, chronic stress is often associated with a reduced capacity for the organism to handle pathogen exposure (43)

Immunostimulants

Feeding immunostimulants can potentially increase disease resistance (45, 46). The idea is that a proactive presentation of a danger signal to the immune system would allow for a better response to a subsequent pathogen exposure, as the constituents of the immune system is already in an "alert" state. However, as briefly covered in the previous sections, pro-inflammatory responses are often associated with negative feedback-loops. If the pendulum is swinging towards an anti-inflammatory milieu at the time of pathogen exposure, the result would be the opposite of what was intended. Although not the norm, anti-inflammatory responses has been reported from immunostimulant administration (47). Exhaustive effects due to high dosages has also been reported (48). In other words, one ought to tread lightly and investigate thoroughly before artificially mediating immune systems as the effects may ricochet.

β-glucan is a polysaccharide of glucose linked by β-glycosidic bonds (46). It is a structural component in cell walls of a wide array of organisms, including species of plants, seaweed, fungi, mushroom, bacteria and most importantly, yeast (46). Depending on its origin, these β-glucan molecules varies in structure, resulting in differences in biological activities (46), where yeast-derived β-glucan has received the most scientific attention. Generally, it would seem as insoluble β-glucans of higher molecular weights tend to be the most biologically active (46). Three routes of administration has currently been employed, being oral administration, injection and immersion (47). While orally is the most practical route of administration, there are some uncertainties in regard to what extent different β-glucans is digested and/or absorbed by the intestine (47). The salmonid intestine certainly has the capacity for uptake, as laminaran (β-1,3-glucan) accumulation in organs such as the spleen has been demonstrated after oral administration (47). β-glucans has been reported to stimulate pro-inflammatory responses in its recipient (45-47, 49) and also anti-inflammatory responses, especially in cases with an ongoing infection (47). It has also been found that feeding low doses of β-glucan to rainbow trout (*Oncorhynchus mykiss*) can potentially alleviate the supressing effects of stress on the immune system (48, 50).

β-glucan is a PAMP and can bind to scavenger receptors (SR), specifically dectin-1, which then mediates a pro-inflammatory response (51). However, the presence of dectin-1 hasn't been found in species other than mammals (52), so the exact binding mechanism of β-glucans in teleost fish is therefore yet to be discovered (47). Engstad & Robertsen (1993) characterized the presence of a receptor in Atlantic salmon macrophages that can recognize and bind yeast β-1,3-glucan (53). β-glucans is commonly used as a feed additive in the aquaculture industry, but its documented effects are somewhat inconsistent, and seems to be affected by an array of variables, such as species, season, concentration, period and means of administration, structure and digestibility of the β-glucan molecule and the immunological state of the recipient (45-48). A further exploration of β-glucan's potential to mediate the immune system is therefore an important field of study.

Nucleotide and vitamin supplementation

Nucleotides are essential compounds for most biochemical processes, and is present as free nucleotides or nucleic acids in amounts correlating to the cellular density of a given food item

(54). Mammalian models have demonstrated the importance of dietary nucleotides on the immune system, including enhanced macrophage phagocytosis, lymphocyte proliferation and disease resistance (54). Dietary supplementation of nucleotides has been shown to increase growth, lysozyme activity, alternative complement activity and immunoglobulin M (IgM) levels in the serum of rainbow trout (55), suggesting that commercial diets used in aquaculture could be benefited from nucleotide supplementation.

Vitamin C (ascorbic acid) is a pronounced water-soluble antioxidant (56). As an antioxidant, ascorbic acid can quench reactive oxygen and nitrogen species generated by cellular processes, such as oxidative burst, and by environmental stress, thus preventing tissue damage (56). Although being limited to water-soluble environments by itself, vitamin C indirectly harbours antioxidative functions in lipids too, through the secondary action of regenerating the active state of vitamin E (α-tocopherol) (56). Beneficial impacts on immune function has also been demonstrated in human models, where vitamin C has, among others, been shown to limit the accumulation of histamine during chronic stress and inflammation and promote leucocyte chemotaxis during common cold symptoms (56). Studies have demonstrated some immunological effects of dietary supplementation of vitamin C in Atlantic salmon parr (57, 58), and more pronounced effects in gilthead seabream (*Sparus aurata*) (59) and rohu (*Labeo rohita*) (60).

Vitamin E comprises four structurally differing tocopherols and four tocotrienols, with slight distinctions in antioxidant activities (61). Tocotrienols exhibit additional neuroprotective, anti-cancer and cholesterol lowering properties in mammalian cells, which is usually not the case for tocopherols (61). The primary function of vitamin E involves the prevention of lipid oxidation, and additional functionalities include enzymatic, transcriptional and structural regulation in cells (61). Research indicates that the functional differences between the eight tocopherol and tocotrienol analogues involves specificity to different signal transduction enzymes and transcription factors (61). α-tocopherol in particular has been a subject of interest for clinical studies in human trials for its potentially beneficial effects in cardiovascular disease (62). Several anti-inflammatory attributes has been discovered, including post-transcriptional limitation of IL-1β production and hampering of NF-κB activity in monocytes (62). Oxidative burst in human monocytes was also inhibited through structural impairment of NADPH oxidase (62). While added dietary levels of vitamin E didn’t effect

immunological parameters in Atlantic salmon (63), enhanced innate immunity has been seen in gilthead seabream (59) and pacu (*Piaractus mesopotamicus*) (64)

Aims and hypotheses

Most of the knowledge base regarding the immune system is built on work encompassing mammalian models, including the leucocyte shift after subjugation to stress (41, 43, 65). To what extent this can be inferred to fish is somewhat unclear, although some literature on the matter exists (66, 67). Further work regarding these mechanisms in fish should therefore be employed. A heightened immunocompetence has been observed in rainbow trout over a 72 hour period following acute stress (50). Following the immunological effects of acute stress over several days should therefore be a subject of interest. There are certain periods where a heightened immunocompetence could greatly benefit the aquaculture industry, for example when a disease outbreak is detected in a nearby farm of shortly after sea transfer. Acute stress exposure may hold promise in eliciting such effects and administration of immunostimulants can potentially serve the same purpose. Effects from oral administrations of immunostimulants has however been highly variable (45). A continued investigation of how these factors influence the immune system in fish is therefore warranted.

The main goal of the thesis was the characterize the immunological effects of a diet supplemented with of mixture of β-1,3/1,6-glucan, nucleotides, vitamin C and vitamin E as well as acute stress on Atlantic salmon post-smolts. This was divided into the following sub-goals:

1. Evaluate effects on the growth performance of the salmon.
2. Evaluate effects on peripheral leukocyte counts.
3. Evaluate effects on head kidney transcription.

The hypotheses of the present study were:

1. The treatment diet will not have any effects on the growth performance.
2. The treatment diet will have a stimulatory effect on the innate immune system.
3. Acute stress will induce a decrease in circulating lymphocytes and increase in circulating phagocytes.
4. Acute stress will have a stimulatory effect on the innate immune system, lasting for several days.

Methods

Feeding trial

150 post-smolts of Atlantic salmon (from Lerøy Midt AS, Lensvik) were transported to NTNU Sealab and used in a feeding trial with a mean initial body weight of 123.6g ± 1.93 (mean ± S.D.). Fish were randomly selected, weighted and placed in six flow-through tanks (1x1m with 400L aerated seawater at about 12.5 °C) (n=25) which were assigned to either a control feed or a treatment feed (see figure 1 and table 1) in triplicates. Individuals with particularly low or high weights were put aside. The experimental diets were provided by DSM Nutritional Products. For a total of 53 feeding days the fish were fed their assigned diet by automatic feed dispensers, aiming at consistently overfeeding in order to avoid restrictions on growth and aggression associated with competition for food. Dissolved oxygen levels, temperature and the load of uneaten feed pellets were monitored daily. The tanks were cleaned twice during the experimental period. At day 29, feeding had to be interrupted for two days due to low oxygen levels and not being able to raise the rate of inflow water. The last day of feeding was the preceding day before the first sampling day (T_0) and stress trial.

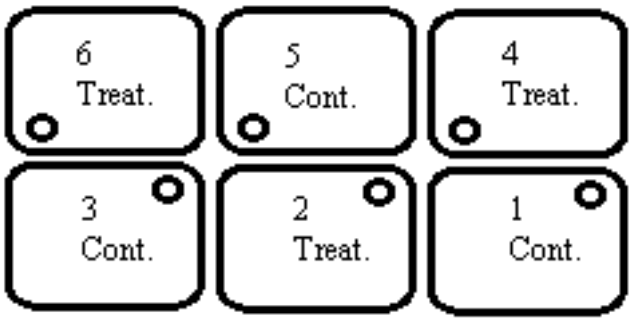

Figure 1. Experimental tank setup for the feeding trial. Cont. refers to the control feed and treat. refers to the treatment feed. Circles in the upper right corners visualizes openings in the tanks.

Stress trial and sampling

5 fish from tank 3 were removed and immediately anesthetized and euthanized with an MS222 overdose (T_0). The remaining 20 fish in tank 3 where then stressed for approximately 10 minutes by chasing with a stick (see table S2). The same process was repeated for tank 2, 1, 6, 5 and 4, respectively. After 23 hours (T_1), 5 new fish were removed from each tank, anesthetized and euthanized for sampling. The same process was again repeated 71 hours

after the initial stress trial (T_2). Finally, the remaining 10 individuals in each tank were anesthetized and only biometric measurements were taken on these individuals.

Table 1. Composition of the treatment and control feed. Health premix, the ingredient separating the two, contained a mix of purified β-1,3/1,6-glucan, nucleotides, vitamin C and vitamin E.

RAW MATERIALS	**TREAT. FEED (% COMPOSITION)**	**CONT. FEED (% COMPOSITION)**
FISH MEAL LT	15,00	15,00
SOYBEAN MEAL 50	7,50	7,50
SOY PROTEIN CONCETRATE	13,00	13,00
RAPESEED MEAL	10,00	10,00
WHEAT	10,10	10,60
WHEAT GLUTEN	11,50	11,50
CORN GLUTEN	7,00	7,00
HEALTH PREMIX[1]	**0,50**	**0,00**
MONO CALCIUM PHOSPHATE (MCP)	1,50	1,50
SOY LECITHIN	0,50	0,50
CHOLINE CHLORIDE 60%	0,60	0,60
PRÉMIX VIT + MIN -DSM OVN VMP 1	0,50	0,50
L-LYSINE	0,50	0,50
DL-MÉTHIONINE FG	0,30	0,30
RAPESEED OIL AT COATING	9,50	9,50
WINTERIZED FISH OIL AT COATING	12,00	12,00
TOTAL	**100,00**	**100,00**

[1]Contains 250 ppm β-1,3/1,6-glucan (DSM), 350 ppm nucleotides, 1000 ppm vitamin C and 400 ppm vitamin E (ppm corresponds to mg/kg diet).

After the MS222 overdose, fish was weighted and the fork length measured. Peripheral blood samples were then taken from the caudal vein using heparinized Vacutest kits (Kima, Arzegrande, Italy). The fish were temporarily laid on ice after blood samples had been collected, and were then moved to the dissection station. Approximately 120 mg of head kidney tissue were dissected and immediately immersed in 1,25 mL RNAlater (Sigma, Poole, UK). Samples immersed in RNAlater were stored in a refrigerator (4 °C) for 24 hours, then placed in a freezer (-18 °C) until further analysis.

Growth analysis

Fulton's condition factor (k) were calculated before and after the experimental period with the given formula:

$$k = \frac{W}{L^3} * 100$$

where W refers to the total weight (g) and L refers to the fork length (cm). Specific growth rate (SGR) were calculated with the given formula:

$$SGR = \frac{(\ln(Bm_f) - \ln(Bm_i))}{D} * 100$$

where Bm_f and Bm_i refers to the final and initial total biomass (g) of the tank and D refers to the total number of feeding days.

Over the course of the experimental period, one mortality was recorded. The individual belonged to tank 3 and no external signs would indicate the cause of the mortality. On the final day of sampling, one individual from tank 1 and one individual from tank 3 where interchanged and their tank of origin could not be determined. In total, three final biometric measurements could not be taken and as these individuals could not be tied to their respective initial measurements, the average initial weight times the number of lost final measurements were subtracted from the calculated initial biomass of the given tanks.

Blood analyses

Haematocrit was immediately measured from the blood samples, using a Compur haematocrit centrifuge. The remainder of the samples were then centrifuged at 1000 g for 3 minutes at 4 °C. A drop from the pelleted cell layer were smeared on an ethanol rinsed microscope slide, dried and stained with Hemacolor (Sigma, Poole, UK) before analysis.

A total of 72 blood smears were used in the cell count analysis, n=12 for each diet and time group. An area of intermediate cell density (few overlapping erythrocytes) in each blood smear was scanned using a NanoZoomer (Hamamatsu Photonics) and opened in the *NDP.view2* (Hamamatsu Photonics) software. Following a predetermined navigational

pattern, seven images depicting an average number of 551 cells were exported from each smear. If areas within the predetermined pattern consisted of irregular cell densities, randomly picked areas on the smear with satisfactory levels of cell densities were supplemented. These images were run through a custom-made algorithm (script provided in Supplementary materials) which marked and counted cell nuclei. The processed images were then reopened in *NDP.view2* where the algorithm's errors were corrected for. Finally, the original image was opened in *NDP.view2* where cell types distinct from mature erythrocytes were characterized and counted. The cell types characterized and counted in this analysis included erythrocytes, immature erythrocytes, ghost cells, partly ghost cells, lymphocytes, plasmacytoid lymphocytes, monocytes, neutrophil granulocytes and thrombocytes. A library of example characterizations is included in the appendix (figure S1). Erythrocytes were counted in order to standardize for the cell density. Counts from individual images that undoubtedly constituted an outlier were removed and then the average proportion of each cell type for each smear was calculated. Mean counts were compared for every cell type (besides ghost cells and partly ghost cells) before stress. In the following analysis, the lymphocyte proportion constituted the cell types of lymphocytes and plasmacytoid lymphocytes, and the phagocyte proportion constituted the cell types of monocytes and neutrophil granulocytes.

Head kidney qPCR

Head kidney tissue samples stored in RNAlater were thawed and RNA isolation were performed using the RNeasy Plus Universal Mini Kit (Qiagen, Norway, Oslo) according to the supplier's protocol, using the Tissuelyzer II (Qiagen) instrument. Approximately 120 mg of tissue was homogenized (instead of the recommended 40 mg), and subsequently diluted 1:3 in order to ensure that every sample contained representative tissues of interest. The isolated RNA concentration and purity were measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The majority of the samples had 260/280 and 260/230 ratios above 2, and none of the samples were therefore excluded. RNA integrity was then determined using the RNA 6000 Nano Kit (Agilent, Norway, Oslo) with the 2100 Bioanalyzer (Agilent) instrument. All the samples were given a RNA Integrity Number (RIN) above 9, the majority in the range of 9,7-10, and none of the samples were therefore discarded.

cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen, Norway, Oslo). 1 µg RNA was used in the reaction and 0,5 µg RNA was used in the no reverse

transcription (-RT) control plate. After cDNA synthesis following a program of 15 minutes of incubation at 42 °C and a 3 minute inactivation step at 95 °C, the samples were diluted 1:10. Quantitative real-time PCR was performed using the LightCycler 480 SYBR Green 1 Master kit (Roche Diagnostics, Germany, Mannheim) on the LightCycler 96 (Roche Diagnostics) instrument. 5 µL of diluted cDNA (or -RT control) were mixed with 3 µL RNase-free water, 10 µL LightCycler 480 SYBR Green 1 Master reagent and 2 µL primer mix (5 µM). qPCR were run on the following program; 10 minutes preincubation at 95 °C, 45 cycles of three step amplification (95 °C for 10 seconds, 55 °C for 10 seconds and 72 °C for 15 seconds) and finally a continuous rise from 65 °C to 97 °C for the melting curve analysis. Target and reference genes included in the analysis are presented in table 2. PCR efficiency were calculated using the *LinRegPCR* software. The reference genes β-actin and EF1AA were used to normalize the data, as suggested from a *geNorm* analysis (68).

Statistics

All the statistical analyses were conducted using the statistical software *R*, if not otherwise stated. The significance level were set a $p < 0.05$ for all the analyses. The SGR data was calculated from weight measurements of 147 individuals, where tanks served as replicates in the analysis (n=3). The data set was fitted with a simple linear model, *lm(SGR~Diet)*, and analysed with a one-way ANOVA test.

Average haematocrit values within tanks for each time point (n=3) was calculated from a total of 89 measurements, as one blood sample could not be taken from an individual belonging to tank 3, 23 hours post stress. A linear model was fitted with the explanatory variables of *diet* and *time after stress*. The data was analysed with a two-way ANOVA test, accompanied by a Tukey multiple pairwise-comparisons test (Tukey HSD).

For the cell count data set, models were first fitted using linear mixed effects models (*lmer*) with the *lme4* package to account for the nested structure of the data set. A partially Bayesian method (69) were instead employed as many of the *lmer* models estimated a group-level variance of zero (singular fit). Therefore, the models were fit with *blmer* models using the *blme* package with the default settings for the maximum penalized likelihood estimator. Models comprised of the fixed effects of *diet* and *time after stress* and the random factor *tank* were fitted as explanatory variables for the different cell types. Firstly, the models were

Table 2. Forward and reverse sequences for the primers included in the qPCR analysis.

Name	*Oligonucleotide sequence (5' > 3')*	*Accession number*	*Target*
IL-1β	*For.* GCTCAACTTCTTGCTGGAGAGT *Rev.* GGGCGCCGACTCCAA	AY617117	Target
IFNγ	*For.* GCTGTTCAACGGAAAACCTGTTT *Rev.* GTCCAGAACCACACTCATCCA	AJ841811, AY795563	Target
IL-10	*For.* CCGTTTGACATCAACGAGTTCATCT *Rev.* CAGCTCTCCCATTGCCTTATACAG	AJ317969	Target
IκBα	*For.* AGAGTGAGGAGGAGTGCATGT *Rev.* CTGCTTCAATTCTGCCCAAATGTAA	AB118099	Target
CD8α	*For.* AGAACGAAACGATGCCCACAT *Rev.* CTGTTGTTGGCTATAGGATGTTGTTG	AY693391, Y693393, AY701521	Target
MHC-I	*For.* GCGACAGGTTTCTACCCCAGT *Rev.* TGTCAGGTGGGAGCTTTTCTG	AF504013-25	Target
TNF-α1+2	*For.* AGGTTGGCTATGGAGGCTGT *Rev.* TCTGCTTCAATGTATGGTGGG	DQ787157.1, DQ787158.1	Target
Lys C II	*For.* ATGGATGGCTACGCTGGAAAC *Rev.* GCCACGTAGGACCTCAGGTCCTG	AF 179305	Target
IFNα	*For. ACTGAAACGCTACTTCAAGAAGTTGA* *Rev. AGGAAAGAGACAAAACGTCATCTGC*	DQ354152.1 DQ354153.1	Target
IL-17A	*For. TGGTTGTGTGCTGTGTGTCTATGC* *Rev.TTTCCCTCTGATTCCTCTGTGGG*	KJ921972.1	Target
HSP70	*For.* CCTCTACATTCATAAACTGCAACT *Rev.* CTGGCTGATGTCCTTCTTGTGT	AJ632154.1	Target
EF1A$_B$	*For.* TGCCCCTCCAGGATGTCTAC *Rev.* CACGGCCCACAGGTACTG	BG933853	Reference
18s rRNA	*For.* CCCCGTAATTGGAATGAGTACACTT *Rev.* ACGCTATTGGAGCTGGAATTACC	BG936672	Reference
EF1A$_A$	*For.* CCCCTCCAGGACGTTTACAAA *Rev.* CACACGGCCCACAGGTACA	AF321836)	Reference
β-actin	*For.* CCAAAGCCAACAGGGAGAAG *Rev.* AGGGACAACACTGCCTGGAT	BG933897	Reference

assessed for possible significant interaction terms by computing the confidence intervals of the parameter estimates. Were no significant interaction detected, models with all possible combination of fixed effects were fitted with maximum likelihood (*REML=F*) and the best model was determined with an AIC test. The total number of individuals used in this analysis were 72, where each group consisted of 12 replicates.

The data set from the qPCR was imported to the *qBase+* software. Individual fish were considered as technical replicates in the analysis, and tanks as biological replicates, in order to avoid running the risk of pseudoreplicating. An unpaired t-test was used to compare the different diet effects for each time point and a one-way ANOVA analysis were conducted for comparing the different time points after stress within each diet. The total number of individuals used in this analysis were 90, considered as 3 biological replicates for each dietary group.

Results

Growth performance

The experimental diet did not significantly affect the growth of the fish ($p = 0.35$), see table 3.

Table 3. Growth performance over the experimental period of 53 feeding days, n=3. Subscript i refers to initial measurements and subscript f refers to final measurements. k is the calculated Fulton's condition factor and SGR is the calculated specific growth rate.

Diet	Weight_i (g) (± SD)	Weight_f (g) (± SD)	k_f (± SD)	Survival (%)	SGR
Control	124,7 ± 11,9	291,5 ± 37,4	1,30 ± 0,1	98,7	1,60
Treatment	122,5 ± 11,9	282,0 ± 38,6	1,28 ± 0,1	100	1,57

Blood analyses

Haematocrit values were significantly higher in the treatment population ($p = 0.015$). Compared to T_0 measurements, haematocrit values were significantly higher 23 hours post stress ($p = 0.002$), while measurements 71 hours post stress were insignificantly different (see table 4). T_2 measurements were also significantly lower than T_1 measurements ($p = 0.02$).

Table 4. Mean haematocrit measurements for each time point, n=3. T_0, T_1 and T_2 corresponds to 0, 23 and 71 hours post stress, respectively.

	Haematocrit (%) (± SD)			
Diet	T_{0A}	T_{1B}	T_{2A}	**Total**
Control	32,1 ± 2,6	36,5 ± 2,5	34,1 ± 2,8	34,2 ± 2,2
Treatment	35,3 ± 2,4	37,6 ± 1,8	35,0 ± 2,6	36,0 ± 1,7*

Note. Statistics: distinct letters (A, B) indicate significant differences between time groups. Asterisk indicate significant difference from control population.

Before stress exposure, the immature erythrocyte proportion of the total blood cell count was significantly higher in the treatment population (table 5). No other cell type was affected by

the diet at any time point. The lymphocyte proportion, constituting lymphocytes and plasmacytoid lymphocytes, was significantly reduced 23 and 71 hours post stress exposure in the control population, and only 23 hours after stress in the treatment population (figure 2). The lymphocyte proportion was significantly higher 71 hours post stress compared to 23 hours post stress for both populations. The phagocyte proportion, constituting monocytes and neutrophils, was significantly higher 71 hours post stress compared to before stress in the treatment population only. A significant dietary interaction with stress was found for thrombocytes 23 hours post stress, where the treatment population had a reduced thrombocyte proportion after stress exposure and the control population had an increased thrombocyte proportion following stress exposure. The thrombocyte proportion was also significantly higher 23 hours post stress in the control population.

Table 5. Proportions of individual cell types from the total peripheral blood cells counted before stress, n=12.

	Control (% ± SD)	**Treatment (% ± SD)**
Immature erythrocytes	0,64 ± 0,23	0,87 ± 0,24*
Lymphocytes	1,54 ± 0,45	1,39 ± 0,46
Plasmacytoid lymphocytes	0,03 ± 0,03	0,02 ± 0,02
Neutrophil granulocytes	0,14 ± 0,11	0,11 ± 0,07
Monocytes	0,01 ± 0,01	0,00 ± 0,01
Thrombocytes	1,32 ± 0,35	1,56 ± 0,35

Note. Statistics: asterisk indicate significant difference from control population.

Head kidney transcription

The experimental diet did not significantly affect the transcription of any of the target genes (see figure 3). The amount of IκBα transcripts where significantly reduced 23 and 71 hours after stress for both the control and the treatment population. The amount of transcript where also significantly higher 71 hours after stress compared to 23 hours after stress. The amount of MHC I transcripts were significantly reduced 23 and 71 hours after stress for both populations. There were no significant differences in the amount of transcript for the two time points after stress in the control population, however, a significantly higher amount of transcript were found 71 hours after stress compared to 23 hours for the treatment population. The amount of Lys C II transcripts were significantly lower 23 and 71 hours after stress, and no significant differences were found between the two time points after stress. This was the case for both the populations. The amount of TNF α1+2 transcripts were significantly reduced 23 and 71 hours after stress in the control population, and only 23 hours after stress in the treatment population. No significant differences between the two time points after stress were

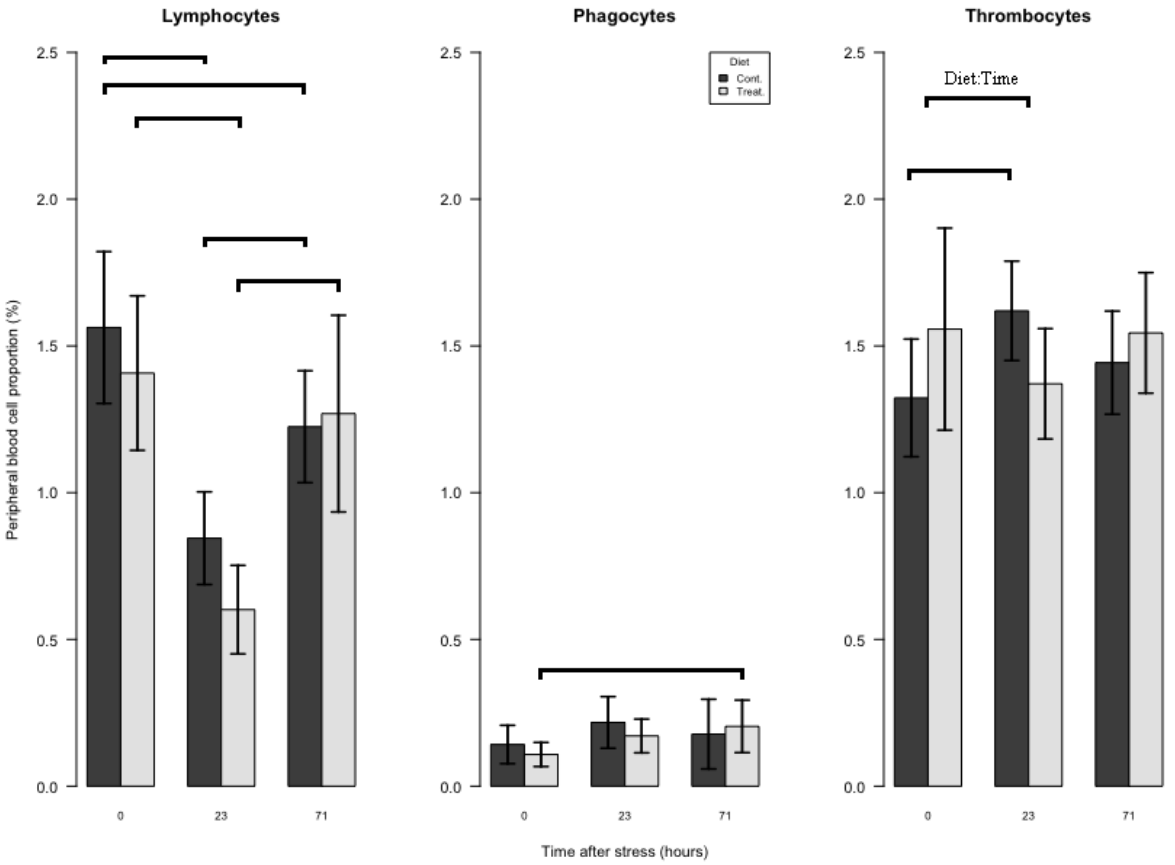

Figure 2. Proportion of lymphocytes, phagocytes and thrombocytes of total blood cell count. Lymphocytes comprises the cell types of lymphocytes and plasmacytoid lymphocytes, while phagocytes comprises the cell types of monocytes and neutrophils. Bar heights represents mean estimates and error bars represents standard errors, n=12. Horizontal bars indicate significant differences between groups, and "Diet:Time" signifies a significant interactions between diet and time after stress.

found in either population. The amount of transcript for hsp 70 were significantly reduced in the two time points after stress for both populations. No differences were found between the two time points after stress.

The amount of IL-10 transcripts were significantly reduced 23 hours after stress in the control population. The same was the case in the treatment population, however, the amount of transcript were significantly higher 71 hours after stress compared to 23 hours in this population. The amount of IFNɣ transcript were significantly reduced 23 hours after stress in the control population. The same was the case in the treatment population, however, the

amount of transcript were significantly higher 71 hours after stress compared to 23 hours in this population. The amount of CD8α transcript were significantly reduced 23 hours after stress in the control population. The same was the case for the treatment population, however, the amount of transcript were also significantly lower 71 hours after stress in this population. Neither stress nor diet did significantly altered the amount of IL-1β transcript in either population.

Transcript data could not be retrieved for the target genes of IL-17A and IFNα. T_1 and T_2 measurements for IL-17A was outside the dynamic range of the analysis, meaning there was not enough transcript to quantify. By looking at the amplification curve of IFNα (see figure S3), it would seem like the experimental population embodied several alleles for the target gene, as roughly half the individuals yielded a linear amplification curve, while the rest yielded an exponential amplification curve.

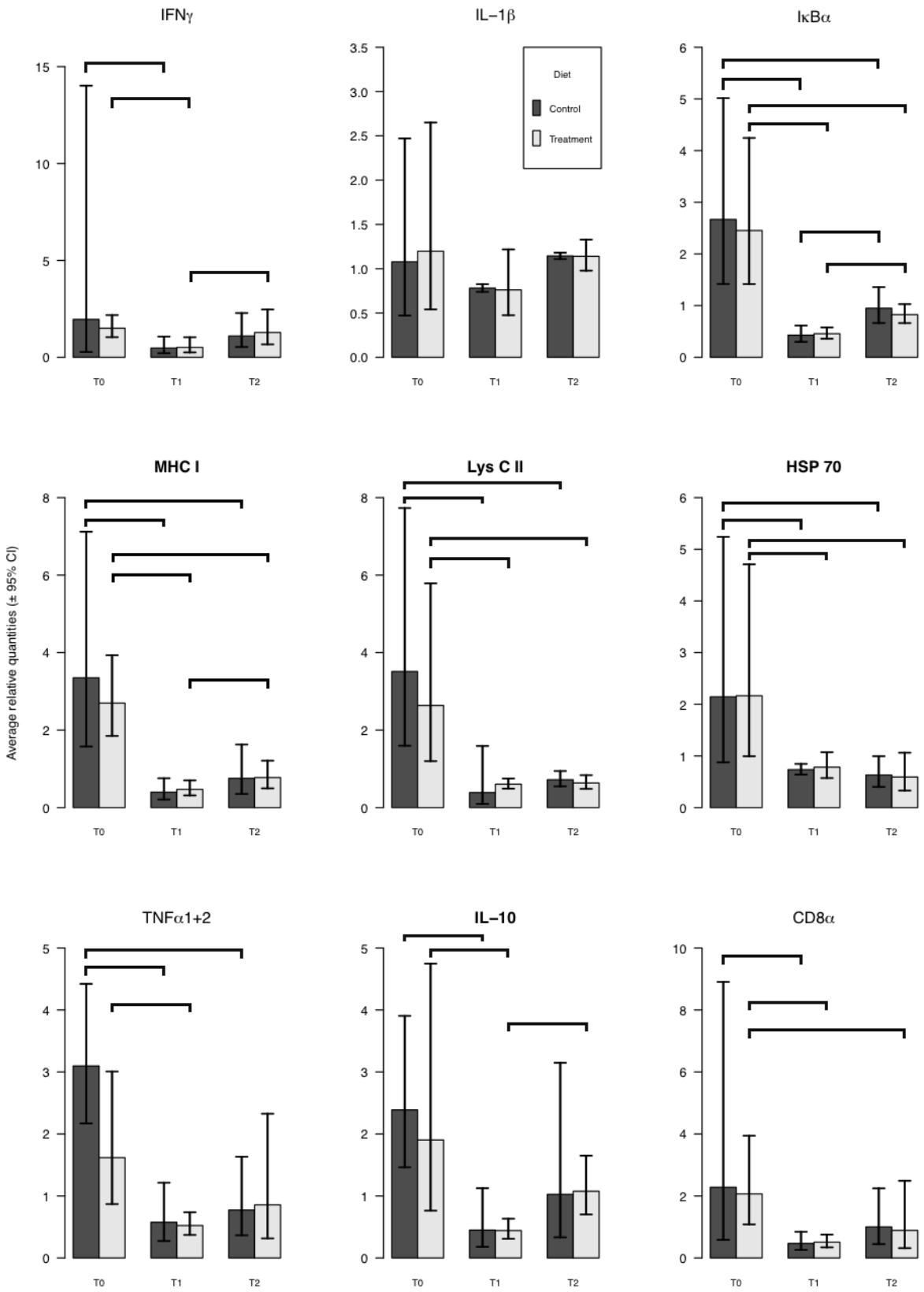

Figure 3. The average relative quantities of the transcripts for the genes of interest in the head kidney tissue samples. T0, T1 and T2 refers to the different sampling times, before stress, 23 hours after stress and 71 hours after stress, respectively. Mean estimates are calculated from 3

biological replicates, each comprised of 5 technical replicates (individual fish), and the error bars corresponds to the 95% confidence interval. Horizontal bars indicate significant differences between the groups.

Discussion

Growth performance

No significant differences in growth were observed between the two dietary groups, which corresponded with the hypothesis. The calculated average k-factors of 1.29 and SGR of 1.58 in this experiment corresponds reasonably well with previous results obtained in feeding experiments on post-smolts of Atlantic salmon (70, 71), with the calculated SGR definitely leaning towards the higher end of the spectrum. This was the case even though overfeeding wasn't consistently achieved for the first 31 feeding days, highlighting very good growth condition or a remarkable capacity for compensatory growth when conditions improve.

Administration of β-glucans has previously yielded non-significant changes, lower and higher growth performances (45). It has been hypothesised that the increased growth sometimes observed is a result of a heightened disease resistance, where upon pathogen exposure, the group fed the immunostimulant was either protected from disease development or had less of a metabolic cost associated with its eradication (45). One might suspect that the heightened leucocyte activity and pro-inflammatory responses that are often reported from immunostimulant administration (45, 47) would infer a reduced growth performance when no pathogen exposure takes place, as these responses would be metabolically costly. Nucleotide supplementation has previously been reported to positively affect growth performances in rainbow trout, in a dose-dependent manner (55). In Atlantic salmon, no effects on growth has been demonstrated in diets with differing levels of vitamin C supplementation (57, 58). Similarly, no effects on growth in Atlantic salmon were observed when diets ranged from vitamin E-depleted to high levels of supplementation (63). In the present trial, the constituents of the treatment diet could potentially have swayed the growth performance in either direction, but given no real pathogen exposure, the treatment diet was expected to insignificantly affect growth.

Blood analyses

Haematocrit values were found to be significantly higher in the treatment population. The contraction of the spleen and subsequent release of blood cells into circulation is a near immediate response following the perception of a stressor (32). No preliminary predictions were made regarding the haematocrit values, as the measurements were primarily taken to ensure that unstressed fish were sampled. However, Zhou et al. 2012 reported a significantly higher red blood cell count in cobia (*Rachycentron canadum*) fed vitamin C supplemented diets (72). Elevated red blood cell levels has also been observed in Atlantic salmon fed a nucleotide supplemented diet, although this difference was not significant (73). These finding could indicate that the constituents of the current treatment diet could have had an effect on the haematocrit levels. Noise disturbance could also have induced a minor stress response during the sampling of the fish. Tank 3, being a control tank, was sampled first, and tank 6, being a treatment tank, was located closest in proximity to the ongoing activity during the sampling procedure. These factors may also have contributed to the observed difference in haematocrit values. The significantly higher haematocrit measured 23 hours post stress remains unexplained, and is likely due to external factors, as the haematocrit should have returned to baseline levels approximately an hour after the stress exposure (74).

Out of the total 3075 leukocytes counted before stress in the present experiment, lymphocytes constituted 48.78%, plasmacytoid lymphocytes 0.75%, monocytes 0.13%, neutrophil granulocytes 3.84% and thrombocytes 46.50% of the total leukocyte population. Previously in Atlantic salmon, Chin & Woo (2005) counted 66.4-75.1% lymphocytes, 1.6-3.6% granulocytes, 0.19-1.1% monocytes and 20.1-31.9% thrombocytes (in the pre-vaccination groups) (75). Hardie et al. (1991) counted 68.0-80.3% lymphocytes, 16.8-23.3% thrombocytes, 2.0-7.5% neutrophils and 1.0-2.8% monocytes (57) and Hardie et al. (1990) counted 59.7-65.9% lymphocytes, 30.3-39.1% thrombocytes, 2.1-2.6% neutrophils and 0.9-1.4% monocytes (63). Comparing the present data with what has previously been reported indicates relatively low lymphocyte numbers while the thrombocyte proportion was higher than normal. Monocyte and neutrophil proportions didn't deviate substantially from the ranges previously reported, although monocyte numbers seems to be at the lower end of the spectrum.

Due to the inherently low numbers of neutrophils, and especially monocytes (57, 63, 75), the recorded data on these cells types were subject to high levels of uncertainty. The same was

the case for plasmacytoid lymphocytes (own observations). On average, 3859 cells were recorded for each of the 72 fish included in the analysis, in which the less common cell types only sporadically appeared. The estimated means for these cell types is therefore likely to be highly influenced by random variation, instead of primarily encapsulating the biological variation within the samples. To which degree the random variation contributed to the high variances of the estimates is difficult to determine, but the strength of the data set on these cell types can either way be regarded as weak. In order to better characterize the effects of stress on peripheral blood cells, lymphocytes and plasmacytoid lymphocytes, as well as monocytes and neutrophils were combined. Sophisticated phagocytic functions has recently been described in thrombocytes (13). However, thrombocytes were decided to be analysed separately from the remaining phagocyte proportion, as this numerous cell type would have accounted for the vast majority of the cells.

In the present trial, no dietary effects on individual leukocyte populations were detected, which contradicted the hypothesis. The treatment diet was expected to have a stimulatory effect on the innate immune system. An enhanced proliferation of neutrophils and monocytes was therefore one of the awaited outcomes. Although leukocyte subpopulations were not distinguished, Lin et al. (2011) reported significantly higher peripheral leukocyte counts in koi fed β-1,3-glucan (76) and Sahoo & Mukherjee (2001) reported significantly higher leukocyte counts in rohu (*Labeo rohita*) following oral administration of β-1,3-glucan (77). Similarly, a β-1,3/1,6-glucan supplemented diet was shown to significantly increase the leukocyte count in channel catfish (*Ictalurus punctatus*) (78). Selvaraj et al. (2005) reported unaltered leukocyte numbers following oral administration and bath treatment of yeast-derived β-glucan in carp (*Cyprinus carpio*), however, intraperitoneal injections of β-glucan significantly increased peripheral neutrophil and monocyte proportions in a seemingly dose-dependent manner (79). The proportion of immature erythrocytes were significantly higher in the treatment population, which initially came as a surprise. However, this observation was likely a result of the significantly higher haematocrit in the treatment population.

Acute stress application was expected to result in a reduction of circulating lymphocytes, while phagocyte numbers were expected to increase. In line with the hypothesis, a clear reduction in the circulating lymphocyte proportion was observed 23 hours post stress, which displayed a gradual return to normal values after 71 hours. A significant increase in phagocyte prevalence was observed 71 hours post stress in the treatment population only. This

observation was in line with the hypothesis, but the overall phagocyte response was much weaker than expected. As elegantly described in Dhabhar et al. 2012, the physiological stress response in rodents induce an initial influx of leukocytes into circulation, followed by a subsequent decrease of lymphocytes and monocytes as they migrate to the skin or sites of immune activation, while circulating neutrophil numbers continue to increase (41). Wojtaszek et al. 2002 described a similar trend in carp, where a reduction in circulating lymphocyte and eosinophil numbers were accompanied by an increase of neutrophil numbers following cortisol administration (66). A reduction of circulating lymphocytes/thrombocytes and an increase in neutrophils/monocytes following stress has also been described in rainbow trout (67).

The thrombocyte prevalence was significantly increased 23 hours post stress for the control population only, and a significant interaction was detected between the diet and the stress application. To the authors knowledge, such an effect has not previously been described. It has been reported that wound-closure was accelerated in common carp following β-glucan treatment (80). It is thus possible that the β-glucan administration in the current study enhanced the migration of thrombocytes to peripheral tissues, yielding an apparent reduction in thrombocytes. However, this does not explain the increased thrombocyte prevalence observed in the control group.

Head kidney transcription

In the present trial, the treatment diet was expected to have a stimulatory effect on the innate immune system. None of the target genes included in the transcriptional analysis of the head kidney appeared to be regulated by any dietary effects, which directly contradicted the hypothesis. A lacking macrophage activation was evident from the equivalent transcription of the pro-inflammatory cytokines IL-1β and TNFα. An upregulated lysozyme transcription would indicate an enhanced phagocytic function in macrophages, but the expression of this target gene was unaltered. IL-10 and IκBα could reveal possible anti-inflammatory responses, but these target genes were also equally expressed in the control and the treatment group. IFNγ being equally expressed imply no activation of T-cells in the head kidney. Similarly, unaltered CD8α expression implies that the treatment diet did not affect cytotoxic T-cell function. Hsp 70 is one of the major chaperones present in most cells where an upregulation could have been a consequence of increased inflammation. MHC I is differentially expressed

in various cell types, but even MHC I expression in MHC I negative cells is heavily regulated by IFNs (81). Given no apparent T-cell activation, it should come as no surprise that MHC I was equally expressed in the two groups.

The treatment diet contained a cocktail of β-glucan, antioxidants and nucleotides which were all assumed to have a potential for immune stimulation. The published literature on β-glucan is abundant, but the results are largely inconsistent, especially when oral administration is employed (45, 47). Intraperitoneal injections of β-glucan has been reported to upregulate the lysozyme activity in head kidney cells, intestinal cells and blood polymorphonuclear cells (82), as well as pro-inflammatory cytokine (IL-1β and TNF-α) transcription in the head kidney of Atlantic salmon (83). Oral administrations of β-1,3/1,6-glucan has yielded significantly upregulated IL-1β and downregulated IgM transcription in head kidney samples of gilthead seabream (84), as well as downregulation of both IL-1β and IL-10 in the head kidney of common carp (85). Nucleotide supplementation has previously been shown to improve the immunocompetence of rainbow trout, by, among others, enhanced serum lysozyme activity (55).

Vitamin C and E has received plentiful scientific attention for its regulatory and anti-inflammatory capabilities in mammalian immunology (56, 62). In Atlantic salmon, Hardie et al. (1991) demonstrated a dose-dependent enhancement of serum complement activity and a heightened mortality rate of individuals lacking in dietary vitamin C when challenged with *Aeromonas salmonicida* (57). Serum protein levels and leucocyte numbers, respiratory burst and erythrophagocytosis activity and specific humoral responses were unaffected by vitamin C supplementation (57). Thompson et al. (1993), on the other hand, reported a heightened specific humoral response in the group fed low levels of vitamin C compared to high doses, after a pathogenic bacterial challenge (58). Leucocyte and plasma bactericidal activity and leucocyte migration were unaffected by dietary vitamin C levels (58). Hardie et al. (1990) tested the effects of differing dietary levels of vitamin E on the immune function of Atlantic salmon parr (63). The vitamin E deficient diet were found to supress serum complement activity, which in turn reduced the phagocytic efficiency in macrophages (63). Dietary levels of vitamin E had no effects on leucocyte numbers, macrophage respiratory burst activity or antibody and lymphokine production in lymphocytes (63). However, clear demonstrations of vitamin C and E stimulating innate immunocompetence is provided in other teleost species (59, 60, 64). Supplementation of vitamin C has been reported to significantly enhance the

respiratory burst activity in gilthead seabream (59) and rohu (60). Supplementation of vitamin E has been reported to enhance complement and phagocytic activities in gilthead seabream (59) and increase the recruitment of macrophages to foreign objects in pacu (64). Ortuño et al. (2001) also demonstrated that a joint vitamin C and E diet synergistically enhanced the respiratory burst activity in gilthead seabream head kidney leukocytes (59).

It is difficult to determine exactly why no dietary effects could be detected. The response to oral administration of β-glucan has previously been varied, and lackluster effects isn't a novelty in the literature (45, 47). While vitamin supplementation has proven stimulatory for the innate immune response in several teleost species (59, 60, 64), most examined parameters remained unaltered in Atlantic salmon, specifically (57, 58, 63). Nucleotide supplementation proved stimulatory in rainbow trout (55), but not at the lowest level of supplementation (0.5 g/kg), which was higher than the nucleotide supplementation in the present experiment (0.35 g/kg). On the other hand, a direct comparison of the nucleotide content in the cited and current control diets cannot be made. Substantial shifts in the nucleotide content of commercial diets may have occurred over the 9 years separating the two experiments. Although there have been discrepancies in the individual effects of each supplement, they have also all been proven to harbour a capacity for immune stimulation. A previous experiment using the same diets as in the current experiment yielded an enhanced phagocytosis and a reduced intracellular oxidative burst in peripheral leukocytes from rainbow trout (50). It is therefore surprising that the simultaneous inclusion of four mediating agents would not provoke any detectable effects in the head kidney transcripts selected as representatives for the immune response.

Most of these previous studies have conducted functional analyses on leukocytes. A similar alteration in activity was expected to be reflected by a regulated gene transcription in the current experiment. However, such comparisons are not always valid, as the quantity of transcripts does not always give a quantitative measurement for the corresponding protein. A dissociation between the transcription of IL-1β and TNFα and their subsequent translation has for example been described (25). Conducting analyses on the protein level could therefore be of interest.

Another potential explanation for the absence of dietary effects from specifically β-glucan could be the presence of tolerance-building mechanisms or exhaustive effects after longer

administrative periods. In the present experiment, the treatment population were fed the supplemented diet continually over 53 days. In a feeding experiment with koi, a peak in effect from β-1,3-glucan supplementation was observed at the 21st day, followed by a decline in effect towards the end of the 56 day feeding trial (76). Similarly, a peak in immunological activity was reported at day 42, followed by a decline towards day 56 in a feeding trial with *Labeo rohita* (86). Supplementation of whole yeast (*Saccharomyces cerevisiae*) to gilthead seabream enhanced phagocytic activity after 4 weeks (final measurement), while oxidative burst activity was only enhanced after 2 weeks (87). Comparable temporal investigations were not found for Atlantic salmon, but these experiments on other species indicates that diminishing returns may occur over longer administrative periods. Complementary to this, fish macrophages has been reported to acquire tolerance to LPS, another PAMP, over repeated exposure (12). Therefore, it cannot be excluded that there may have been more pronounced dietary effects during earlier stages of the experimental period.

The transcriptional data in the present experiment was expected to reflect an enhanced innate immunocompetence for several days after the stress application. An upregulated expression of the pro-inflammatory cytokines IL-1β, TNFα and IFNγ, as well as an upregulated transcription of lysozyme could indicate such an effect. However, all the target gene transcripts, except IL-1β, was significantly reduced 23 hours after stress. An enhanced immunocompetence has previously been demonstrated in rainbow trout following acute stress, in terms of enhanced phagocytosis (88) and plasma lysozyme activity (89). Verlhac-Trichet & de Billy (2013) demonstrated a significant increase in the phagocytic activity of peripheral leukocytes in rainbow trout following acute stress application, which was sustained for a 72 hour period (50).

Some aspects of this observation could be explained by a stress-induced release of leukocytes from the head kidney, similar to the contraction and release of blood cells from the spleen (32). The reduction in the number of transcripts would then be a result of a lower leukocyte representation in the samples. Such an observation has been made in gilthead seabream, where head kidney monocytes/macrophages and granulocytes migrated into circulation following stress application, where a full recovery wasn't observed until 4 days post stress (90). Similar mechanisms has been indicated in rainbow trout (67). The current observation would also indicate a similar release of T-cells as the CD8α transcript follows the same

pattern. A stress-induced upregulation of IL-1β transcription could possibly negate the loss of transcript due to the loss of monocytes and macrophages.

Some contradicting findings are however present. MHC I and hsp 70 is expressed in almost every cell (17, 38). MHC I is differentially expressed in different cell types, with lymphoid cells harbouring the highest degree of expression (81). Despite this, an equally dramatic reduction in transcript as genes being solely expressed in leukocytes, such as lysozyme, would seem unlikely. Restraint stress has been demonstrated to significantly increase the hsp 70 transcription in several rat tissues (91), while handling did not affect hsp 70 expression in the liver of rainbow trout (92). The significant reduction in the hsp 70 transcript in the current study therefore strongly suggests that the loss of leukocytes could not be the sole explanatory factor. The absence of leucocytes could however have contributed to a reduction in free radical prevalence, which may result in a lower hsp 70 transcription. If the reference genes used to normalize the data were heavily upregulated following stress, a similar pattern as observed in the current qPCR data could have been artificially generated. However, such as effect was not evident from examining the reference genes.

The general trend observed 71 hours post stress could mirror a slow return of leukocytes to the head kidney. Jeney et al. (1997) reported a stress response of a reduced amplitude in rainbow trout fed β-glucan (48). A similar observation was made in Verlhac-Trichet & de Billy (2013) (50). Regarding the amount of transcript to be an indirect measure of the amount of leucocytes present in the head kidney, the amount of MHC I, TNFα, IL-10 and IFNγ transcript would indicate a faster recovery in the treatment population. This being the case as the measured amount of transcript were either significantly higher 71 hours post stress than at 23 hours post stress, or insignificantly different from the amount of transcript measured before stress. The amount of CD8α transcript would in this regard indicate the opposite. It should be noted that these effects are primarily generated from discrepancies in variances, while the mean estimates only show minor differences.

From the current data it is not possible to establish proof of an upregulated innate immune response in the head kidney. It can be assumed that the migration of leukocytes into peripheral tissue would have reduced the mRNA of leukocyte-related proteins, yielding an apparent loss of activity. An upregulation of the immune response at 23 and 71 hours post stress would therefore not be visible as long as the number of cells had not returned to pre-

stress levels. To test the hypothesis that stress upregulates the innate immunity, leukocyte subpopulations would have to be prepared by differential centrifugation and then analysed. This was initially attempted, but several lines of challenges and time constraints made this inapplicable in the current thesis.

The choice of statistical models and data processing employed may be considered as conservative approaches. The full sample size was only harnessed in the cell count analysis, while all the other analyses had to compress the majority of the samples in order to not overstep the boundaries set by the nested structure of the experimental design. As significant tank effects were detected in the qPCR data and some discrepancies were indicated in the haematocrit data, disregarding the effect of the tanks would not seem appropriate. Committing type II errors are heavily emphasized in this regime, with the trade-off being a low probability of committing type I errors. It should also be noted that the validation of the mixed-effect models was somewhat of a redundant process, as the random factor only included 6 groups. Validating linear models including only 3 replicates imposed similar challenges. Whether or not the data sets were in accordance with the model assumptions could therefore not be properly determined.

Conclusion

In conclusion, the current study has shown that, under the present experimental conditions, a diet supplemented with β-1,3/1,6-glucan, nucleotides, vitamin C and vitamin E did not affect growth, peripheral leukocytes or any of the chosen gene transcripts in the head kidney. No dietary stimulation of the innate immune system was therefore observed. There was clear evidence that acute stress exposure lead to a reduction in circulating lymphocytes, presumably due to a migration into peripheral tissues. The circulating phagocyte proportion displayed a generally weak response to acute stress, where a significant increase was observed 71 hours post stress in the treatment group only. An interesting interaction between acute stress and dietary effects was observed in circulating thrombocytes, but could not be explained. It was apparent that the stress exposure induced changes in the head kidney that would not allow for pre- and post-stress comparisons. No stress-mediated stimulation of the innate immune system could therefore be determined. Controlling for the cellular composition of the samples would possibly yield more interpretable results.

Supplementary materials

Table S1. Haematocrit measurements for each time point and tank. T_0, T_1 and T_2 corresponds to 0, 23 and 71 hours after stress, respectively.

		HAEMATOCRIT (%) (± SD)		
TANK	**Diet**	**T_0**	**T_1**	**T_2**
1	Control	32,4 ± 3,4	36,8 ± 1,5	34,6 ± 4,4
2	Treatment	35,0 ± 3,7	37,2 ± 1,9	33,4 ± 2,7
3	Control	31,4 ± 0,9	34,1 ± 2,1	33,2 ± 1,9
4	Treatment	34,4 ± 1,5	38,6 ± 1,1	34,6 ± 2,3
5	Control	32,6 ± 3,3	38,2 ± 2,0	34,6 ± 1,8
6	Treatment	36,4 ± 1,1	37,1 ± 2,0	37,0 ± 1,9

Table S2. Duration of the chasing stressor for the different tanks.

Tank	***Stress duration (minutes)***	***Diet***
1	10	Control
2	11	Treatment
3	11	Control
4	10	Treatment
5	11	Control
6	11	Treatment

The following script were used in the *Octave* software to automatically count the majority of the cells within the exported images:

```
tic

pkg load image;

### Load image and create binary image
antall_fisk = 16
antall_bilder = 7


fiskematrix = cell(antall_fisk, antall_bilder);
for fisk = 1:antall_fisk
   for bilde = 1:antall_bilder
      filnavn = sprintf('Originalbilder/%d/%d.%d.jpg', fisk, fisk, bilde)
```

```
Im40 = imread(filnavn); # load image
h = waitbar(0, "please wait..."); # create loading bar
bigIm40 = imresize(Im40,2); # create big version of image
waitbar(0.1,h); # update loading bar
binaryImage = im2bw(Im40,0.5); # create binary-image of loaded image

### Remove cell walls
negativeBinary = 1-binaryImage; # create inverse of the binary (for erosion)
se = strel("square", 5); # define a strel to use to erode image (remove cell-walls)
negativeBinary = im2bw(imerode(negativeBinary, se)); # erode image (remove cell-walls)

### Clean up image
#removing smaller regions/regions with fewer than 50 pixels (noise)
negativeBinary = bwareaopen(negativeBinary, 100);

### Detect ellipses
waitbar(0.2,h); # update loading bar
negativeBinary2 = imresize(negativeBinary,2); #to successfully detect ellipses, need larger
matrix
waitbar(0.4,h); # update loading bar
#bigBinary = imresize(binaryImage,2);
#positiveBinary=1-negativeBinary;
#imshow(positiveBinary);
#positiveBinary2 = imresize(positiveBinary, 2);
waitbar(0.6,h); # update loading bar
# detect ellipses using the "regionprops"-function
structEllipses = regionprops(negativeBinary2,
{'Centroid','MajorAxisLength','MinorAxisLength','Orientation'});

### Display original image
figure("visible", "off");
#imshow(bigBinary);
imshow(bigIm40); # show big version of original image
waitbar(0.8,h); # update loading bar

# Display all the ellipses detected
t = linspace(0,2*pi,50);
hold on
for k = 1:length(structEllipses)
   a = structEllipses(k).MajorAxisLength/2;
   b = structEllipses(k).MinorAxisLength/2;
   Xc = structEllipses(k).Centroid(1);
   Yc = structEllipses(k).Centroid(2);
   phi = deg2rad(-structEllipses(k).Orientation);
   %x = a*cos(t) - b*sin(t);
   %y = a*cos(t) + b*sin(t);
   x = Xc + a*cos(t)*cos(phi) - b*sin(t)*sin(phi);
   y = Yc + a*cos(t)*sin(phi) + b*sin(t)*cos(phi);
   plot(x,y,'r','Linewidth',2)
end
```

```
hold off
savename = sprintf("Resultater/%d.%d.png", fisk, bilde)
saveas(gcf, savename)

length(structEllipses)
fiskematrix(fisk,bilde) = length(structEllipses)
close(h);
end
end
print(fiskematrix)
 # close loading bar
toc
```

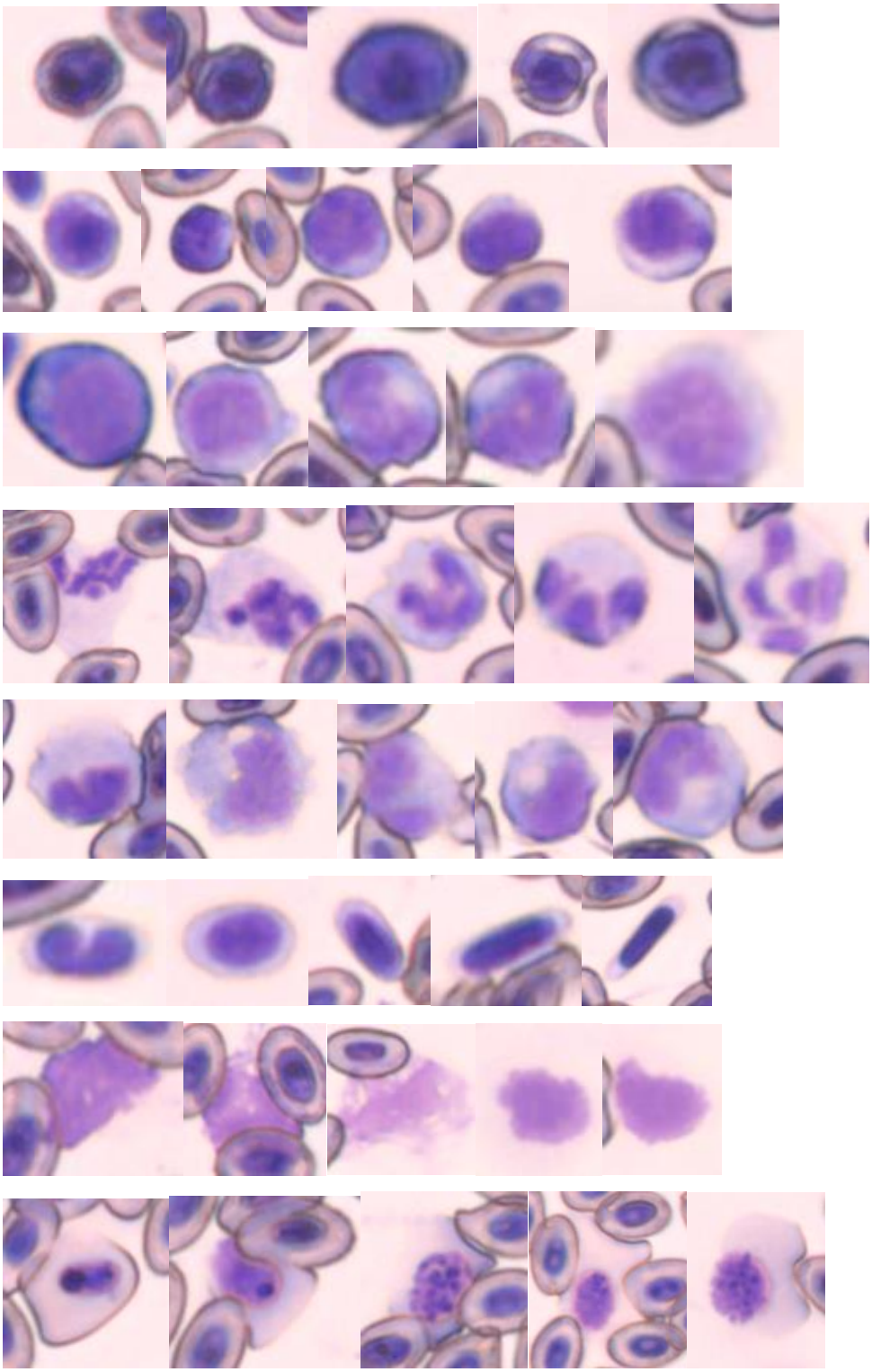

Figure S1. Five examples for each of the characterized blood cells. In descending order; immature erythrocyte, lymphocyte, plasmacytoid lymphocyte, neutrophil granulocyte, monocyte, thrombocyte, ghost cell and party ghost cell. Note that cell sizes in the figure poorly reflects the actual cell sizes, and size comparisons between the cells should not be made.

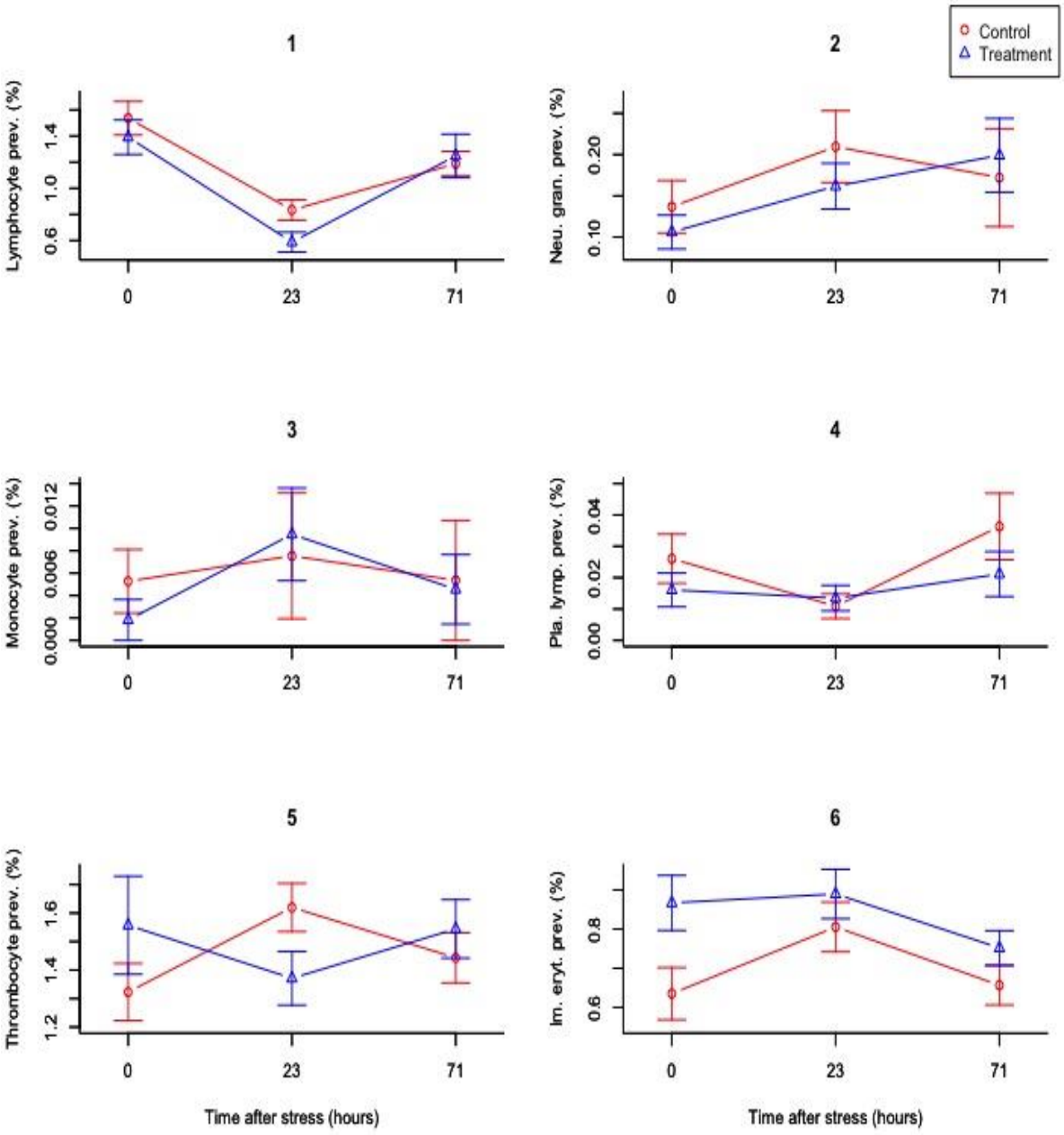

Figure S2. The percentage of circulating lymphocytes (1), neutrophil granulocytes (2), monocytes (3), plasmacytoid lymphocytes (4), thrombocytes (5) and immature erythrocytes (6) in the two populations over the three time points. The central point corresponds to the estimated mean of the group and the error bars corresponds to the standard error of the estimate, n=12.

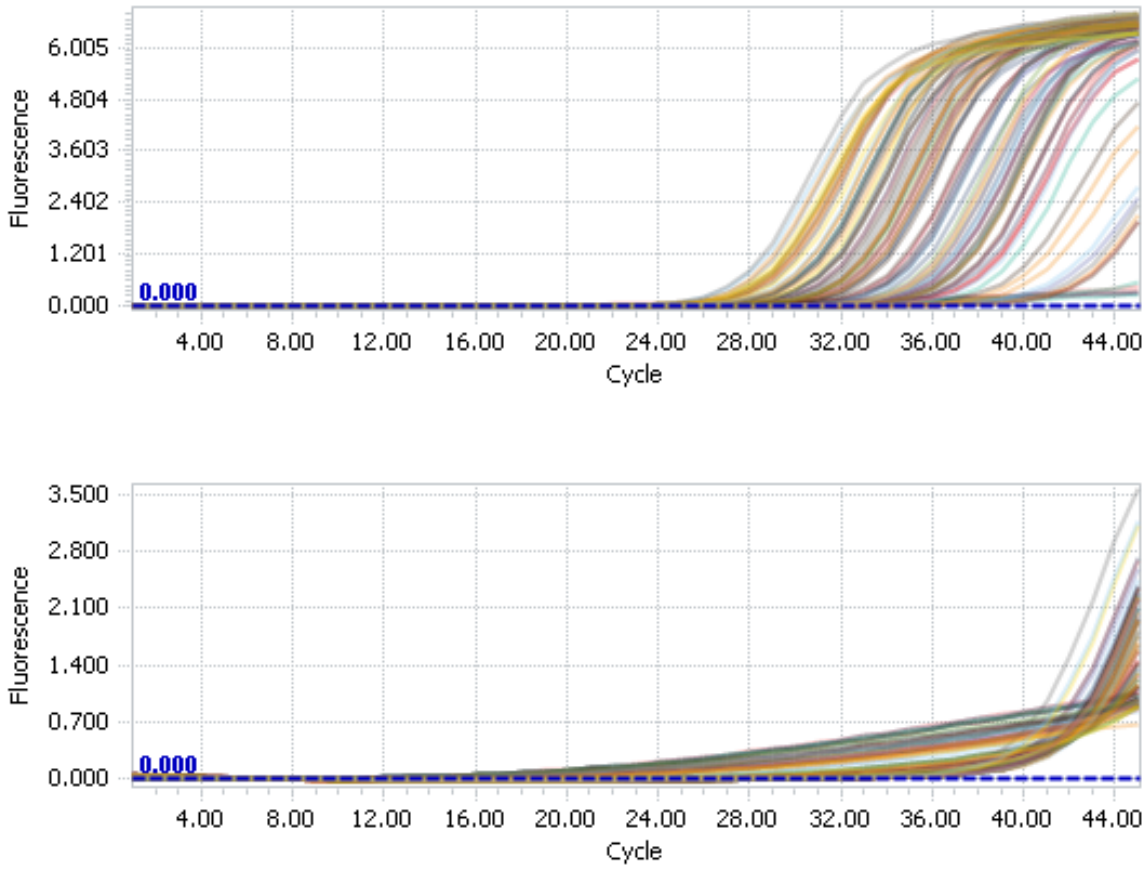

Figure S3. Amplification curves for IL-17A (upper) and IFNα (lower). Images are exported from the *LightCycler 96 SW 1.1* software.

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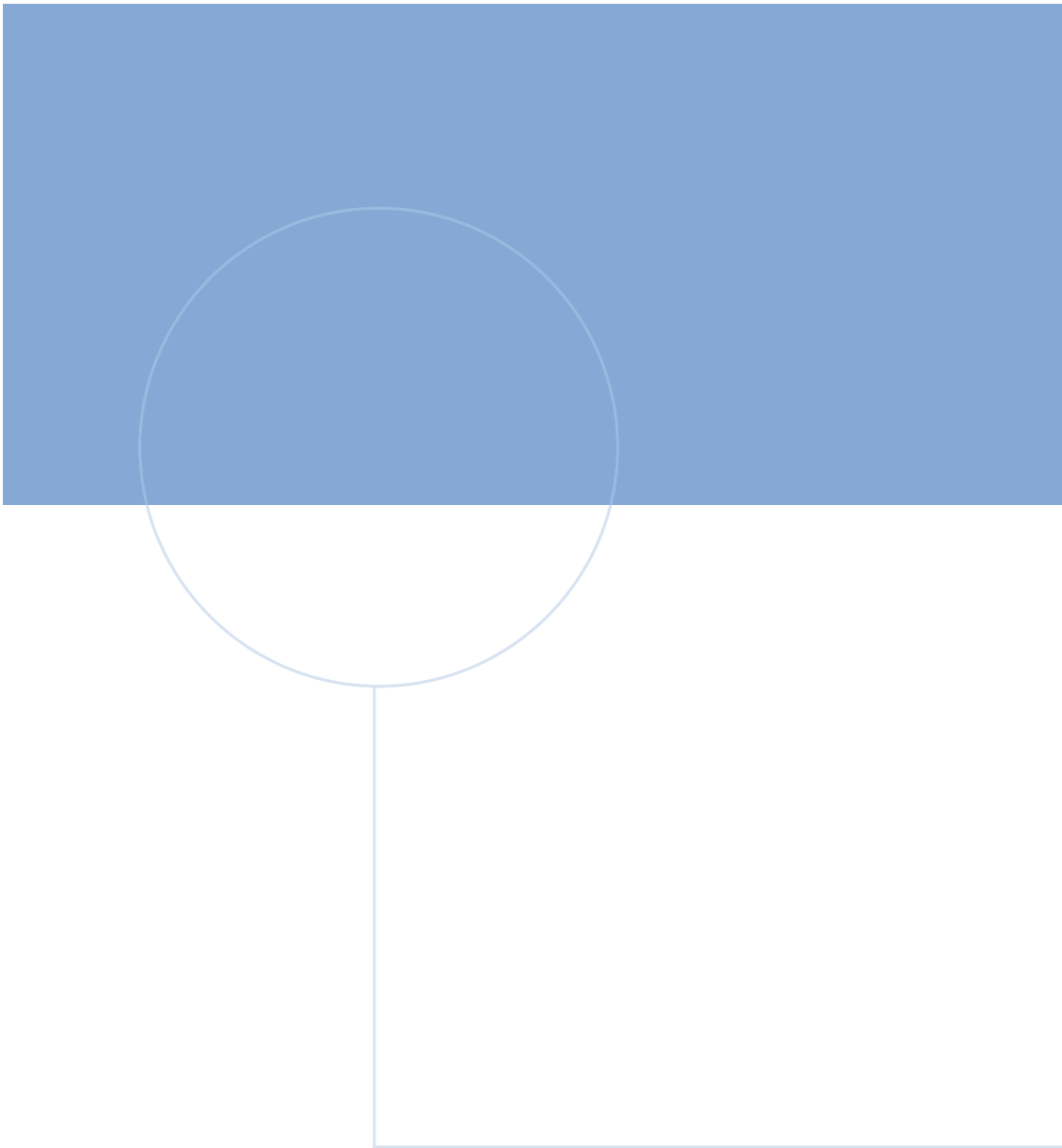

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