UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

MINERAL CARRYOVER FROM SHELLED MUSSEL MEAL IN THE SPOTTED WOLFFISH (*ANARHICHAS MINOR*)

Potential of dietary mineral supplementation on growth, stress and health

Terese Hjelleset

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Supervisor: James Hinchcliffe, Department of biological and environmental sciences

Examiner: Michael Axelsson, Department of biological and environmental sciences

Contents

Abstract

The potential of Swedish aquaculture is currently not fulfilled as Sweden is still a net importer of fish with low production and narrow species diversity. In recent years, there has been increasing interest in expanding and diversifying Swedish aquaculture to make it more sustainable and competitive. Introducing new species and novel sustainable feeds can contribute to reducing the carbon footprint and the pressure on wild fish populations. A promising potential species for cold-water aquaculture is the spotted wolffish. Commercial cultivation is compromised by the lack of an optimized diet, which may be related to health issues like nephrocalcinosis. Mussel meal is a promising protein source that can be produced locally in Sweden and contribute towards a new Swedish feed sector. Including shells in mussel meal can increase production efficiency, reduce waste, and may provide minerals. This project aimed to evaluate shelled mussel meal as a potential feed ingredient by assessing the effect of shell inclusion on growth, health, and stress in spotted wolffish. We measured osmolarity, ion- and acid-base balance in plasma, cortisol, glucose, lactate, hemoglobin and hematocrit, as well as insulin-like growth factor 1, free fatty acids and ghrelin. We also assessed specific growth rate, condition factor, weight gain and average weight and length. The results show that growth performance was not impaired by shell inclusion; the average final weight was 1162.72g ± 30.82 for fish fed shelled mussel meal, and 1145.17g ± 32.39 for fish fed musselmeal. The inclusion of shells did not impair the maintenance of the homeostasis, plasma stress biomarkers or growth biomarkers. This indicates that shelled mussel meal is a potential sustainable ingredient in fish feed that does not impair the overall wellbeing of spotted wolffish.

Sammanfatning

Svenskt vattenbruk är en liten sektor i jämförelse med andra nordiska länders produktion och har stor potential att expandera. Idag består 90% av produktionen av regnbågslax. Under de senaste åren har intresset vuxit för att utöka både produktionsmängd och artdiversitet i syfte att förbättra svensk vattenbruks konkurrenskraft och hållbarhet. Genom att utnyttja ett större antal arter och hitta nya, hålbara alternativa foder kan koldioxidutsläpp och överfiske av vilda populationer reduceras. En av de arter med hög potential inom kallvattenodlingar är den fläckiga havskatten. Kommersiell odling begränsas idag av att artens näringsbehov inte har blivit fullständigt studerat, vilket misstänks orsaka hälso- och välfärdsproblem, som nefrokalcinos. Musselmjöl är en lovande proteinkälla för fiskfoder som kan produceras lokalt i Sverige och därmed bidra till att utveckla den svenska fodersektorn. Inkludering av musselskal i fodret kan öka produktionseffektiviteten, minska avfall, och potensiellt tillföra viktiga mineraler. Detta projekt syftade till att utvärdera musselmjöl med skal som en potentiell fiskfoderingrediens genom att uppskatta effekten av skaltillsats på tillväxt, hälsa, och stress i fläckig havskatt. Vi analyserade osmolaritet, jonkoncentrationer och pH i plasma, stressbiomarkörerna kortisol, glukos, laktat, hemoglobin och hematokrit, samt insulin-like growth factor 1, fria fettsyror och ghrelin. Tillväxt blev mätt med spesifikt tillväxthastighet, kondisjonsfaktor, viktökning och genomsnittlig vikt och längd. Reultaten visade ingen negativ effekt på tillväxt; den genomsnittliga slutvikten var 1161.72g ± 30.82 för fisk som fått foder innehållande skal och 1145.17g ± 32.39 för fisk som fått foder utan skal. Våra resultat påvisade inga signfikanta skillnader påvärkade inte fiskens förmage att upprethålla homeostas, biomarkörer för stress eller biomarkörer för tilväxt. Detta indikerar att musselmjöl med skal är en potentiell hållbar ingrediens i fiskfoder, och att det inte påverkar den fläckinga havskattens hälsa och vælfærd negativt.

1. Introduction

1.1. Aquaculture in Sweden

Today, the aquaculture industry is the fastest growing animal food production industry, accounting for 46% of the total fish production globally, including molluscs, crustaceans and other aquatic animals (FAO, 2020). The major fish producer of the world is China, accounting for 35% of the total global fish production in 2018, while European fish production accounts for 10% of the total and is the only continent whose average production (million tons/year) has decreased gradually over the last few decades (FAO, 2020). The dominating aquaculture nation of the Nordic countries is by far Norway, producing 1 453 042 tons fish including molluscs and crustaceans in 2019 (OECD, 2022). In stark contrast, Swedish aquaculture produced 11 502 tons in 2019 (OECD, 2022), and has repeatedly during the past decade had the lowest production of the Nordic countries (FAO, 2020). Swedish aquaculture has great potential due to the access to high quality water resources, high veterinary status and well developed infrastructure (Wenblad et al., 2013), but still fish import costs exceed export earnings, resulting in a trade deficit (OECD, 2021).

In 2020, Swedish aquaculture produced 9900 tons of fish including molluscs and crustaceans, for human consumption, where almost 90% (8700 tons) was rainbow trout (*Oncorhynchus mykiss*), 11% (1100 tons) was Arctic char (*Salvelinus alpinus*) and around 23% (2300 tons) was mussels (Jordbruksverket, 2020). The trade deficit, low production numbers and narrow species diversity are some of the factors driving the increasing interest in expanding and diversifying Swedish aquaculture. By developing local aquaculture systems, the dependence on produce and transportation from dominating aquaculture nations across the globe can be reduced. Wild fisheries must be reduced to protect the stagnating wild fish populations, which means that fish production will rely more heavily on cultured fish in the future. Self-sustained production of food fish and aquafeed can reduce the carbon footprint and provide local jobs (Árnason et al., 2015; Wenblad et al., 2013). The development of new sustainable feed formulas, and the introduction of new aquaculture species are both important in order to accomplish a more competitive and sustainable Swedish food production industry (Wenblad et al., 2013)

1.2. Spotted wolffish, *Anarhichas minor*

A species of increasing interest in cold-water aquaculture is the spotted wolffish, *Anarhichas minor*. The spotted wolffish is a marine bottom-dwelling finfish native to the North Atlantic and Barents sea (Le François et al., 2021). Favorable traits like high market value, high growth rate, late sexual maturation and strong osmoregulatory capacity make it a promising potential species for cold-water aquaculture (Knutsen et al., 2019). Wolffish are robust animals that thrive at high densities and can tolerate relatively large ranges in water quality parameters like oxygen and carbon dioxide. Early development of the adaptive immune system results in a low disease susceptibility. Infection caused by the *Aeromonas salmonicida* is the only bacterial disease reported in farmed wolffish (Foss et al., 2004; Le François et al., 2021). Generally, the spotted wolffish are fed high-protein feeds, but the nutritional requirements have not been fully investigated (Foss et al., 2004).

One of the major challenges currently compromising spotted wolffish culture is the lack of knowledge and access to an optimized diet (Foss et al., 2004; Le François et al., 2021; Templeman, 1986). It has been suggested that the nutritional requirements, like minerals, are not fully met in captivity, resulting in health and welfare issues like calcareous deposits in the kidneys (nephrocalcinosis) and build-up of partly mineralized fatty material under the skin surface (Béland

et al., 2020; Foss et al., 2004). Nephrocalcinosis is rare in wild fish, and may be caused by selenium in the diet (Béland et al., 2020). Wolffish get their name from their canine like teeth designed to crush hard prey. In the wild, spotted wolffish mainly prey on hard-shelled organisms like echinoderms, molluscs and crustaceans (Templeman, 1986). Further research on the nutritional requirements and development of feed for spotted wolffish is in order to improve culturing, health and welfare of the species.

1.3. Feeding the aquaculture industry

As of now, fishmeal is the main protein source in the manufacture of aquafeeds because of the many desirable nutritional and physical properties. High protein content, high digestibility, high palatability and the nutritional characteristics make fishmeal the most reliable protein source in aquafeed (Glencross et al., 2007; Jannathulla et al., 2019; Turchini et al., 2019). As fishmeal is usually derived from wild fisheries, the availability is decreasing with the stagnating, over-exploited wild fish populations, and the price increases subsequently. Replacing the fishmeal with less limited resources is important for a sustainable and economically successful aquafeed production that meets the increasing demands of the expanding aquaculture industry (Turchini et al., 2019).

The evaluation of alternative aquafeed ingredients over the past decades have resulted in more sustainable and low cost feed formulas of high nutritional value, but fishmeal is still often added to the feed (Glencross et al., 2007). Previous research trials have focused on supplementing aquafeed with alternative raw materials like soybean, microbial mass, microalgae, and mussel meal. However, many of the alternatives fail to match the nutritional characteristics of fishmeal, like protein content, amino acid balance, fatty acid content and mineral content (Árnason et al., 2015; Carter & Hauler, 2000; Delamare-Debouteville et al., 2019; Knutsen et al., 2019). The use of several plant protein sources in aquafeed is limited due to poor digestibility and the presence of antinutrients (Jannathulla et al., 2019). Additionally, production of terrestrial plants for fishmeal competes with terrestrial plant production for human consumption, as well as water resources and land area (Tacon & Metian, 2015).

1.4. Mussel meal

The evaluation of alternative protein source has proven mussel meal to be a valid potential alternative ingredient in fish feeds. The nutritional characteristics of mussel meat have been proven similar to those of fishmeal, with a high protein content, a sufficient amino acid profile that meets the nutritional demand of fish, and it contains the essential omega 3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Mussels are organisms of low trophic levels that can be cultured specifically to remove nutrient overload in eutrophicated areas and are used to close the nutrient loop in co-cultures with other species and are thus sustainable to produce. They don't compete with terrestrial food production and resources like land area and water resources (Árnason et al., 2015), and they can be cultured in Swedish and Nordic waters, reducing transportation costs and pollution. The mussels used in mussel meal are often too small (<5cm) to be marketed for human consumption, and may make up 30 – 50% of the total mussel production (Árnason et al., 2015; Berge & Austreng, 1989). Utilizing the waste from mussel production in feed manufacture can contribute to a more circular economy as resources are recycled instead of the linear "take-make-dispose" economy.

1.5. Inclusion of shells in mussel meal

Normally, the shells from mussel production are viewed as a waste product, and the de-shelling process makes mussel meal production an energy costly process (van der Heide et al., 2021). By including shells in the feed, the waste from mussel production is reduced and there is potential to reduce the energy cost of de-shelling. Shell inclusion in mussel meal increases the volume of the pellet and allows for larger production, making it more cost effective. The mussel shells have a high ash content mainly consisting of $CaCO_3$. They can therefore provide valuable minerals like calcium, phosphorus and magnesium (Hertrampf & Piedad-Pascual, 2003) when included in feeds. Minerals are required by all animals for normal growth and development. The formation of exoskeletons, endoskeletons, teeth and scales, as well as several other physiological processes depend on mineral availability in the feed and the surrounding water (Gatlin, 2007; Hertrampf & Piedad-Pascual, 2003). Therefore, replacing fishmeal with shelled mussel meal could have several benefits for aquaculture in both socio-economic and health and welfare aspects.

Previous studies including shelled mussel meal in fish feed have suggested that protein digestibility may decrease as a result of a high ash content, and that the high calcium content can reduce the availability of other important minerals like zinc, which may impair growth performance (Berge & Austreng, 1989; Langeland et al., 2016). Fish fed the diet with the highest mussel inclusion rate (45%) showed a reduced growth performance compared to the control diet without mussels, however no significant differences between the groups were reported. The overall digestibility of dry matter also decreased with increasing inclusions of whole mussels and enlarged livers were observed in the fish. However, no significant differences between condition factor (K) in the diet groups were found, and no negative effects were observed from the high calcium content. Berge and Austreng (1989) concluded that it is possible to use ground, whole blue mussels in fish feed.

1.6. Stress, health and welfare in fish

Defining stress is not always easy as the term is used to describe both a stressor and stress responses. A stressor can be defined as an endogenous or exogenous stimulus that may comprise the homeostasis of an individual and can encompass anything from a change in water temperature to a novel sound. Aquaculture related stressors such as temperature changes, poor water quality, hypoxia, high rearing densities, poor nutrition, vaccination, and transportation may threaten the homeostasis of captive fish (Lays et al., 2009; Wendelaar Bonga, 1997). The stress response alters the homeostasis of the body through mobilization and reallocation of energy (Wendelaar Bonga, 1997), and is grouped into the primary, secondary and tertiary stress response.

The primary stress response includes changes in neuroendocrine and endocrine activity. In teleost fish, this includes activation of two main neuroendocrine routes (Wendelaar Bonga, 1997). The first one is the sympathetic nervous system, where the hypothalamic-sympathetic-chromaffin (HSC) cell axis releases catecholamines (adrenaline and noradrenaline) into the circulation from the chromaffin cells in the head kidney (figure 1). Activation of the hypothalamic-pituitary-interrenal (HPI) axis results in corticosteroids, mainly cortisol, release into the circulation from the interrenal cells in the head kidney (figure 1) (Wendelaar Bonga, 1997).

The secondary stress response includes the physiological effects caused by the primary stress response, such as respiratory, behavioral, cardiovascular, metabolic and immune processes. Catecholamines increase the cardiac output, blood oxygen transport capacity, gluconeogenesis, glycogenolysis, glucose release from the liver and free fatty acid (FFA) mobilization, among other

effects (Wendelaar Bonga, 1997). Glucose and FFAs are the two most important energy substrates in animals, and elevated plasma levels are seen subsequent to cortisol and catecholamine secretion (Ruane et al., 2001). Cortisol is a hormone with various important functions in osmoregulation, immune function, reproduction and growth, as well as playing an essential role in the primary stress response through hydromineral balance regulation and energy metabolism (Mommsen et al., 1999; Wendelaar Bonga, 1997). Disturbances of the hydromineral balance is one of the primary effects of stress in fish (Lays et al., 2009) as well as increased hematocrit and hemoglobin coupled to increased oxygen demand (Hudson et al., 2008; Roche & Bogé, 1996).

The tertiary stress response includes the effects of long-term or chronic stress. Prolonged exposure to a stressor can have detrimental effects on the fish homeostasis, which can cause symptoms as behavioral changes, immune system deficiencies and abnormal or decreased reproduction, growth and development (Lays et al., 2009; Sneddon et al., 2016; Wendelaar Bonga, 1997).

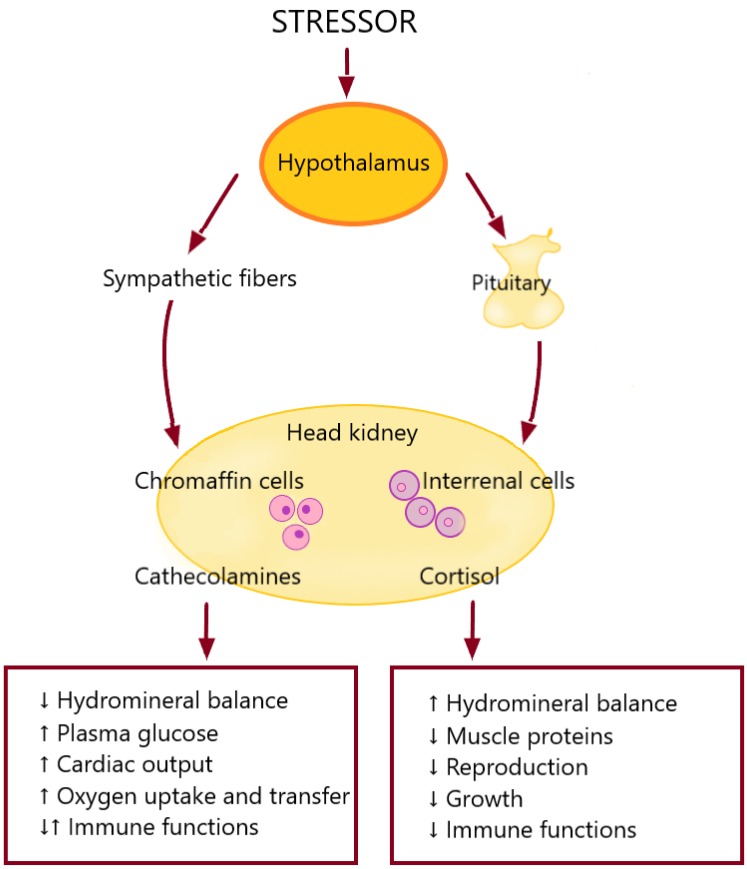

Figure 1:Simplified schematic of the stress response through the two main neuroendocrine routes (left: HSC axis, right: HPI axis). ↑ indicates upregulation, ↓ indicates downregulation.

Generally, spotted wolffish are not easily stressed in captivity compared to other species. Studies have shown that the spotted wolffish have, similar to other sedentary, benthic species, a relatively slow and weak primary stress response to a range of stressors with varying length and severity (Lays et al., 2009). Their low cortisol response together with low glucose plasma levels can be coupled to the sedentary lifestyle and a passive coping style, instead of the “fight or flight” response

which is more typical for pelagic fish. This non-stressed behavior is one of several favorable traits that make the spotted wolffish suitable for farming (Lays et al., 2009).

1.7. Growth in fish

Growth in fish can be defined as the change in size (length or weight) over time (Dutta, 1994). It is a continuous process as many species increase in both length and weigh their entire life (Dutta, 1994; Hinchcliffe et al., in prep). Length growth and weight growth are two fundamentally different processes. Length growth means hard tissue growth, mostly skeletal, and is a permanent process where the growth zones don't close (Dutta, 1994). Weight growth encompasses muscle, fat and gonadal growth and is reversible. The relationship between length and weight is called condition factor (K). K gives insight into the wellbeing of the fish as it is a good index to compare if the fish are growing well (Dutta, 1994), or for example the effects of different treatments. The K decreases during starvation, but also during sexual maturation as energy is mobilized for gonadal growth and development, and during the smoltification process in salmon (Björnsson et al., 1989). Therefore, K cannot always be directly indicative of the condition of the fish. The specific growth ratio (SGR) expresses growth as the percentage of change in body size per day and is a parameter commonly used by aquaculturists to measure growth in fish (Dutta, 1994; Hopkins, 1992). The smaller the fish, the higher the SGR, and as fish grow older the SGR declines (Dutta, 1994).

1.7.1. The GH/IGF1 axis

Insulin-like growth factor 1 (IGF-1) is the predominant endocrine growth regulator in most vertebrates and plasma concentrations can be positively correlated to an individual's growth rate (Hack et al., 2018). IGF-1 can be produced in all tissues in the body; however, the primary source is the liver. Hepatic IGF-1 is secretion is stimulated by growth hormone (GH) from the anterior pituitary gland. Upon secretion, IGF-1 binds to IGF-1 receptors in target tissues, mainly muscle and bone tissue, and stimulates somatic growth. IGF-1 also has a negative feedback effect on the pituitary production of GH (figure 2). The activity of IGF-1 is regulated by IGF binding proteins (Igfbp) that modulate the availability of IGF hormones to their respective receptors (Hack et al., 2018).

GH secretion from the anterior pituitary gland under the control of the hypothalamus is regulated by GH releasing hormone (GHRH), which stimulates production and secretion of GH, and somatostatin (SSTN), which inhibits GH secretion. GH is the ligand to the GH receptor that stimulates growth, cell division and cell regeneration directly through activation of tyrosine kinases, but also indirectly via IGF-1 (Brooks & Waters, 2010; Hartman et al., 1993). GH stimulates protein synthesis, shifting the metabolism towards protein growth while decreasing the lipid storage through lipolysis where is broken down to fatty acids and glycogen. This results in leaner, more muscular tissue (Björnsson, 1997).

In mammals and teleost fish such as tilapia and goldfish, GH release from the pituitary gland is also stimulated by ghrelin (Kang et al., 2011). Ghrelin is an appetite stimulating hormone mainly produced in the intestine, also working as a regulator of somatic growth, locomotor activity and energy balance. In contradiction to other teleost species, the effect of ghrelin in rainbow trout is anorexigenic, reducing plasma IGF-1 and GH levels. Decreased ghrelin levels in rainbow trout were positively correlated to SGR and CF (Jönsson et al., 2007), but there is still a knowledge gap for the role of these hormones in spotted wolffish.

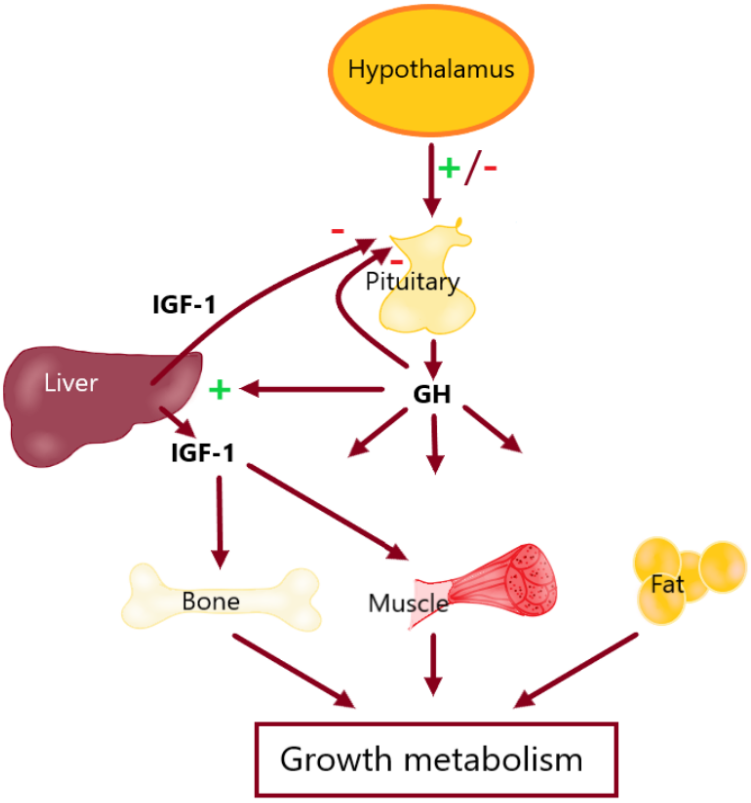

Figure 2: Simplified schematic of the GH/IGF-1 axis. + indicated stimulatory signals, - indicates inhibitory signals.

1.8. Aims and objectives

The aim of the present project was to develop a knowledge base for a new sustainable protein source for Swedish aquaculture. The project will assess the value of shelled mussel meal as an ingredient in aquafeed for spotted wolffish and the potential health implications of mineral carryover with regards to the growth, health and welfare status of the fish. Specific objectives are to:

1. Establish the effect of including shell in mussel meal on growth status by assessing specific growth weight, weight gain and condition factor with the hypothesis that shell inclusion impairs growth performance
2. Investigate the effect of mineral carry-over from mussel meal on blood health, stress and growth parameters by analyzing stress and growth biomarkers, acid-base balance, and ion-balance with the hypothesis that health and welfare is not impaired from dietary mineral supplementation
3. Optimize and validate two radioimmoassays for plasma ghrelin and plasma IGF-1 in spotted wolffish that can help determine the effect of shell inclusion on health and welfare parameters in plasma.

2. Materials and methods

2.1. Fish, holding conditions and experimental design

Spotted wolffish (*Anarhichas minor)* were provided by Aminor AS (Halsa, Norway) and reared at Nord University research station, Mørkvedbukta (Bodø, Norway). The fish were fed Otohime feed (PTAqua, Sandyform, Dublin). The fish were cultivated in a flowthrough system in PE-coated fiberglass tanks (1200L) with vacuum aerated and filtered (200µm) seawater (7,5^{o}C, 34‰) from 250m depth in Saltenfjorden. The photoperiod was set to 12L:12D. Initial samples and weight and length measurements were taken on all fish prior to allocating them into the experimental tanks.

180 adults of approximately 500 – 800g (877.05g ± 18.88) were randomly distributed into six tanks with the same holding conditions as in the pre-experimental period, with a total of 30 fish per tank. Three tanks were fed a control diet which contained experimental mussel meal (MM) and three tanks were fed an experimental diet containing mussel meal with added shell (MS). The fish were fed in excess three times on a daily basis. Everyday recordings of water temperature, oxygen levels, CO^2, and mortality (ammonia, nitrite, nitrate, iron, HCO_3^-) were made. Controls on the water flow rate (200L/h), pH and the fish welfare were made throughout the experiment.

2.2. Experimental feed composition

The two iso-calorific, iso-nitrogenous and iso-lipophilic diets were formulated. The diets were extruded into 5 mm pellets by the Swedish agricultural university (SLU, Uppsala, Sweden). Prior to use, diets were stored at 4 °C and in the dark. During the course of the experiment, diets were stored at room temperature, in air-tight and light protected containers. The compositions of the control diet, mussel meal, and the experimental diet, mussel meal with shells, are shown in table 1.

Table 1: Feed composition of the experimental diet (shelled mussel meal) and the control diet (mussel meal). The shell content and ash content are highlighted in bold font.

Feed compositions

Ingredient (%)	Experimental diet	Control diet
Mussel meal	20.0	20.0
Fishmeal	44.0	45.0
Wheat gluten	7.0	7.0
Fish oil	10.5	10.5
Wheat meal	12.0	15.0
Mineral premix	1.0	1.0
Monocalcium Phosphate	1.0	1.0
Titanium dioxide	0.5	0.5
Shell	**4.0**	**0.0**
Total (%)	100.0	100.0
Proximate composition		
DM %	84.1	84.0
Ash %	**17.5**	**13.7**
Crude protein (%)	48.7	49.7
GE (MJ/kg)	20.0	20.1
Lipid %	14.4	14.5
Ca %	**3.8**	**2.3**
Lysine %	4.1	4.2
Methionine %	1.2	1.2

2.3. Approval by ethical committee

The experiment was approved by the Animal Welfare Committee at FBA, Nord University and was conducted in accordance with the Norwegian animal welfare act (LOV-2009-06-19-97) and the regulation on the use of animals in research (FOR-2015-06-18-761).

2.4. Data collection methods

Weight and length measurements were taken at week 0 (n = 90), 7 (n = 88) and 13 (n = 87). The fish were anaesthetized in overdose with Benzocaine for the start point samples, Finquel vet. for the midpoint samples and Tricaine Pharmaq for the endpoint samples. At the end of the experiment, they were sacrificed by a blow to the head before blood was sampled with a heparinized syringe (7.5g lithium heparin / 1ml Millipore water) from the caudal vein. The blood was then centrifuged with a tabletop spinner and the plasma was extracted into Eppendorf tubes in five different batches to prevent repeated re-freezing and rethawing of samples. A total of 36 fish were sampled, 18 fish from each feeding group. Growth performance parameters were calculated as following.

Specific growth rate, SGR (%/day) = 100 x (Ln [(W_F/W_i)]/Days), where W_F = final body weight (g) and W_i = initial body weight (g). SGR shows the percentual weight gain per day.

Condition factor: K = 100 x (W/L^3), where W = body weight (g) and L = body length (cm). K shows the relationship between body weight and body length.

Weight gain: %WG = 100 x [(W_F – W_i)/ W_i], where W_F = final body weight (g) and W_i = initial body weight (g). %WG shows how much weight the fish has gained between two time points.

2.5. Analytical procedures

Plasma IGF-1 was measured using a radioimmunoassay according to the protocol developed by GroPep Ltd, as described by Björnsson et al., 2018. A test of parallelism was performed with different plasma concentrations (3x, 2x, 1x, 0.5x and 0.25x) and two different secondary antibody dilutions (1:20 and 1:40). The undiluted plasma samples 3x, 2x and 1x were prepared with 75µl, 50µl and 25µl plasma before the extraction step with 50µl, 25µl and 100µl acid ethanol respectively. The 0.5x and 0.25x samples were diluted with Milli-Q water before the extraction step. The secondary antibody was either diluted 1:20 or 1:40 in RIA buffer before it was added to the sample tubes. The test of parallelism was performed on pooled plasma samples from both spotted wolffish and Atlantic wolffish of different ages. After the test of parallelism, the analysis was conducted with undiluted (1x) spotted wolffish plasma and 1:40 diluted secondary antibody. The IGF-1 primary antibody (Anti-Barramundi IGF-1 antiserum (Rabbit)) used was purchased from Agrisera, Vännäs, Sweden, and the secondary antibody (Anti-rabbit IgG Sigma R0881) and gamma-globulin (IgG from rabbit serum, Sigma I8140-10mg) was purchased from Sigma-Aldrich, St. Louis, USA.

Plasma ghrelin was measured with a radioimmunoassay according to the protocol described by Hosoda et al., (2000) and modified by Jönsson et al., (2007). A test of parallelism was performed using acidified and non-acidified plasma in a dilution series with 1x, 0.5x, 0.25x and 0.125x dilutions with RIA-buffer. The non-acidified, undiluted (1x) plasma was used for the RIA analysis. The primary antibody (Anti-rat ghrelin [1-11] antisera) was gifted from Dr. Hiroshi Hosoda, Department of Biochemistry, national Cardiovascular Center Research Institute, Osaka, Japan. The secondary antibody (Anti-rabbit IgG Sigma R0881) was purchased from Sigma Aldrich, St. Louis, USA. The tracer (NEX388010UC), an iodinated human ghrelin, was purchased from Perkin-Elmer, USA.

Plasma cortisol was measured with a radioimmunoassay according to the protocol described by Young, (1986), modified by Sundh et al., (2011). A beta-counter, Wallac 1409 liquid scintillation counter (LKB Instruments, Turku, Finland), was used to determine the radioactivity in the samples. The standard stock solution (5µg/mL cortisol prepared from hydrocortisone) was purchased from Sigma-Aldrich, St. Louis, USA. The tracer (tritiated hydrocortisone-[1, 2, 6, 7–3H (N)] was purchased from NEN Life Sciences Products, Boston, USA and the antibody (sheep anti-cortisol) was purchased from Guildhay Ltd., Guildford, UK.

Osmolality was measured with a freezing point depression method using The Advanced Micro-Osmometer Model 3300 with deionized water (0 mOsm/kg) as a reference. Ion concentrations were measured using 60µl plasma diluted 1:2 in an ion analyzer. Glucose, lactate and free fatty acids were measured using commercially available kits purchased from Sigma Aldrich, St. Louis, USA. The colorimetric assays were conducted according to the company's protocols. The protocols were

adapted for a 96 well microplate spectrophotometer (SpectraMax 190 Microplate Reader), and the samples were measured at 340nm (glucose), 450nm (lactate), 570nm (FFAs).

Hematocrit (Hct) was measured in hematocrit capillary tubes (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) centrifuged at 10000 g for 5 minutes. Hemoglobin (Hb) was analyzed with a handheld hemoglobin analyzer. (HemoCue 201+, Ängelholm, Sweden). The mean corpuscular hemoglobin concentration (MCHC) was calculated with the following equation.

MCHC = Hb/Hct, where Hb is the hemoglobin concentration [g/dl] and Hct is the hematocrit value [%].

2.6. Statistical analysis

Statistical analyses were made in SPSS (IMB SPSS Statistics for Windows, Version 28.0.1.1 (14), IBM Corp, Armonk, NY, USA). To determine which statistical test for comparison of means to use, all data were tested for normality with the Shapiro-Wilk test of normality and through visual inspection of histograms, as well as for heterogeneity of variance with the Levene's test. For normally distributed data, an independent, two-sided students t-test was used for testing the differences between means in the two treatment groups. For non-normally distributed data, independent two-sided Mann-Whitney U-test was used. The significance levels used in all tests was 0.05.

3. Results

3.1. Growth

Initially, the MS diet group had a lower mean weight (864.61g ± 22.85) compared to the MM diet group (889.50g ± 24.37), but at the end of the experiment, the average weight of the MS diet group (1161.72g ± 30.82) exceeded that of the MM diet group (1145.17g ± 32.39) (figure 3A). A general trend towards better growth performance was observed in the fish fed MS. Overall, K, WG and SGR (figure 3B) was higher in the fish fed MS than in the fish fed MM. The students t-test and Mann-Whitney U-test showed no significant differences in body weight, length, K, WG or SGR between the treatment groups ($p>0.05$) at week 7 and 13 of the experiment (appendix B). The biometric and growth performance data at all timepoints is presented in table 2.

Table 2: Body weight [g], body length [cm], condition factor [K], weight gain [%] and SGR [%/day] of spotted wolffish fed mussel meal and shelled mussel meal at week 0, 7 and 13. At week 0, 90 fish were measured, at week 7, 89 fish were measured, and at week 13, 88 fish were measured. The values are presented as mean ±SEM. No statistical significances were observed between the two diets (p>0.05).

Biometrics and growth performance

Time	Diet	Body weight [g]	Body length [cm]	K	WG [%]	SGR[%/day]
Week 0	MM	889.50 ± 24.37	40.73 ± 0.33	1.29 ± .0.02	-	-
	MS	864.61 ± 22,85	40.06 ± 0.28	1.32 ± 0.02	-	-
Week 7	MM	990.56 ±27.58	41.41 ± 0.32	1.37 ± 0.02	19.28 ± 4.86	0.23 ± 0.09
	MS	992.84 ± 27.81	40.89 ± 0.29	1.42 ± 0.02	21.72 ± 2.83	0.29 ± 0.08
Week 13	MM	1145.17 ± 32.39	43.87 ± 0.33	1.33 ± 0.02	39.38 ± 6.00	0.30 ± 0.05
	MS	1161.72 ± 30.82	43.74 ± 0.29	1.36 ± 0.02	42.41 ± 5.84	0.34 ± 0.04

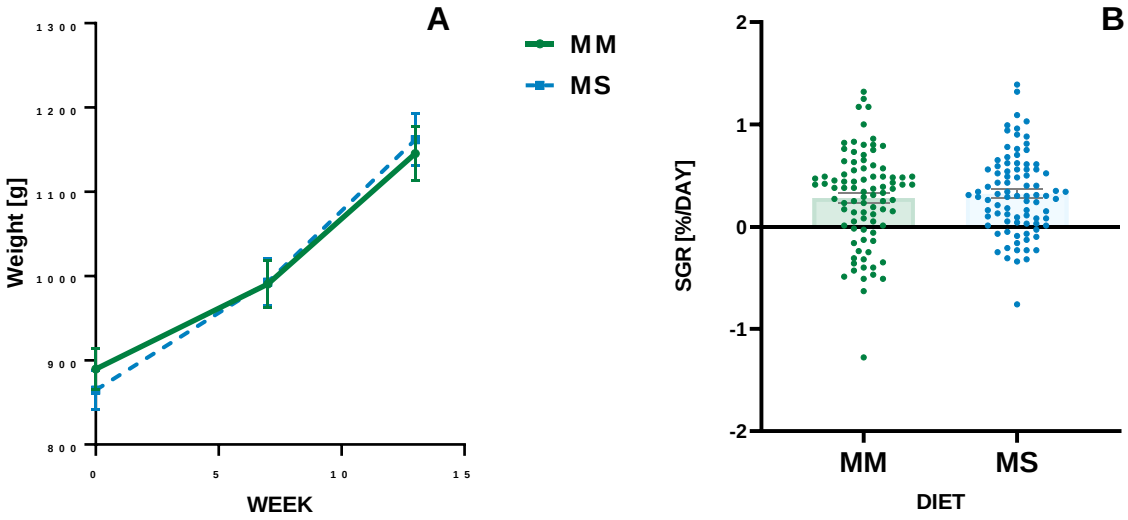

Figure 3: Mean (±SEM) values for (A) weight [g] at week 0, 7 and 13, and (B) SGR [%/day] at week 13. For spotted wolffish fed mussel meal (MM) and shelled mussel meal (MS). No significant differences between weight or SGR at any timepoint was observed with the statistical comparison of means.

3.2. Plasma biochemistry

Overall, no significant differences were observed with the students t-test or Mann-Whitney U-test for any of the measured parameters between the treatment groups (appendix B). A trend towards higher IGF-1 levels were observed in the fish fed MS (528.29 pg/mL ± 104.80) compared to the MM group (376.85 pg/mL ± 34.77), however the nature of the data shows that two outliers contribute to this increased mean (figure 4B). The stress biomarkers cortisol, glucose and lactate do not differ significantly between the groups (p>0.05). Fish fed MS have slightly lower average

cortisol levels at an average of 33.20 ng/mL ± 5.38 (figure 4A) compared to the MM group with an average of 34.32 ng/mL ± 4.80. Simultaneously, fish fed MS had higher average glucose levels (2.72 mg/mL ± 0.41) compared to fish fed MM (2.58 mg/mL ± 0.21). Lactate levels are higher in fish fed MM, but the difference is not significant ($p = 0.08$). Generally, ion concentrations are slightly higher in blood plasma from fish fed the mussel meal diet but none of the differences are significant. Hematocrit levels differ slightly between the two groups ($p = 0.72$). The appetite stimulating hormone ghrelin did not differ significantly between the feeding groups (figure 4C).

Table 3: Plasma biochemistry of spotted wolffish fed with mussel meal (MM) and mussel meal with shells (MS). The samples were taken in week 13 of the experiment. Values are given in mean ± SEM. None of the parameters differ significantly between treatments ($p>0.05$).

Plasma Biochemistry

	n	MM	MS
Glucose [mmol/L]	MM: 18, MS: 17	1.46 ± 0.12	1.54 ± 0.23
FFAs [mmol/L]	MM: 18, MS: 17	0.12 ± 0.02	0.12 ± 0,02
Lactate [mmol/L]	MM: 18, MS: 17	0.69 ± 0.07	0.53 ± 0.06
mOsm/kg	MM: 18, MS: 17	347.00 ± 5.14	338.82 ± 2.53
K^+ [mmol/L]	MM: 12, MS: 11	3.93 ± 0.15	3.85 ± 0.14
Na^+ [mmol/L]	MM: 16, MS: 11	162.16 ± 3.54	154.55 ± 2.93
Cl^- [mmol/L]	MM: 16, MS: 11	125.30 ± 3.69	116.29 ± 2.49
Ca^{2+} [mmol/L]	MM: 17, MS: 11	1.52 ± 0.03	1.55 ± 0.02
n-Ca^{2+} [mmol/L]	MM: 17, MS: 11	1.61 ± 0.03	1.54 ± 0.02
T-Ca^{2+} [mmol/L]	MM: 17, MS: 11	3.38 ± 0.06	3.25 ± 0.05
Hemoglobin [g/dL]	MM: 13, MS: 14	3.83 ± 0.08	3.89 ± 0.18
Hematocrit [%]	MM: 13, MS: 14	20.46 ± 0.43	19.93 ± 0.85
MCHC [g Hb/mm^3]	MM: 13, MS: 14	0.19 ± 0.01	0.20 ± 0.01
pH	MM: 12, MS: 11	7.40 ± 0.00	7.40 ± 0.00

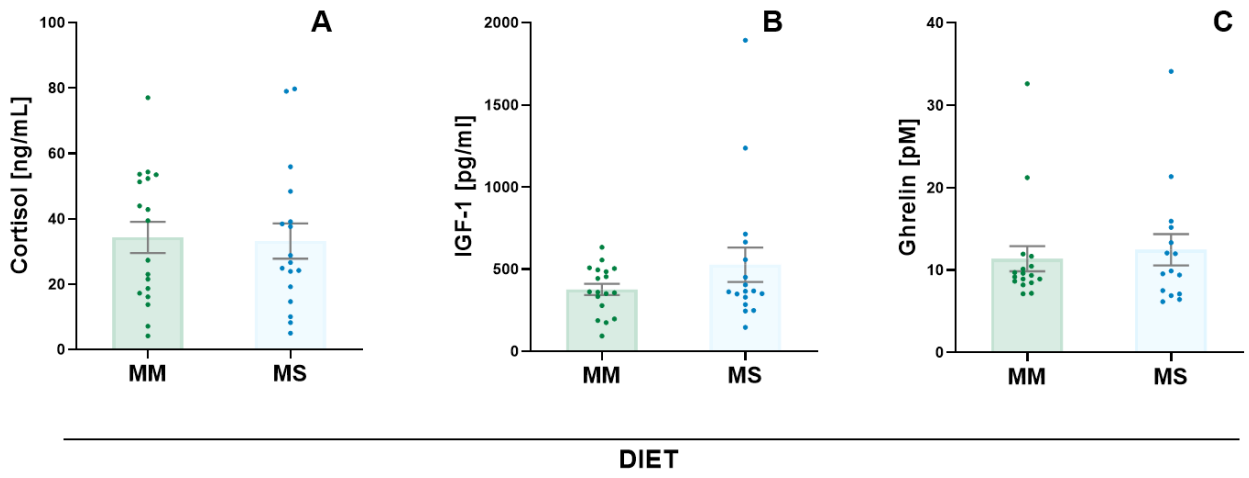

Figure 4: A: Mean (± SEM) values of (A) plasma ghrelin [pM] and (B) plasma IGF-1 [pg/mL] for spotted wolffish fed mussel meal (MM) and shelled mussel meal (MS) at week 13 of the feeding trial. No significant differences between the treatments were observed on either cortisol, ghrelin or IGF-1 levels.

4. Discussion

4.1. Shelled mussel meal does not impair growth performance in spotted wolffish

The average final weight of the 2 year old spotted wolffish was 1145g ± 32.39 for the MM diet and 1161g ± 30.82 for the MS diet group. Moksness, (1994) reported average final weights of 2-year-old spotted wolffish cultured at fluctuating temperatures between 5.8 – 13.7ºC to be 1580g. These fish were fed a common dry pellet. Moksness, (1994) also estimated that under optimal conditions (<7ºC), the spotted wolffish can be raised to 5.0kg after 2 years.

The total SGR (day 0 – 85) was 0.30% ± 0.05 for the fish fed MM and 0.34% ± 0.04 for the fish fed MS. These values are lower than the SGR values reported by Jonassen, (2002), who observed SGR values between 0.41% and 0.47% in spotted wolffish fed feeds with high-fat and low-fat diets. However, these fish were juveniles, and the average start weight was 450 and 474g. In the current experiment, the fish were adults, and the start weight was 877g ± 18.88. Growth rates decrease in fish with age (Moksness, 1994), and this comparison is therefore not accurate. Knutsen et al., (2019) reported an overall SGR of 0.26% in juvenile spotted wolffish fed different inclusions of the microalgae *N. oceanica*. As these fish were younger and the start weight was 625g ± 7.44, we could expect the SGR to be higher than for the wolffish in the current experiment. Tremblay-Bourgeois et al., (2010) reported SGR values between 0.34% and 0.90% for juvenile spotted wolffish reared at 10, 20 and 40 kg/m^2 densities. The SGR values in the current experiment shows that the fish grew well on both mussel meal and shelled mussel meal, with no impairment caused by the increased ash content from shell inclusion. A relatively large proportion of the fish in the current experiment had negative SGR values (figure 3B), which highlights how the broodstock and diet is not optimized yet for spotted wolffish. Growth ratio of spotted wolffish has been proven heavily dependent on the feed intake (Foss et al., 2002), which may suggest that not all fish were feeding at normal rates. A

possible establishment of a feeding hierarchy may explain the wide distribution of SGR in the fish (Hinchcliffe et al., in prep).

IGF-1 and ghrelin has never before been measured in spotted wolffish. The assays were optimized to fit the respective standard curves and can therefore be used for future research on this species. IGF-1 and ghrelin plasma levels were not affected by the inclusion of shells as none of the parameters differed significantly between the feeding groups. The role of ghrelin in fish species is still not fully elucidated, however it is considered an appetite stimulating hormone and the present results indicate no reduction in appetite caused by the addition of shells. Unpublished analysis of plasma ghrelin in Atlantic wolffish show results ranging from 2.56 pmol/L ± 1.20 – 4.24 pmol/L ± 1.70 (Hinchcliffe et al., in prep), which is significantly lower than the obtained results from the present study (MM: 11.38 pmol/L ± 1.53, MS: 12.47 pmol/L ± 1.89). Since ghrelin also plays a role in GH secretion, and therefore in growth regulation, these results may correspond with the higher growth rates of spotted wolffish compared to Atlantic wolffish. FFAs have also not been measured in spotted wolffish before and in general, there remains a paucity if the screening of plasma from the scientific literature. The values did not differ significantly between the two diets. FFAs have been measured in Atlantic wolffish, where values between 0.03 nmol/ml ± 0.01 and 0.11 nmol/ml ± 0.06 were observed (Hinchcliffe et al., in prep), while the current experiment detected values at 0.12 mmol/L for both diets. FFAs are released as a consequence of catecholamine and GH secretion and may reflect the growth process or the possibly ongoing stress response.

4.2. Feeding spotted wolffish with shelled mussel meal does not negatively affect plasma stress biomarkers

Plasma glucose levels were not significantly different between the experimental groups. Glucose levels in spotted wolffish have previously been observed between 0.3 and 0.4 mmol/L (Lays et al., 2009), which is very low compared to other pelagic, actively swimming species like salmonids, carp and cod (3 – 10mmol/L), and also low compared to other sedentary species (0.2 – 1mmol/L). Glucose levels in the present study range between 0.77 – 3.93 mmol/L, which is relatively high compared to the values reported for spotted wolffish by Lays et al., (2009). Cortisol mobilizes glucose, and the low levels observed in the spotted wolffish may indicate a low capacity of energy mobilization when exposed to a stressor. Lays et al., (2009) found significant elevations of cortisol levels in spotted wolffish exposed to hypoxia, while the glucose levels only increased during the recovery period after the hypoxia challenge. The low levels could also be interpreted as a low energy mobilizing capacity during stress exposure. Low plasma cortisol levels (<10 ng/ml) were reported in spotted wolffish rapidly taken from the stock tank and killed by a blow to the head. Cortisol levels ~ 30 ng/ml were considered as a significant response. Plasma cortisol levels as high as 170 ng/ml were reported after *in vivo* Adrenocorticotropic hormone (ACTH) injection in spotted wolffish, and proves the capacity of cortisol synthesis in this species (Lays et al., 2009). Glucose levels peaked at the same time as cortisol levels, confirming that cortisol plays a role in glucose mobilization in this species.

Le François et al., (2013) reported cortisol levels between 5 – 45 ng/ml in a study where the fish were exposed to acute (handling) or chronic (confinement) stressors. Pre-stress cortisol values were on average 5.07 ng/ml ± 1.79, and the highest value of 45 ng/ml was observed at a rearing density of 40 kg/m^2. At a rearing density of 30 kg/m^2, cortisol levels were 30 ng/ml. In the present experiment, plasma cortisol levels ranged between 4.23 and 79.74 ng/ml, with an average of 34.32 ng/ml ± 4.80 for fish fed MM, and 33.20 ng/ml ± 5.39 for fish fed MS. These values are similar to

the plasma cortisol values observed in spotted wolffish exposed to hypoxic conditions and a rearing density of 30 kg/m^2 (Lays et al., 2009; Le François et al., 2013), however the present values are not close to the cortisol levels caused by ACTH injection. This may indicate an ongoing primary stress response, but the cortisol levels between the diet groups did not differ significantly and can therefore not with certainty be caused by the diet.

During the experiment, some fish in all tanks were infected with the cold-water ectoparasite Costia necatrix, which mainly affects skin and gills of fish and may cause symptoms like pale skin, scratching, mucus production, gill swelling and suffocation (Savage, 1935; Wedekind, 1999). The fish were treated with formalin, which is a highly effective chemotherapeutic against ecto-parasite infections of fish skin, gills and fins. Formalin treatment has been proven to cause damage to gills and hematopoietic organs of fish (Tavares-Dias, 2021), and changes in plasma parameters like chloride, calcium, cortisol, glucose, lactate, hemoglobin and hematocrit have been observed in species like Atlantic salmon (*Salmo salar*), rainbow trout (*Salmo gairdneri*), Coho salmon (*Oncorhynchus kisutch*) and pirarucu (*Arapaima gigas*) (Andrade-Porto et al., 2017; Nieminen et al., 1983; Powell et al., 1996; Wedemeyer, 1971) after formalin exposure. Differences in rearing conditions and the fact that this is a novel species make the results inconclusive. Additionally, the lack of information about stress responses caused by diet limits our knowledge on how mussel meal and shelled mussel meal may affect stress biomarkers in plasma. Complementary analyses like white blood cell count (WBC) are necessary to determine the state of the immune response.

The increased lactate value in fish fed MM did not significantly differ between the fish fed MS ($p = 0.084$). Lactate levels in spotted wolffish have previously only been measured by (Knutsen et al., 2019), who reported similar values as the present study (0.41 – 0,86 mmol/L). Increased lactate values are seen during anaerobic respiration, for example when the fish is very active, and is not a direct indicator of stress.

4.3. Shelled mussel meal has no significant effect on blood plasma biochemistry

Regarding plasma osmolality, acid-base balance and ion concentrations, no significant differences were found between the treatment groups. Osmolality, Na^+, K^+ and pH were all within the normal range of spotted wolffish (Osmolality: 330 – 369 mOsm/kg (Foss et al., 2001; Knutsen et al., 2019; Magnussen et al., 2008), K^+: 2.60 – 4.56 mmol/L (Imsland et al., 2009; Knutsen et al., 2019; Tremblay-Bourgeois et al., 2010), Na^+: 127 – 185 mmol/L (Imsland et al., 2009; Magnussen et al., 2008; Tremblay-Bourgeois et al., 2010), and pH: 7.12 – 7.42 (Imsland et al., 2009; Knutsen et al., 2019). As cortisol increases the Na^+-K^+-ATPase pump activity in intestinal cells, a decrease in osmolality can be expected if cortisol levels are high. The results from this study show normal osmolality and confirm the strong osmoregulatory capacity of the species.

Ca^{2+} levels in spotted wolffish has, to our knowledge, only been measured by Knutsen et al., (2019), who reported levels between 0.91 – 1.05 mmol/L. The Ca^{2+} levels in the present study exceed these values (MM: 1.52 mmol/L ± 0.03, MS: 1.55 mmol/L ± 0.02). These levels are lower compared to other marine species like the gilthead sea bream (*Sparus auratus)* (Abbink et al., 2004), turbot (*Psetta maxima)* (Ruyet et al., 2003) and sablefish (*Anoplopoma fimbria)* (Kim et al., 2017). A study on tilapia (*Oreochromis niloticus*) showed that plasma Ca^{2+} levels in nephrocalcinosis affected fish (3.66 mmol/L) were significantly lower than those of healthy fish (4.35 mmol/L) (Chen et al., 2003). Nephrocalcinosis affected rainbow trout fed diets containing different levels of

selenium showed no changes in plasma Ca^{2+} levels (Hicks et al., 2006). The lack of knowledge on the relationship between dietary calcium and nephrocalcinosis, as well as an established reference range for healthy and nephrocalcinosis affected spotted wolffish should be addressed in future research.

Hematocrit levels were higher than the control values for unstressed wolffish (18.6 – 19.5 % (Tremblay-Bourgeois et al., 2010)) and matched those of juvenile spotted wolffish stressed by air immersion (21.8 – 22.1% (Tremblay-Bourgeois et al., 2010)). Generally, active species have higher hemoglobin and hematocrit values than less active fish. As spotted wolffish are sedentary, not actively swimming species, it is expected that the hemoglobin and hematocrit levels in plasma are low. Increased hematocrit correlates positively with increased cortisol levels and is considered a reliable indicator of stress (Hudson et al., 2008). In the bottom-dwelling Antarctic fish (*Trematomus bernacchii)*, hematocrit levels of 22 ± 1% were considered high and indicative of a stress response (Hudson et al., 2008). In order to fully interpret Hb and Hct, complementary hematological parameters like red blood cell count (RBC) and mean corpuscular volume (MCV) need to be taken into consideration.

Cl^- values were lower than the normal range of 134 – 163 mmol/L (Foss et al., 2001; Knutsen et al., 2019; Magnussen et al., 2008). In another study performed on rainbow trout fed a diet containing ground, whole blue mussels, it was stated that the high NaCl content in the mussel shell could disturb alter the homeostasis of the fish (Berge & Austreng, 1989), however the diets in the present experiment is not prepared similarly.

5. Conclusion

No significant differences in SGR, WG, K and final weight and length were observed between the fish fed mussel meal and the fish fed shelled mussel meal. This means that shell inclusion in mussel meal does not impair the growth performance of spotted wolffish. No significant differences in the stress and growth biomarkers cortisol, glucose, lactate, ghrelin, IGF-1 and FFAs were caused by dietary shell inclusion. The osmoregulatory function was also unaffected by the shells as there were no significant differences in osmolarity, ion concentration or acid-base balance between the diet groups. This means that there is a function for shell waste from mussel meal culturing, which can contribute to a more cost-effective and sustainable feed production. The function of hormones like ghrelin and IGF-1 are still not fully understood in spotted wolffish. Additional research on body tissues like liver, muscles, kidneys, and intestine are required to fully assess the effect of mussel shells in the feed. Causes of nephrocalcinosis should be investigated further, as well as the nutritional demand of spotted wolffish. Blood plasma parameters like minerals, amino acids and a full hematological profile including RBC, WBC and MCV should also be taken into consideration in order to fully interpret the results.

The results from the present study suggest that shelled mussel meal is a potential alternative ingredient in fish feed that may replace a proportion of fishmeal without comprising the overall wellbeing of the fish.

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Appendix A: Popular Science Summary

It's The World's Most Underrated Aquaculture Species – but How Do We Feed It?

The snaggle-toothed spotted wolffish has many names: the world's ugliest fish, devil fish, sea wolf and intriguingly; the world's most underrated aquaculture species. Despite its frightening appearance, the wolffish is a social and calm animal perfect for farming. A big question surrounding the spotted wolffish is how to feed them. Maybe feeding them shells can give us some answers!

Why do we need the spotted wolffish?

Swedish aquaculture has low production, narrow species diversity and fish import costs exceeding export earnings. These are all clear indications that Swedish aquaculture needs to expand and diversify. This is where the spotted wolffish comes in; the high market value, high growth rate and robust physique make it a promising species for cold-water aquaculture. The nutritional demand of this fish remains a mystery and is possibly causing health and welfare issues like kidney stones.

Testing novel, sustainable fish diets

In this experiment, two diets for the spotted wolffish were tested: one diet containing mussel meal with shells, and one diet with only mussel meat. Mussels are a sustainable replacement for the commonly used fishmeal, which is a finite resource usually made from wild fish. Shells from mussel production are viewed as a waste product, but they contain valuable minerals for normal growth and development. In the wild, spotted wolffish eat hard-shelled organisms including mussels, and hopefully some of their health and welfare issues in captivity can be improved by mineral supplementation. Using shells in the feed can reduce shell waste from mussel production while increasing the size of the pellets. Therefore, replacing fishmeal with shelled musselmeal could have benefits for aquaculture in several aspects.

Can we feed shells to wolffish?

The results show that including shells in the feed does not reduce growth in the spotted wolffish. No disturbances of blood ions or pH were found, and no indications of stress or disturbances of growth hormones were detected. This means that including shells in their feed is possible, and we are one step closer to solving the mystery of what the spotted wolffish needs to eat to be happy and healthy. Additionally, we have a use for shell waste and a possible sustainable feed alternative that can help meet the demands of the growing aquaculture industry. We are still waiting for more results to fully evaluate the effect of shells on health and welfare, but the conclusion from my experiment is: **so far, so good!**

Appendix B: Statistical Analysis of Data

Table 4: Statistical analysis of all analyzed plasma, stress and growth parameters. The equality of means between the diets was tested with a Students t-test for the normally distributed data, and Mann-Whitney U-test for the not-normally distributed data. The Student and Mann-Whitney p-values are given assuming equal or unequal variances depending on the p-value from the Levene's test for equality of variances. Mann-Whitney p-values are not corrected for ties. *Unequal variances **Not-normally distributed. MM = mussel meal, MS = mussel meal with shells.

Statistical analysis

	Levene's	Shapiro-Wilk		Test for Equality of Means	
	p-value	p-value		Student p-value	Mann-Whitney p- value
		MM	MS		
mOsm/kg	0.201	<0.001**	0.166	-	0.245
K^+ [mmol/L]	0.158	0.830	0.221	0.711	-
Na^+ [mmol/L]	0.152	0.402	0.276	0.134	-
Cl^- [mmol/L]	0.159	0.041**	0.709	-	0.121
Ca^{2+} [mmol/L]	0.210	0.538	0.277	0.139	-
pH	0.666	0.802	0.861	0.920	-
nCa^{2+} [mmol/L]	0.221	0.399	0.120	0.099	-
TCa^{2+} [mmol/L]	0.240	0.435	0.435	0.102	-
FFAs [mmol/L]	0.959	0.651	0.009**	-	0.909
Glucose [mmol/L]	0.224	0.075	<0.001**	-	0.597
Lactate [mmol/L]	0.405	0.074	0.495	0.084	-
Ghrelin [pM]	0.492	<0.001**	0.004**	-	0.682
Cortisol	0.885	0.250	0.078	0.887	-
IGF-1 [pg/ml]	0.038*	0.953	0.005**	-	0.546
Hb	0.010*	0.625	0.661	0.758	-
Hct	0.007*	0.021**	0.525	-	0.720

MCHC	0.026*	0.017**	0.005**	-	0.076
CF week 0	0.922	<0.001**	0.409	-	0.120
CF week 7	0.458	0.703	0.173	0.458	-
CF week 13	0.248	0.013**	0.372	-	0.124
Weight [g] week 0	0.649	0.473	0.701	0.457	-
Weight [g] week 7	0.889	0.318	0.801	0.889	-
Weight [g] week 13	0.708	0.399	0.778	0.708	-
Length [cm] week 0	0.290	0.093	0.587	0.290	-
Length [cm] week 7	0.279	0.110	0.577	0.279	-
Length [cm] week 13	0.272	0.232	0.433	0.272	-
% SGR week 0	0.345	0.555	0.767	0.610	-
% SGR week 7	0.842	0.815	0.133	0.944	-
% SGR week 13	0.272	0.108	0.777	0.516	-
% WG week 0	0.784	<0.001**	<0.001**	-	0.830
%WG week 7	0.733	0.003**	<0.001**	-	0.637
%WG week 13	0.914	<0.001**	<0.001**	-	0.822