A circular economy approach for sustainable feed in Swedish aquaculture: A nutrition and physiology perspective

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UNIVERSITY OF GOTHENBURG

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For my Gran,

"The last ever dolphin message was misinterpreted as a surprisingly sophisticated attempt to do a double-backwards-somersault through a hoop whilst whistling the 'Star Spangled Banner', but in fact the message was this: *So long and thanks for all the fish.*"

- Douglas Adams, The hitchhiker's guide to the Galaxy

POPULÄRVETENSKAPLIG SAMMANFATTNING

Resultaten som presenteras i den här avhandlingen visar på möjligheter, men också på ett fortsatt behov av kunskap och innovation för att etablera en miljövänlig och resurseffektiv produktion av grå havskatt (ofta kallad kotlettfisk) och Europeisk hummer. De två arterna är uppskattade hos konsumenten och har högt marknadsvärde i Sverige. Målet med arbetet var att bidra till en kunskapsbas om deras odlingsbiologi; med djurens välfärd, en cirkulär ekonomimodell och minimal miljöpåverkan i åtanke i enlighet med FN:s agenda 2030.

En av de viktigaste utmaningarna inom vattenbruket är frågan om ersättning av fiskmjöl som proteinkälla i fodret då efterfrågan nu har överträffat utbudet på grund av den snabba utvidgningen av vattenbruket. Avhandlingen visar att man kan producera högvärdigt foderprotein från outnyttjade biprodukter från sjömatsindustrin, vilket också kan innebära ett värdehöjande av råvaran. Metoden som används är den så kallade pH-skiftprocessen, en ny teknik som optimerades i avhandlingen, som man kan använda för att producera ett högkvalitativt protein från biprodukter från sjömatsindustrin.

En av de största utmaningarna med att odla hummer är att öka överlevanden under larvstadierna, vilket krävde att vi tog fram kunskap om fodrets optimala sammansättning för de tidiga livsstadierna. Larverna växer och överlever bäst när de får äta på sina artfränder, och i dagens hummerodlingsindustri ser man därför en hög grad av kannibalism, vilket naturligtvis inte är gångbart (om inte larverna hålls separat). En rad olika studier gjordes; bland annat beskrev vi näringsinnehållet hos hummerlarverna i detalj, för att ge ett underlag för att utveckla ett artspecifikt foder. Vidare undersökte vi lämpligheten hos ett tiotal olika slags foder med lokala råvaror, bland annat med proteinisolat från biprodukter, som formulerades specifikt för att hitta ett foder som kan användas för att odla hummer kommersiellt. Det viktigaste resultatet var att vi fann att en diet som innehöll en andel räkor, skapad av lokala biprodukter från industrin, var den bästa källan och mest lovande kandidaten till ett hållbart hummerfoder.

Näringsbehovet för havskatten var också okänt och därför undersökte andelen protein i fodret som behövdes för optimal tillväxt, vilket visade sig vara relativt mycket - minst omkring 50% protein. Vi studerade också hur havskatt tål olika vattentemperaturer. Temperatur är en viktig faktor inom vattenbruket och havskatt kan överleva i varmt (15°C) vatten. Men, det sker på bekostnad av högre metabolism och minskad tillväxt, även om fisken inte visade några symptom på att vara stressade enligt de indikatorer som användes, t ex stresshormonet kortisol. Vi rekommenderar att odla havskatt vid låga temperaturer (≤11° C) för att få god tillväxt och välfärd. Sammantaget verkar dock havskatt vara en bra art att odla, de är relativt lätta att hantera och motståndskraftiga mot stress.

Resultaten kan bidra med kunskap och nyttiggöras i samhället för ett miljömässigt hållbart vattenbruk och produktion av nyttig mat i Sverige. Exempel är strategier för diversifiering av odlade arter och nya innovationer (foder). Genom en ökad användning av biprodukter från sjömatsindustrin i foderproduktionen kan fisket av vildfångad foderfisk som ansjovis, eller användandet av t ex sojamjöl i djurfoder, minskas. Kunskapen kan också överföras till andra arter och kontexter och bidra till en tryggad livsmedelsförsörjning och utveckling av ett resurssnålt vattenbruk - även i andra delar av världen.

DISSERTATION ABSTRACT

One major challenge in aquaculture is the issue of fishmeal replacement as a protein source in aquafeeds. It is agreed that the rate of demand has now outpaced the rate of supply due to the rapid expansion of aquaculture. In Sweden, work is being done to establish a knowledge base for the development of sustainable marine aquaculture, focusing on two species: Atlantic wolffish, *Anarhichas lupus* and European lobster, *Hommarus gammarus,* as well as on two novel protein ingredients. The goal of this thesis was to contribute to a knowledge base for the farming biology and culture operations of the two species, with a circular economy model and minimal environmental impact in mind in line with UNs agenda 2030.

In paper I, work was done to optimize the pH-shift process, a novel protein extraction technology with potential to produce a highly concentrated protein ingredient from industrial seafood by-products. Three combinations of herring by-products were chosen along with two different process settings and differences in final proximate composition were characterized. Results showed the alkaline version of the process gave significantly higher protein yields and all forms of by-products were deemed as promising.

Paper II initiated four feeding experiments (novel feed types, feeding regime and feed size and cannibalism effects) on growth and survival, to inform and update husbandry protocols in *H. gammarus*. Overall, we found that feed offered six times daily, small-grade dry feed (250–360 µm) and larvae fed different proportions of dry feed and/or conspecifics in both communal and individual rearing systems all improved growth and survival rates. This underlines the impact of cannibalism on survival in H*. gammarus* larviculture.

In paper III we examined the suitability of locally produced, novel protein sources from by-products on the growth of recently metamorphosed post-larva. We found that, that a diet containing a proportion of shrimp, created from local industry by-products, was the best source of a sustainable lobster feed for the emerging lobster aquaculture sector.

The nutritional requirements of Atlantic wolffish are not known. In paper IV six experimental diets were formulated to test differing protein increments, 35-60%. We found that there was a high protein requirement in the diet (50-60%) but observations suggested that individual wolffish were able to compensate for this by increasing individual feed intake.

The aim of paper V was to establish the stress response of Atlantic wolffish exposed to an acute and chronic temperature challenge. Overall we found evidence confirming a stress response in selected parameters, suggesting that at 15°C, the high allostatic load of this temperature leaves no scope for growth. However, no evidence of a primary stress response (cortisol) could be found, suggesting that cortisol may not be a good parameter to measure welfare in this species in future studies and aquaculture operations.

Keywords: Sustainability, aquaculture, aquafeeds, wolffish, European lobster

PAPERS INCLUDED IN THIS THESIS

This thesis is built around the work of the following papers, which are referenced throughout according to their roman numerals:

Paper I: Hinchcliffe, J., Carlsson, N. G., Jönsson, E., Sundell, K., & Undeland, I. (2019). Aquafeed ingredient production from herring (*Clupea harengus*) by-products using pH-shift processing: Effect from by-product combinations, protein solubilization-pH and centrifugation force. *Animal feed science and technology*, *247*, 273-284.

Paper II: Powell, A., **Hinchcliffe, J**., Sundell, K., Carlsson, N. G., & Eriksson, S. P. (2017). Comparative survival and growth performance of European lobster larvae, *Homarus gammarus,* reared on dry feed and conspecifics. *Aquaculture research*, *48*(10), 5300-5310**.**

Paper III: Hinchcliffe, J*. Powell, A*. Langeland, M. Vidakovic, A. Undeland, I. Sundell, K. & Eriksson, S. Comparative survival and growth performance of European lobster, *Homarus gammarus* post larvae reared on novel feeds. Accepted and published online in *Aquaculture research October 2019*

Paper IV: Hinchcliffe, J. Roques, J. Roos, J. Langeland, M. Hedén, I. Sundh, H. Sundell, K. Björnsson, B.T. & Jönsson, E. (2019) Effect of dietary protein level on growth and health of juvenile Atlantic wolffish (*Anarhichas lupus*). *Manuscript*

Paper V: Hinchcliffe, J*. Roques, J*. Ekström, A. Hedén, I. Sundell, K. Sundh, H. Sandblom, E. Björnsson, B.T. & Jönsson, E. (2019). Effects of environmental warming on growth, metabolism and stress responses in Atlantic wolffish (*Anarhichas lupus*). *Manuscript*

*Paper III and paper V, both authors contributed equally to the work

CONTENTS

ABBREVIATIONS

ANF	**Anti-nutritional factor**
AS	**Aerobic scope**
AAS	**Absolute aerobic scope**
FAS	**Factorial aerobic scope**
FCR	**Feed conversion ratio**
FD	**Freeze-dried shrimp pellet**
GBF	**Gut barrier function**
GC-MS	**Gas chromatography–mass spectrometry**
H	**Herring meal based feed**
HA	**Herring meal based feed + astaxanthin supplement**
HG	**Herring meal based feed + glucosamine supplements**
HAG	**Herring meal based feed + astaxanthin and glucosamine supplements**
MDS	**Moult death syndrome**
MMR	**Maximum metabolic rate**
NKA	**Na^+K^+ATPase activity**
MS-222	**Tricaine methanesulfonate**
n-3 PUFA	**polyunsaturated omega-3 fatty acids**
OD	**Oven-dried shrimp pellet**
ODS	**Oven-dried shrimp pellet + immune supplement**
PAS	**planktonic artemia supplement**
PIT tag	**Passive integrated transponder tags**
PL	**Post larvae**
S	**Shrimp meal based feed**
SBM	**Soybean meal**
SDG	**Sustainable development goal**
SGR_W	**Specific growth rate for weight**
SGR_L	**Specific growth rate for length**
SMR	**Standard metabolic rate**
T_{opt}	**Optimal temperature for growth**

1.0 INTRODUCTION

1.1 We live in an era of overexploitation

The global population is expected to increase up to 9.3 billion people by 2050 (FAO 2018). Thus, a considerable growth in food and feed production, worldwide, is required in order to meet this rising demand. One important objective highlighted by the sustainable development goal 2 – zero hunger - is that all people should have access to nutritious food (United nations development programme, 2019). At the same time, food production should not impair biodiversity and food sectors should become more diverse. Both land plant cultivation and terrestrial animal farming require large arable land areas and a large freshwater supply – both of which remain a scarcity in many countries (IPCC, 2018). The crucial question mankind must answer, this century, is how humanity will be able to supply the food needed by the global human population beyond 2050, considering the antagonistic interaction between the growing population and the anthropogenically driven climate and mass extinction crisis…once the limits of terrestrial food production have also been taken into account. In addition to the constraints and methods of terrestrial food production (IPCC, 2018), global fisheries landings have stagnated (FAO 2018), contributing further to the current food production crisis. Under this scenario, aquaculture has become the fastest growing food sector this century (FAO 2018). At the global scale, a robust aquaculture sector has the potential to improve the resilience of the world's food production (Troell *et al.,* 2014a). However, in order to do so, aquaculture needs to address important challenges that will hinder its future development; some of the most important are the development of sustainable feeds for fed aquaculture, as well as diversification of species and environmental friendly farming systems (Troell *et al.,* 2014a).

1.2 Sustainability

"Sustainability", has become a buzzword embedded into every core of modern society, becoming fundamental to all modern day political, economic and societal rhetoric; in this era of "fake news", never has the meaning of a word become so important. The Brundtland report - also called *our common future* – that was published by the UN secretary in 1983 and provided the most commonly accepted and used definition of sustainability: A "development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (Brundtland, 1987). In its broadest sense, "sustainability", can be defined as a system's capacity to continue over a long period of time (Cambridge dictionary 2019). Its usage can be traced back to conflicts over the increasing evidence of global scale environmental risks, such as climate change (Geissdoerfer *et al.,* 2017). Following on, the 2005 world summit on sustainable development identified sustainable development goals as "economic development", "environmental development" and "social development" (UN general assembly, 2005). The three have since become fundamental to sustainable development, leading to the inauguration of "the three pillar concept", see figure 2.

The 2030 Agenda for sustainable development was adopted by all United Nations Member States in 2015 and provides a shared blueprint for sustainable development from now into the foreseeable future (United nations development programme 2019). At its heart are the 17 Sustainable Development Goals (SDGs), which are an urgent call for action by all countries to solve global societal challenges in a global partnership (Figure 1). Each goal has its own specific targets that will, overall contribute towards its overall objective.

Figure 1: The sustainable development goals, image taken from (United nations development programme, 2019)

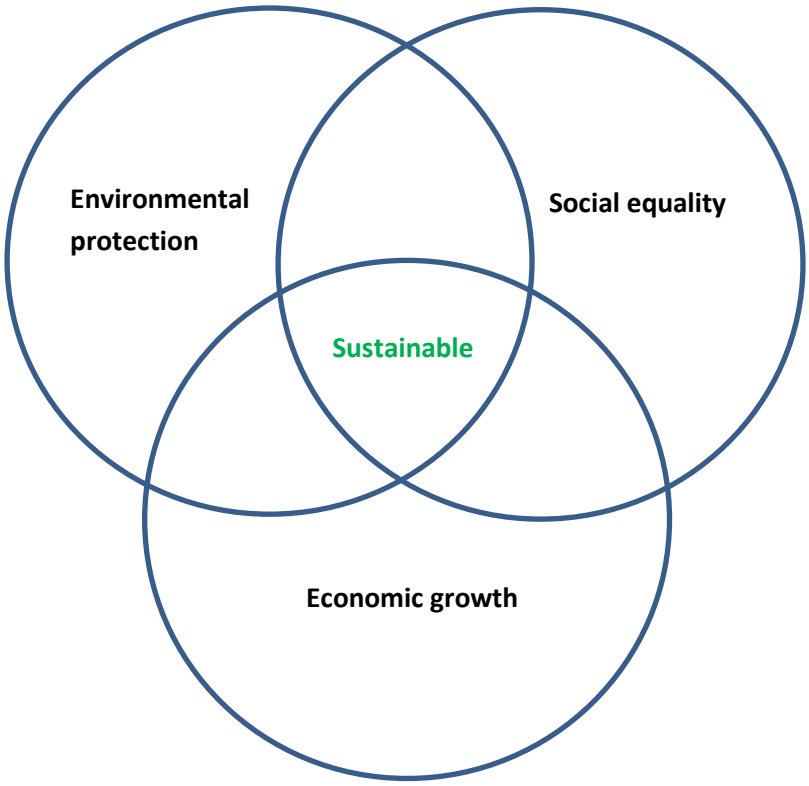


Figure 2: The three pillars on sustainable development as they are today. **Economic growth** is the pillar that represents economic development. **Environmental protection,** the pillar that represents environmental development and protection has transpired globally over the last 20 years. However, climate change, the large scale biodiversity loss and large scale pollution that we see today clearly shows we are far from successfully with this policy. The **Social equality** pillar focuses on the social well-being of people. The Brundtland report made a significant step with linking environment and social development. The social pillar also includes cultural perspectives that do not necessarily have a quantitative value, and are often interconnected to environmental and economic perspectives.

1.2.2 Circular economies

The concept of the Circular Economy has been gaining momentum since the late 1970s (Geissdoerfer *et al.,* 2017), and has emerged recently as a policy goal in the context of rising resource prices and climate change (Gregson *et al.,* 2015). The overall aim is to move away from the linear economic model (Figure 3); in a circular economic model, "wastes" become resources to be recovered and revalorized, through recycling and re-use (Gregson *et al.,* 2015). With an estimated one third of all food produced for human consumption being lost or wasted (FAO, 2011), the UN agenda 2030 goals called for a reduction in food loss during production (UN 2030 goal 12), with promotion of circular economy's becoming a core concept that is now manifested in EU strategies and agendas (Geissdoerfer *et al.,* 2017; EU, 2019)

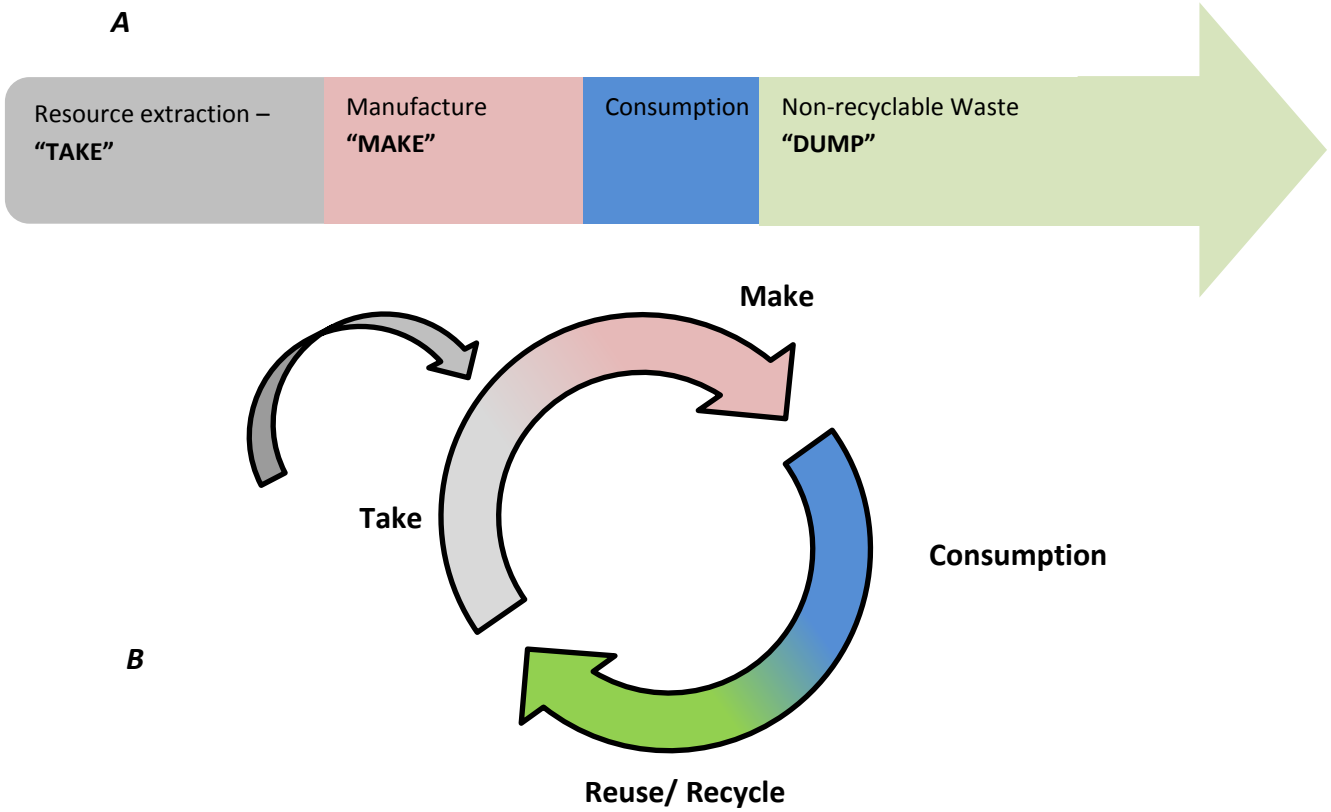


Figure 3: A) traditional linear economy, showing the "take, make, dump" model. B) A circular economy model showing resource extraction, manufacture and consumption with recycling/reuse as a core concept as opposed to non-recyclable waste in the linear model.

1.3 The blue revolution

The blue revolution refers to the intense growth in global aquaculture that has taken place since 1960. This rapid increase has been due to a large spike in human population growth, at the same time as wild fisheries have stagnated (FAO, 2018; Figure 4). The 2030 Agenda sets aims for the contribution and conduct of fisheries and aquaculture towards food security and nutrition, and the sector's use of natural resources, in a way that ensures sustainable development in economic, social and environmental terms (SDG 2, 9, 12 and 14). The contribution of aquaculture to total, global seafood production (aquaculture and capture fisheries combined), has increased at a rapid pace. In 2000 the contribution of aquaculture to seafood production was 25.7%, in 2012. When I was first starting to become engaged in aquaculture as a bachelor student, production was 35% (FAO 2012); now for the first time ever, aquaculture is on the verge of reaching 50% of total, global seafood with 47% productivity reached in 2016 (FAO 2018). Global aquaculture production in 2016 was 110.2 million tonnes, with the first-sale value estimated at USD 243.5 billion (FAO 2018).

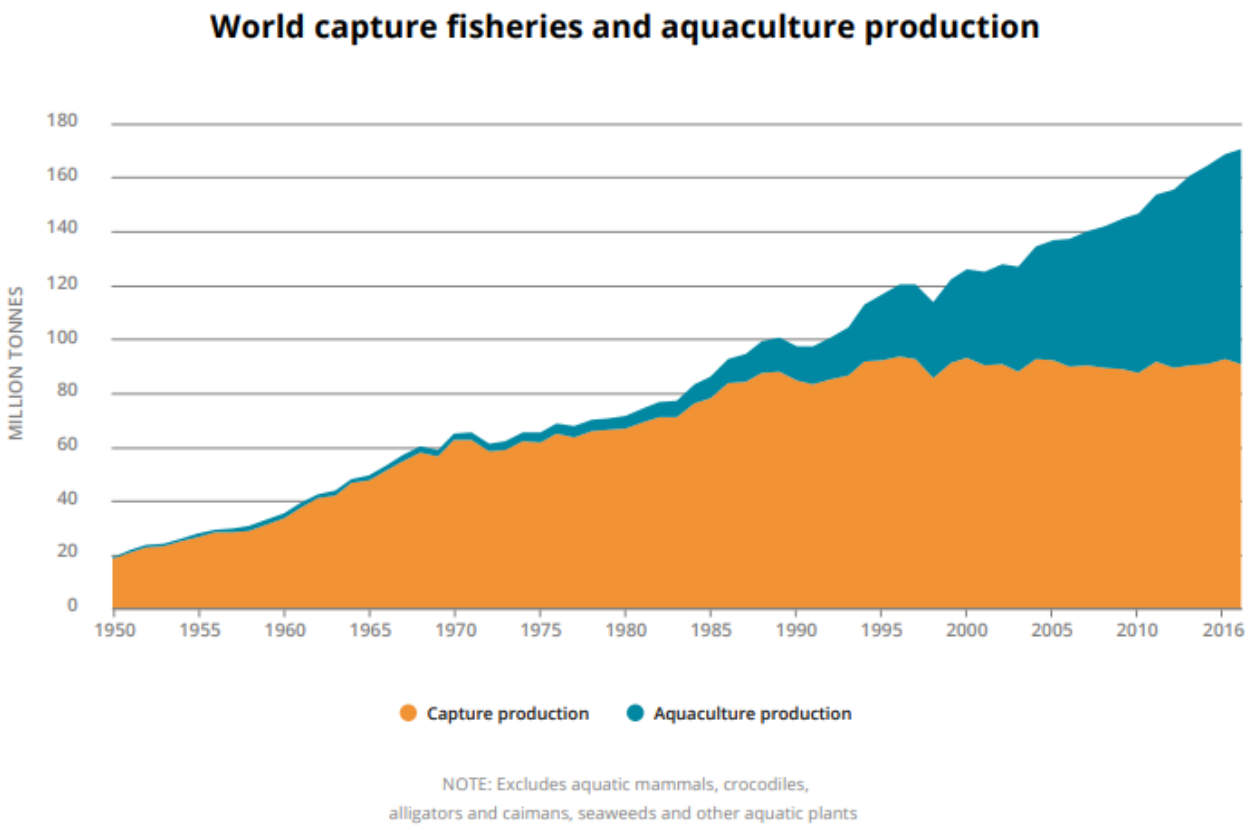


Figure 4: The rapid rise of aquaculture and the stagnation of capture fisheries, taken from the FAO (2018).

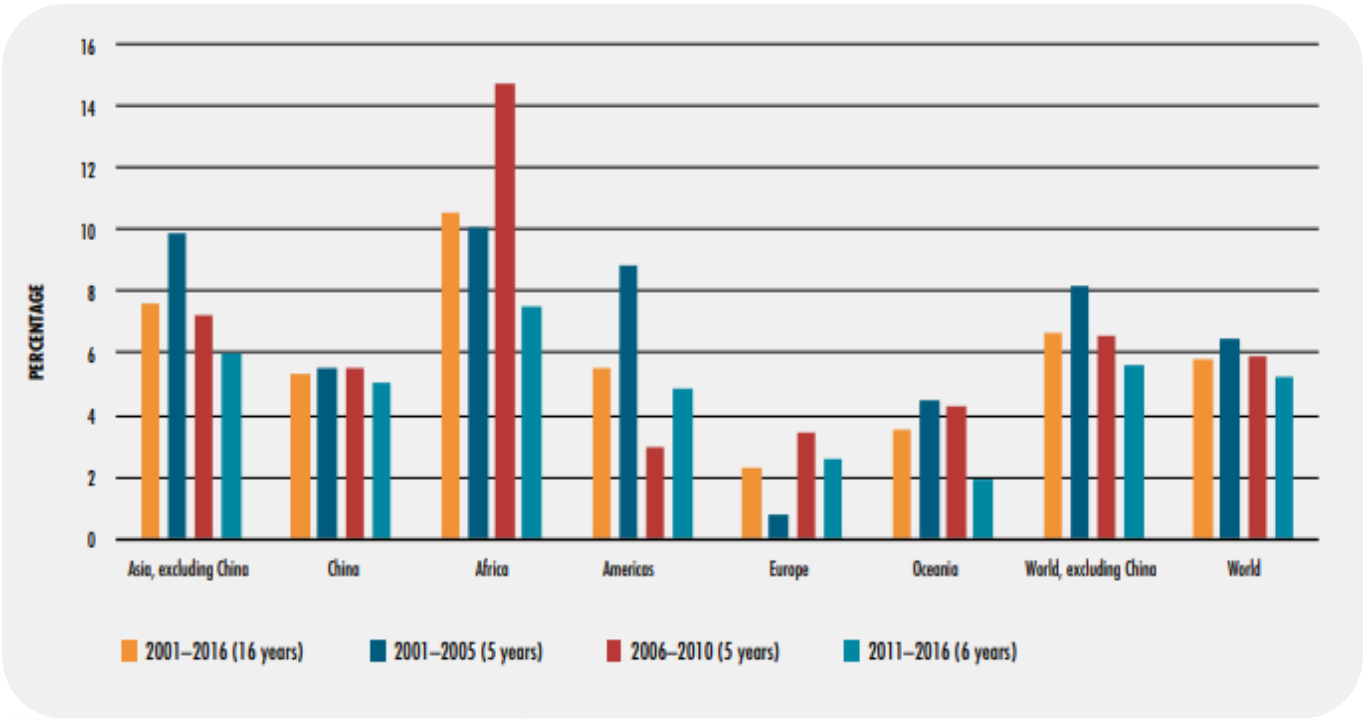
1.3.2 The shift towards seafood

Generally, seafood is low in saturated fats, carbohydrates, and cholesterol, whilst containing high amounts of protein, essential micronutrients, including various vitamins, minerals, and polyunsaturated omega-3 fatty acids (n-3 PUFA's; FAO 2018). Thus, even in small quantities, provision of seafood can be effective in addressing food and nutritional security, especially in developing countries with rapid population growth (The World Bank, 2013).

Table 1: Summary of fish supply and consumption taken from The World Bank, (2013)

Production	**Total fish supply**		**Food fish consumption**	
	2008	**2030**	**2006**	**2030**
Capture fisheries	89,443	93,229	64,533	58,159
Aquaculture	52,843	93,612	47,164	93,612
Total	142,285	186,842	111,697	151,771

A



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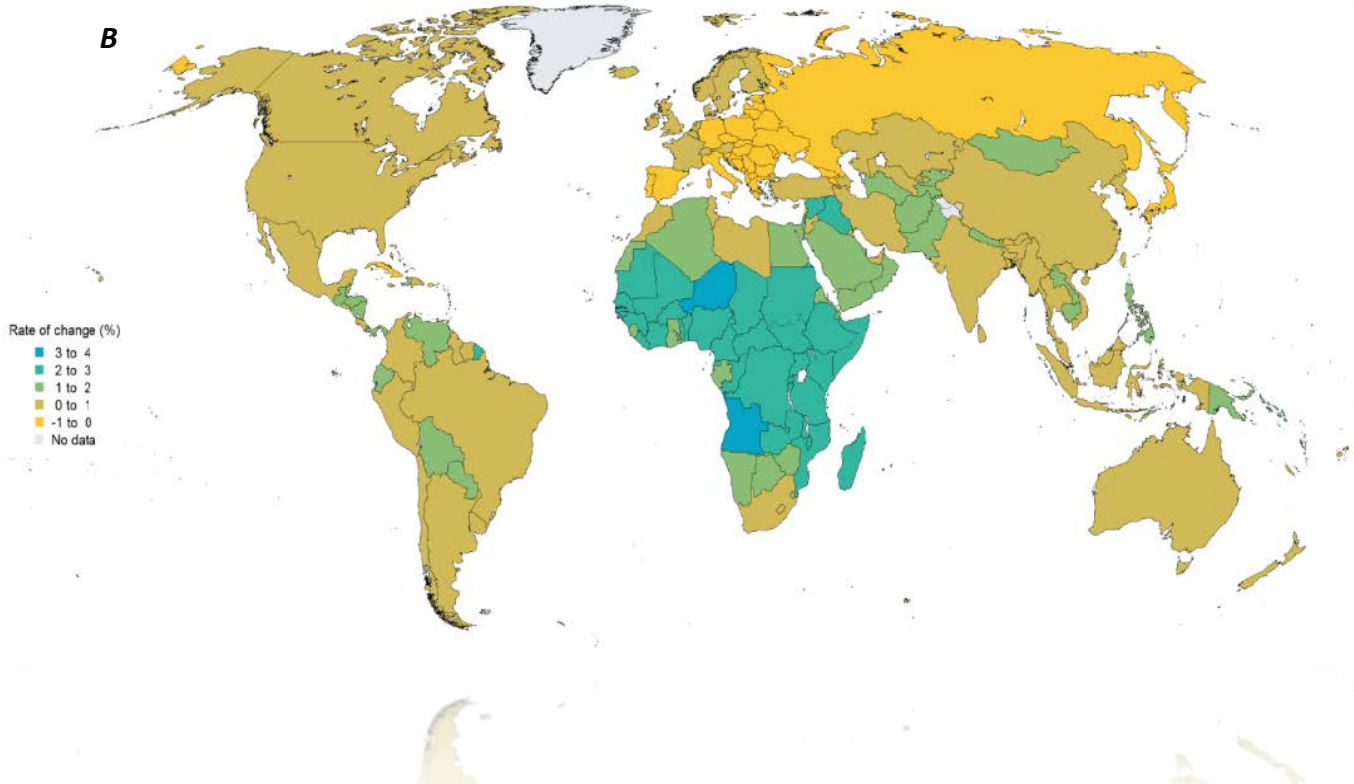

Figure 5: A) Average annual growth rate of aquaculture by volume (excluding aquatic plants), taken from FAO (2018). *B)* Expected average rate of human population change in the years 2025-2030 (medium variant projection). Data and map provided by (United nations population division, 2019)

As illustrated in Figure 5, while the level of overall aquaculture development varies greatly among and within geographical regions, there is a clear correlation with areas of fastest human population growth and areas with highest annual growth rate of aquaculture. Thus, aquaculture growth will have two growth scenarios that will occur this century:

1) **Population-driven demand:** The need for affordable protein, while nutritious, in rural regions with high population growth
2) **Market-driven demand:** The desire to produce high quality protein and fats to increase food security and dietary diversification in developed countries.

1.4 "The aquacalypse"

As described above, aquaculture is projected to be the prime source of seafood by 2030 (The World Bank, 2013; FAO 2018) and as reviewed in this thesis, the demand continues to grow, whilst capture fisheries have reached their maximum take. But, for an aquaculture system to be truly sustainable, it must conform to the Agenda 2030 SDGs and in the wider perspective, the three sustainability pillars that were described above. Despite the importance of fish to economic development and food provision, public debate in relation to aquaculture is dominated by concerns over resources and environmental sustainability (Ashley, 2007; Jenkins *et al.,* 2009; Mood and Brooke, 2010; Cashion *et al.,* 2016). A strong historical dependence of aquaculture on marine ingredients derived from capture fisheries as key feeds has been presented as a major challenge for the sector (Jenkins *et al.,* 2009; Mood and Brooker 2010; Cashion *et al., 2017*). However, this is not the major challenge facing aquaculture today; the real major challenge facing aquaculture is the need to improve public perception whilst decreasing its environmental footprint (Troell *et al.,* 2014a). Greenhouse gas emissions from aquaculture remain relatively small, estimated to be 15% of those from agriculture (Waite *et al.,* 2014) but emissions have been growing due to increased usage of feeds (FAO 2018). Therefore, reducing fishmeal and fish oil use and feed conversion ratios (FCRs) can be important in minimizing emissions (Hasan and Soto 2017).

"The aquaculture revolution, the blue revolution, has not always been green. We have to make the blue revolution greener. We need a turquoise revolution!"

—Thierry Chopin, Professor and expert in integrated multi-trophic aquaculture systems. University of New Brunswick

1.5 Aquaculture in Sweden

Swedish aquaculture has followed a modest expansion compared to geographical regions like Africa and Asia, with China being by far the largest producer in the world (FAO 2018). Nonetheless, production has increased from an average of 6000 tonnes in the 1990s to 15,000 tonnes in 2017 (Statistics Sweden, 2018). Aquaculture in Sweden is dominated by fresh water production of rainbow trout, which contributes to an estimated 88% of total production (Sweden statistics 2018). In contrast to this, marine aquaculture is comprised mainly of the edible mussel (*Mytilis edulis*) with stable annual harvests around 2000 tonnes (Statistics Sweden, 2018). The Swedish coastline has not been traditionally utilized as a food production area, unlike its neighbour Norway, and so introducing a new food production system to this area, such as marine aquaculture, would meet specific challenges. These challenges can only be met by novel strategies, which take local geography, local biology, economic factors and societal needs into account. The investigation "Det växande Vattenbrukslandet 2009" (Statens offentliga utredningar) followed national strategy "*Svenskt vattenbruk – en grön näring på blå åkrar, strategi 2012–2020" (Swedish aquaculture – a green industry on blue fields, strategy 2012-2020)* was launched in 2012 to stimulate and promote the sector since it was recognized to be underdeveloped. One of the results of these national initiatives was a feasibility study on the culture of marine fish conducted by the previous Aquaculture Centre West at the University of Gothenburg (since 2016, SWEMARC, the Swedish mariculture research centre),which highlighted the potential European lobster, *Homarus gammarus,* as promising candidate species for Swedish aquaculture (Albertsson *et al.,* 2012). Concurrently with this conclusion, of Wolffish sp, *Anarhichas minor* and *Anarhichas lupus* were also chosen as candidate species. Currently, there is large knowledge on both species' biology and their life cycles giving them a large potential for aquaculture development.

1.5.2 European lobster aquaculture

The European lobster has a pelagic larval phase consisting of four zoa stages before metamorphosing into a benthic post larvae stage (PL), where individuals eventually grow into adults (Factor, 1995; Rötzer and Haug, 2015). During the larval period, juveniles feed readily on natural and artificial feeds (Drengstig and Bergheim, 2013), however with differing successes. Temperature is crucial for growth in this species, for example, larval period in 20°C water is around 12 days (Drengstig and Bergheim, 2013) but at 15°C, larval period can last up to 35 days (Bartley *et al.,* 1980). Furthermore, European lobsters can reach 250–300 g in 24–30 months as long as constant 20 °C water is provided (Kristiansen *et al.,* 2004).

1.5.2.2 Potential for the European lobster in Swedish aquaculture

Today, European lobster is considered one of the most expensive seafood products in the world (Powell and European centre of lobster excellence 2016). The EU production of European lobster arises from wild caught landings which rarely exceed 5,000 tonnes per year, compared to American lobster, which are nearly 60,000 tonnes per year in Maine alone (Powell and ELCE, 2016). In Scandinavia the annual landings have declined sharply, this has led to elevated market prices due to an increasing gap between supply and demand (Powell and ELCE 2016). Hence, European lobster is a promising candidate for closed-cycle aquaculture if its culture can be optimised.

1.5.2.3 Bottlenecks for aquaculture

Currently in European lobster hatcheries there is a large degree of cannibalism during larval stages. This significantly decreases juvenile biomass, yet the survival of juveniles strongly depends on cannibalism (Powell and ELCE, 2016). Due to large growth variation and high losses due to cannibalism, cultured PL and adults have to be kept in individual containers. Therefore, the ideal system for rearing lobsters individually should be relatively cheap to maintain and operate, based on automatic feeding and consume little space (Figure 6).

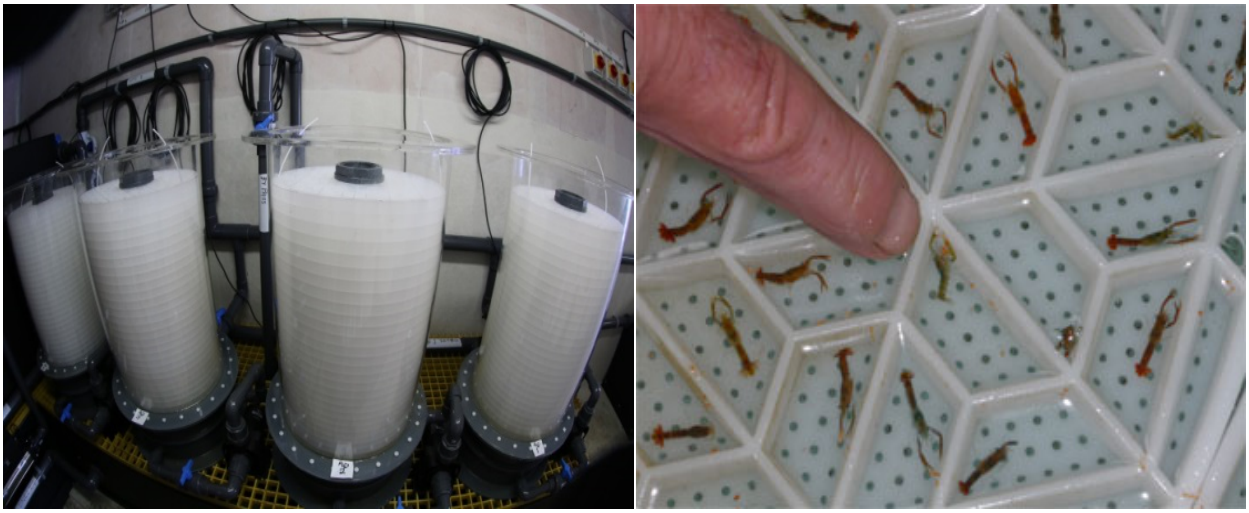

Figure 6: Images are courtesy of ELCE (European lobster centre of excellence https://www.nationallobsterhatchery.co.uk/elce/). Image on the left shows a recirculating aquahive system used to house layers of Orkney cells (image on the right).

Our understanding of the nutritional requirements of Homarid lobsters has expanded little since the early 80's (Conklin, 1995; Drengstig and Bergheim, 2013), and so there still remains a large paucity of information in the literature regarding an optimum feed for European lobster. For the successful cultivation to take place, an innovation in feed that is optimal for the nutrition of the animals and for the sustainability of the industry is warranted. For example, decreasing the level of protein in the diet to reduce costs can confer a financial benefit, but can lead to weight loss and decreased incidence of moulting (Drengstig and Bergheim, 2013). So far, the most suitable diets for juvenile lobsters are either live frozen brine shrimp, *Artemia salina* or pieces of raw shrimp (Personal communication within ELCE), but the cost of obtaining these, either from the wild or under culture conditions, restricts their use. Therefore, formulated feeds are preferred due to their consistent quality, economic viability, and low incidence of bacterial infections and ease of storage.

The reported optimum protein levels for lobsters fed artificial formulations are dated and require a new investigation; values have varied widely in the literature, with differing results: 60% (Castell and Budson, 1974), 53% (Gallagher *et al.,* 1976), 37% (Lucien-Brun *et al.,* 1985), and 35% (Boghen and Castell, 1981). The variation in the results obtained may be due to differing experimental conditions and the protein quality, making it challenging to interpret. Suboptimal feed can cause a variety of challenges when rearing lobsters, for example "Moult death syndrome" (MDS) which causes mortality by entrapment in the exuviae (Conklin, 1995). The feeds presented above may also have contained insufficient phospholipids, which could potentially explain the conflicting protein ranges. Conklin *et al.,* (1980) stated that the inclusion of soy lecithin in the purified diet of crustaceans is critical for survival and its absence can reduce survival dramatically (55% survival within 30 days). The review by Coutteau *et al., (*1997) highlighted the need to understand the interaction between protein sources and phospholipids in diets of crustaceans.

1.5.3 Atlantic wolffish aquaculture

The Atlantic wolffish, *Anarhichas lupus,* is a benthic species, distributed in the North Atlantic and Swedish west coast. It displays very similar characteristics to the more northern distributed spotted wolffish, *Anarhichas minor*, but the two species have some stark differences in biological aspects, one heavily cited example is the difference in growth rates, which is faster in *A. minor* (Moksness, 1994; Foss *et al.,* 2004). The natural diets of both species are well known and have been shown to be very diverse in the literature (DFO, 2013; González *et al.,* 2006; DFO, 2013;). *A. lupus* have a more diverse prey spectrum which mainly consists of echinoderms (29%) fish (28%) crustaceans (26%) and molluscs (9%), whereas *A. minor* preys mainly on fish (50%; Gonzalez *et al.,* 2006). In nature, reproduction occurs during late summer and early autumn and fertilization in the species is thought to be internal (Falk-Petersen *et al.,* 1999). There is a consensus amongst the literature that optimal temperature for growth (T_{opt}) decreases with increasing fish size (Foss *et al.,* 2004). T_{opt} and survival in the earliest juvenile phase has been estimated to be 10.3°C (Falk-Petersen *et al.* 1999; Hansen and Falk-Petersen, 2002), whereas more recently, Árnason et al. (2019) estimated the T_{opt} at 12.1°C at the same size of fish.

Figure 7: Juvenile *A. minor*, reared at commercial facilities at Aminor, Bodö, Norway. Image is courtesy of B.Th. Björnsson.

1.5.3.2 Potential in Swedish aquaculture: bypassing the need, for live feed

So far, the only commercial culture of a wolffish species is in Norway on *A. minor*. The spotted species is thought to be the more promising candidate for cold water aquaculture due to faster growth rates observed in captivity and in literature (Moksness, 1994; Foss *et al.,* 2004). However, Árnason *et al.,* (2019) highlighted the hidden growth potential in *A. lupus* and concluded that it should not be ruled out as a candidate species due to its clear potential for selective breeding and domestication. In addition, the Atlantic wolffish is the only native wolffish species to Sweden. It also a culturally popular fish for angling, commercial fishing and eating, especially on the Swedish west coast, however, the species is currently classified as endangered (Artdatabanken).

In general, wolffish display a wide range of attractive characteristics for aquaculture, both species have been proposed as promising candidates for cold water aquaculture (Falk-Petersen *et al.,* 1999; Le François *et al.,* 2002; Foss *et al.,* 2004) due to, a non-aggressive behavior, few disease problems and high fillet yield. Wolffish species also have the ability to be farmed in dense aggregations, contributing to space saving operations. Le François *et al.,* (2013) showed that juvenile spotted wolffish do not exhibit chronic cortisol responses with increasing density, with the optimum density being 30 kg/m^2. Wolffish eggs have a large incubation time (see next section), however their development differs somewhat from other lipid rich egg producing species such as salmon. Larval wolffish exhaust their yolk reserves upon hatching and are ready to feed on formulated feed at hatching (Falk-Petersen *et al.* 1999; Hansen and Falk-Petersen, 2001). Most fish species are reliant on endogenous yolk reserves for nutrition for some weeks after hatching. The nature of egg and larval development in the species is very favorable for aquaculture. The hatching of well-developed, large fry ready to be fed on formulated food allows wolffish cultivation to dodge some bottlenecks often experienced in production of marine larvae. Overall, this can provide a large economic shortcut to any potential industry which farms wolffish.

1.5.3.3 Bottlenecks for aquaculture

The nutritional requirements of both wolffish species have not been thoroughly investigated, with a lack of information regarding what the optimal nutrient composition of the diet should include, despite the hypotheses made about their natural diets. In general, for the spotted wolffish, protein-rich feed (55–62%) have been used (Foss et al. 2004; Aminor personal communication 2016), but good growth rates have also been obtained using feed with a lower (45–50%) protein content (Foss *et al.* 2004). Jonassen, (2002), investigated growth in juvenile spotted wolffish fed one low fat (15%) and one high fat (20%) diet and showed that fish in the low-fat group had a 13% higher final mean weight at the end of the experiment, indicating a negative relationship between dietary fat level and growth. Recently, Knutsen *et al,* (2019a, 2019b), included the microalgae**,** *Nannochloropsis oceanica* and *Scenedesmus obliquus* in the diet of *spotted* wolffish.

The nutritional requirements of Atlantic wolffish have been less investigated, Steffanusson *et al.,* (1993) and Strand *et al.,* (1995), both performed screening studies on Atlantic wolffish and suggested that the species has a high protein requirement, but neither study was exclusively designed to test this. The paucity of studies shows that detailed investigations, regarding dietary requirements and alternative protein sources are warranted. As this thesis has previously demonstrated, the growth rates of Atlantic wolffish have been shown to be slower than the spotted wolffish species (Foss *et al.* 2004). For a large scale operation, the reported growth performances of *A. lupus* (Stefanussen *et al.,* 1993; Moksness, 1994; Strand *et al.*, 1995; Árnason *et al.,* 2019), is poor and even the fastest growing groups in the recent study by Árnason *et al.,* (2019), were predicted to require three years from hatch to grow to a mean weight of about 1.5 kg. Another important issue is that the egg incubation period is labour-intensive and space consuming which lasts for several months (Foss *et al.* 2004). This represents a vulnerable stage, where temperature fluctuations, mechanical disturbances and infections may cause high mortality and induce pre-mature hatching (Hansen and Falk-Petersen, 2001). However, growing research in the subject has now identified the basic demands linked to reproduction, larval development and juvenile growth and a complete production line has been established for the species *ca.* 10 years from when the first eggs were artificially fertilized (Foss *et al.* 2004; Beirão and Ottesen, 2018; Dupont Cyr et al. 2018).

1.5.4 How can farming both species align with the UN 2030 sustainability goals?

The potential farming of both these species will fit well within the agenda 2030 goals for several reasons (SDG 2†, 9, 12 and 14). Both species can increase food security for Sweden and diversify the domestic food market (SDG 2† and 9). They are deemed to have a high market demand within Scandinavia and are therefore accepted among the local communities. The lobster fishery demand and the fact that the wolffish is a popular angling fish shows how cherished they are within the local culture (SDG 9 and 12). Farming both species will show encouragement to eat seafood and sustainably farm using local resources; therefore fitting within the 3-pillar sustainable development framework that this thesis established earlier. However, whilst the two species are nutritious (SDG 2† & 3), there also global challenges that aquaculture needs to overcome and are described in the following pages. We as humans need to rethink the production into a responsible circular model (goal 12) without exhausting resources for feed (goal 12 & 14), whilst, maintaining animal welfare.

†Goal 2 refers to reducing world hunger. Wolffish and lobster farming will fall into "market driven demand", a potential industry will not end world hunger. However goal 2 contains several targets which a wolffish and lobster industry, will contribute to. In this case, the knowledge base established will generate methods and ideas that are generic and can direct food production systems in a more resilient and resource efficient direction. Finally, these are two model species that were selected for Sweden, but the insights gained here and the circular approach employed have the potential to be applied for other species deemed promising, or already existing in other localities/countries.

1.6 Aquafeeds: facts and challenges

A fundamental footprint in any fed aquaculture production system is feed (Troell *et al.,* 2014a). Feed represents more than half of the total production cost in fish farming and any changes in the cost of feed will have a proportionately large impact on the total cost of production (Árnason *et al.,* 2009). In 2016, of the 171 million tonnes of total fish production, 88% was utilized for direct human consumption (FAO 2018). Whereas, the greatest part of the 12% used for non-food purposes (about 20 million tonnes) was used for fishmeal and fish oil (FAO 2018). A major challenge, during animal production is the need for sustainable protein sources for feed. It is agreed that supplies of fishmeal and oil will not keep pace with the increase in worldwide aquaculture (Miles and Chapman, 2005; Gatlin *et al.,* 2007; Glencross *et al.,* 2007; Olsen and Hasan, 2012; Turchini *et al.,* 2019), with the amount of captured fish destined for non-food purposes estimated to decrease in the future (Olsen and Hasan, 2012; FAO 2018). The high demand for the limited amount of fishmeal available, together with natural variations in the supply, is illustrated in the price increases during the last couple of decades (Figure 8: FAO 2018).

1.6.2 Replacing the golden standard

Fishmeal is, generally, a highly regarded source of feed proteins due to its favorable nutritional profile and excellent amino acid composition (Gatlin *et al.,* 2007), but it is heavily dependent on small dark muscle fish for production. Many different species are used for fishmeal and fish oil production, small pelagic species predominating. Many of the species used, such as anchoveta (*Engraulis ringens*), have comparatively high lipid yields but are rarely used for direct human consumption (FAO 2018). But production rates fluctuate according to changes in the catches of these species. Anchoveta catches, for example, are dominated by the El Niño phenomenon, which affects stock abundance, and ultimately the cost of fishmeal. Over time, adoption of good management practices and the implementation of certification schemes have decreased the volumes of catches of species targeted for reduction to fishmeal (IFFO personal communication 2015; FAO 2018). Production peaked in 1994 at 30 million tonnes and has followed a fluctuating but overall declining trend since then (FAO 2018). Coupled with this, the inclusion rates in compound feeds for aquaculture have also shown a clear downward trend as they are used more selectively, largely as a result of supply and price variation (Olsen and Hasan 2012; FAO 2018). For example, fishmeal and fish oil inclusion rates in Atlantic salmon diets fell from 65 to 24% and from 19 to 11%, respectively, between 1990 and 2013 (Ytrestøyl *et al.,* 2015). Whereas, FCRs have been reduced over the past 25 years largely because of better feed formulations, feed manufacturing and improved husbandry protocols.

If the aquaculture sector is to maintain its current average growth rate, the supply of nutrient and feed inputs will need to grow at a comparable rate. While this may have been readily attainable when the industry was still in its infancy, this will not be the case in the future as the sector and grows into a major consumer and competitor for feed resources.

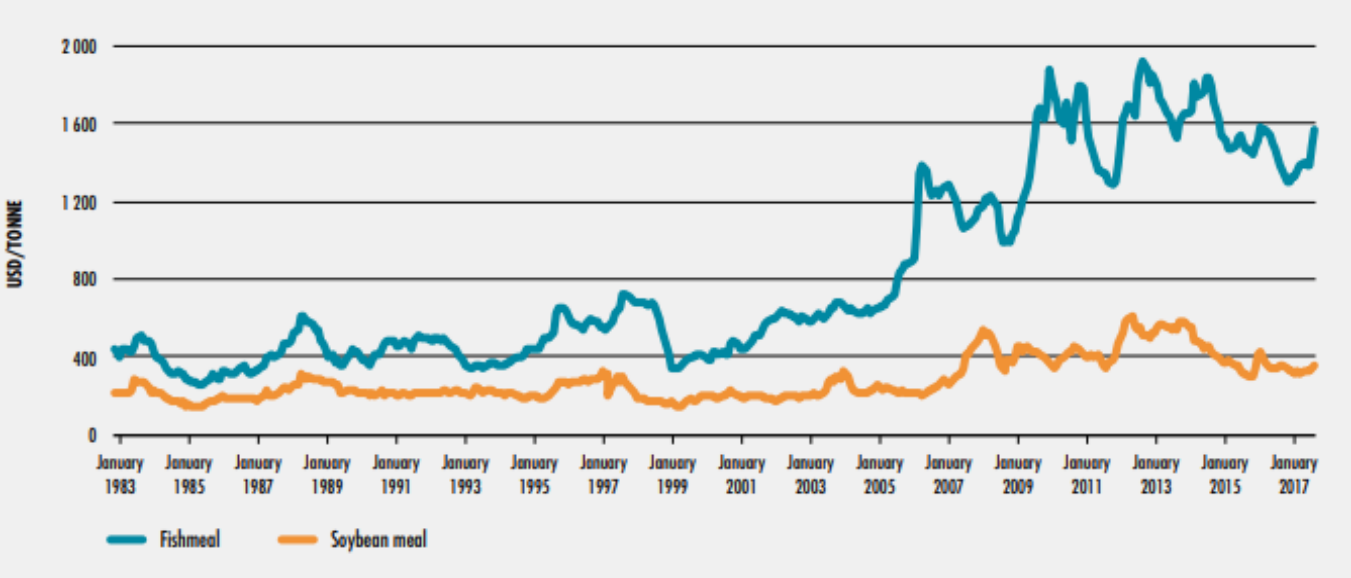

Figure 8: Price development of fishmeal and soybean meal since 1983 taken from FAO 2018. NOTES: Data refer to cost, insurance and freight prices. Fishmeal is considered to be of all origins. SOURCE: Data from Oil World and FAO's GLOBEFISH project.

1.6.3 Alternative protein sources in aquaculture

To become a satisfactory alternative feed component an alternative source to fishmeal must be able to supply comparable nutritional value to the consuming organism, whilst also being economically viable to ensure a competitive cost (Gatlin *et al.* 2007; Table 2). A wide range of raw materials are now used routinely in aquaculture feeds throughout the world. However, the main alternative protein sources, at present, are currently plant based. The most popular alternative is soybean meal (SBM) which has been favoured due to its economic viability and successful applications when used in feeding trials for a wide range of species including trout and salmon (Refstie, 2007), cod (Hansen *et al.,* 2007). Halibut (Berge and Helland, 1999), Red sea bream (Biswas *et al.,* 2007), American lobster (Floreto *et al.,* 2000) and many more. However, the use of plant-derived ingredients in aquafeed has its limitations, and will be discussed in the following section.

1.6.4 The Achilles heel of plant ingredients

While the nutrient composition of plant based meals is often the positive selling point of these raw materials for fish, several drawbacks limit their implementation into aquafeeds. The reduction in growth and health performance in response to high inclusion levels of dietary plant proteins has been reported in several aquatic animals such as rainbow trout (Kaushik *et al.,* 1995; Snyder *et al.,* 2012), Atlantic salmon (Krogdahl *et al.,* 2003; Refstie *et al.,* 2000; Torstensen *et al.,* 2008; Waagbø *et al.,* 2013), black tiger shrimp (Richard *et al.,* 2011) and many more. Most plant protein resources contain a variety of anti-nutritional factors (ANF), bioactive compounds which have evolved as a grazing deterrent in terrestrial plants (Krogdahl *et al.,* 2010). The influence of these ANF on fish can be considerable and varied (Glencross *et al.,* 2019). Several different classes and modes of actions exist which include tannins and saponins, bitter tasting compounds that reduce feed intake (Kumar and Singh, 1984; Francis *et al.,* 2002). An additional drawback of saponins is that they increase the permeability of the small intestine mucosal cells, therefore, facilitating the uptake of potentially harmful materials to which the gut would normally be impermeable (Johnson *et al.,* 1986; Knudsen *et al.,* 2008). Protease inhibitors, which inhibit the proteolytic activity of certain enzymes (Glencross *et al.,* 2019) and lectins, proteins that possess specific affinity for carbohydrate moieties (Sharon and Lis, 2003); which causes reduction in absorption of nutrients in the gastrointestinal tract. Overall, not only does the use of high-levels of plant protein increase incidence of ANFS, but they can also cause nutritional malnourishment issues, due to deficiency in essential amino acids (EAA) such as lysine and methionine, when compared to fish meal (Table 2; Gatlin *et al.,* 2007).

Table 2: Nutrient content of fish meal and targeted ranges for alternate ingredients, modified from Gatlin *et al.,* (2007).

Nutrient (%)	Fishmeal	Targeted ranges for alternative ingredients	Soybean meal
Protein	65-72	48-80	48
Lipid	5-8	2-20	0.9
Fibre	<2	<6	4.2
Ash	7-15	4-8	5.8
Starch	<1	<20	N.D
Arginine	3.75	>3	3.153
Methionine	1.75	**>1.5**	0.68
Threonine	2.5	>2.2	1.766
Lysine	4.72	**>3.5**	3.08
Omega-3 fatty acids	~2	*	3.2

1.7 Marine by-products

In the past, by-products were often thrown away as waste, used directly as feed for aquaculture and livestock, or used in silage and fertilizers (FAO 2018). However, by-products have been gaining attention recently, as they can represent a significant source of nutrients and can now be used more efficiently as a result of improved processing technologies. There has also been a shift in how we view "waste" as the circular economy model becomes more integrated in society. Overall, this represents a growing industry that is in line with EU strategies to reduce linear economies and stimulate blue growth, as well as SDG12. It is expected that there will be no increased usage of whole fish, for fishmeal production, caught by fisheries in the future; any increase will need to come from use of by-products (FAO 2018). It is now estimated that by-products account for about 25-35% of the total volume of fishmeal and fish oil produced (Jackson, 2012; FAO 2018). However, it is clear that there are large regional differences. For example, by-product use in Europe is comparatively high at 54% (Jackson and Newton, 2016). Yet, an under-discussed topic in the literature is the potential negative impact of by-products on the nutritional value of fishmeal as feed. Fishmeal produced from fish by-products will represent 34% of world fishmeal production in 2030, compared to 30% in 2016 (FAO 2018). But no model currently predicts the effects of the use of fish by-products on the composition and quality of the resulting fishmeal or on animal growth and welfare. Possible effects include lower protein and increased ash content in comparison with products obtained from whole fish. This difference in composition may hinder increased use of fishmeal in feeds and needs to be investigated further.

1.7.2 The pH-shift process: A novel protein extraction process

The pH-shift process, originally developed by Hultin and Kelleher (1999), has been shown to be an efficient means for protein recovery from fish muscle raw materials (Undeland *et al.,* 2002; Nolsøe and Undeland, 2009). The process has the advantage over, for example, enzymatic protein hydrolysis in that the proteins are not cleaved or subjected to elevated temperatures during the process, allowing them to retain their technical functionality such as gelation ability (Undeland *et al.,* 2002). In short, the process is based on the knowledge that muscle proteins can be solubilized in water at high (*ca.* 11) or low (*ca.* 3) pH. When proteins are solubilized, they can be separated from insoluble matter such as bones, skin, and to some extent lipids, using centrifugation or filtration (see methods). Purified solubilized proteins can then be isolated by isoelectric precipitation and then dewatered using a second centrifugation or filtration step. It is thought that the most promising use of this process is on more unrefined materials, such as by-products. To date, a series of pH-shift process-based studies have tested the use of fishery by-products as a potential source of protein (Chen and Jaczynski, 2007; Pires *et al.,* 2012; Shaviklo *et al.,* 2012; Chomnawang and Yongsawatdigul, 2013; Panpipat and Chaijan, 2016; Zhong *et al.,* 2016; Abdollahi and Undeland, 2019)

Results have been successful with up to 90% protein yield being reported from trout by-products (Chen and Jaczynski, 2007). However, at the start of this thesis project, there were no previous studies on pH-shift processing of herring by-products, and also not on evaluating the pH-shift produced protein isolate as an aquafeed ingredient.

1.7.3 Mussel meal: recycling nutrients to produce novel protein

As the production and processing of bivalves have increased, efficient use of the total product has become important, not only to maximize financial return, but also to address waste disposal problems. Little attention to date has been focused on meal from molluscs, and in particular, mussel meal as a potential replacement for fishmeal in aquafeeds. Blue mussels have a high protein content and amino acid content that is similar to fish meal (Jönsson, 2009; Jönsson and Elwinger, 2009). In addition to their favorable nutrient profile, the farming of blue mussels is considered environmentally friendly. Blue mussels are filter feeders and thereby they remove nutrients from the water, improving the water quality of a coastal area and also increasing the carrying capacity of the specific area. However, the shells have to be removed in order to obtain a suitable protein level, otherwise ash content is too high and that will in turn lower digestibility. Nevertheless blue mussels are reported to have potential to act as both a dietary protein source (Berge and Austreng, 1989; Vidaković, 2015; Vidakovic *et al.,* 2016; Langeland *et al.,* 2016) and as a feed attractant in fish (Nagel *et al.,* 2014). In Sweden, of the total mussel produced, only 50% are deemed edible for human consumption (personal communication with Swedish mussel farmers), due to damage which occurs during harvest or size. This represents a promising source of protein if viable shell removal can be established.

1.7.4 Evaluation of alternative protein sources

Increased usage of non-marine based feedstuffs coupled with lower fishmeal and fish oil inclusion rates in aquafeed are likely to influence the nutrient content of farmed aquatic products, particularly their fatty acid profiles (Sprague *et al.,* 2016). Marine by-products, such as fish carcasses, which are increasingly used to produce fishmeal and fish oil, still represent an underutilized source of nutrients and micronutrients. SBM remains the most commercially viable alternative to fishmeal in the current market, however the presence of anti-nutritional factors and lack of EAAs are large shortcomings. Of urgent importance is that increased use of plant ingredients in feeds has forced aquaculture production to shift alignment from aquatic resources to terrestrial resources, which occupies large carbon footprints and is heavily dependent on a freshwater supply (FAO 2018). Additionally, these products, for example soy beans, are grown for our own consumption; so it is of key importance to reduce competition with human food resources for sustainable production of aquafeeds.

A common misconception is that there needs to be a single ideal replacement for fishmeal. However, this simply transfers risk from one raw material to another (Turchini *et al.,* 2019). A more appropriate strategy is to enable the use of a broad range of raw materials that enables flexibility in the formulation process and adapt to changes in supply and price as they arise, (Glencross *et al.,* 2007; Turchini *et al.,* 2019), whilst supporting the development of locally sourced ingredients which can enhance food security and local economies.

1.8 Growth physiology of animals

Efficient growth and a high end product (muscle) quality of the farmed animals are major goals in aquaculture. Animals have different physiological capacity for growth depending on their life history strategies. This thesis will briefly describe the growth physiology of fish and crustaceans before proceeding with the next sections. In both groups of organisms, growth is regulated by a complex interplay between genetic, physiological, environmental, nutritional and social factors which go beyond the scope of this thesis. However it is important to know that such interplay exists and this is acknowledged in specific cases in papers II-V.

1.8.2 Fish growth

In vertebrates, growth can be defined as hyperplasia (increase in cell number) and /or hypertrophy (increase in cell size) which is coupled with a positive change in the energy content of the organism (Johnston, 2000). However, the growth pattern of fish can differ in certain aspects compared to vertebrate growth in general. Most fish species exhibit indeterminate growth and thus continue to grow throughout life. Growth can usually be measured over time with specific growth rate (SGR), expressed as percentage gain in weight or length per day (see methods section). But weight and length growth should be seen as two different processes, weight growth refers to soft tissue growth (muscle, fat deposition and gonadal growth) and is reversible whereas length growth (skeletal growth) is permanent and determines the functional size of the organism (Johnston, 2000). Teleosts have various strategies to accumulate lipid reserves, two categories are commonly used to classify these strategies in the literature (Sheridan 1994; Leaver *et al.,* 2008; Kling *et al.,* 2012). Fish are either classified as “lean”, characterized by low muscle fat (1-2%) but high HSI (6-7%) and liver lipid content (Larsson & Fänge 1977), therefore the liver represents the major energy storage (Kling *et al.,* 2012). In contrast, “fatty fish”, have a relatively small liver (1% HSI) and low liver fat, but high mesenteric fat and high muscle lipid content; in these fish the muscle and mesenteric fat represent the major energy reservoir (Kling *et al.,* 2012).

1.8.3 Crustacean growth

Crustaceans do not grow in a linear (or exponential), continuous fashion like many fish species. Exoskeletons are hard and rigid, so they are not expandable and unable to follow the growth rate of soft tissue. In order to grow, crustaceans need to shed their exoskeleton; this process is known as moulting. As growth is limited to these moulting periods, this results in in stepwise growth trajectories. Moulting occurs regularly throughout life (albeit frequency decreases with age) and is controlled by ecdysone, a steroid hormone which regulates moulting in arthropods (Factor, 1995). The period between one moult and the next is called the intermoult, and during this time no exoskeletal growth occurs. However, internal soft tissue growth will continue. Therefore, growth rates in crustaceans can be determined by the interaction of two fundamental processes; moult increments (the increase in size between successive moults, usually defined by the increase in carapace length) and intermoult duration (the time interval between two successive moults). Hence, incorporating both processes into a growth model enables us to obtain a better estimation of growth parameters in crustaceans

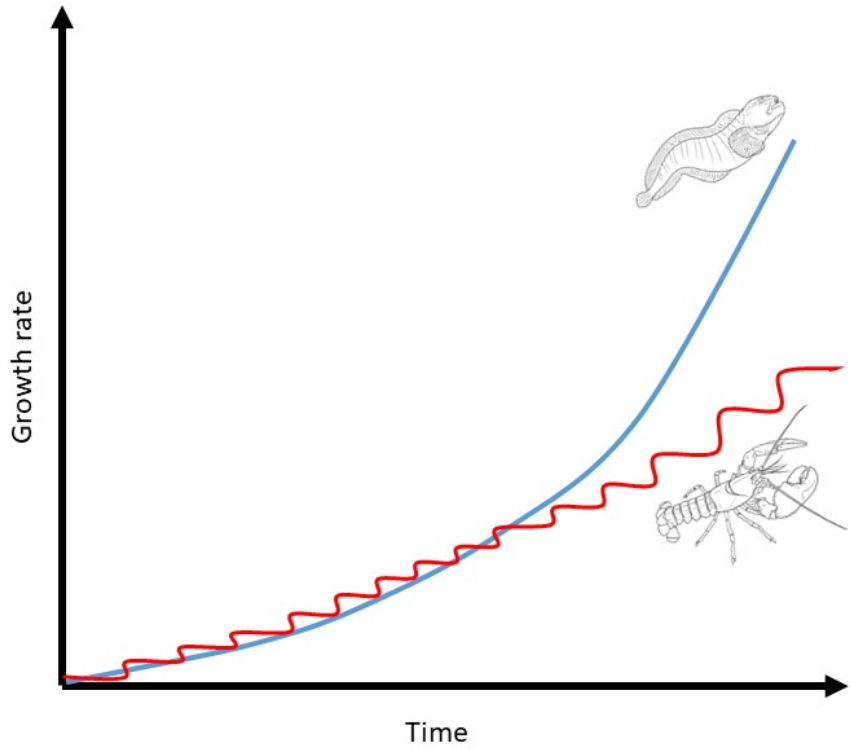

Figure 8: Generalised growth models for teleost fish and crustaceans in juvenile phases.

1.9 Husbandry conditions

Optimum husbandry conditions and welfare are essential practices in aquaculture operations. When working with novel species it is important to establish high levels of both these practices.

1.9.2 Feeding rations

If food supply is reduced below the optimum level, there is invariably a reduction in growth rate. Due to the fact that feed represents more than 50 percent of aquaculture costs, particularly in intensive farming systems, many studies are carried out to determine the best feeding regime for cultured animals, with varying results. For example, in crustacean larval development, a reduction in feed leads to an extended duration for each larval stage (Hartnoll et al., 2001, and references within). Overall, different sizes, species and the diverse environmental and management conditions used in aquaculture all require different feeding rations. Often the most important factor which determines a feed ration is the feed intake (consumption) of the animal being fed. If a fish has a naturally low feed intake such as 0.5% (of total tank biomass), e.g., 50 g of feed with a tank biomass of 10 kg, and it is fed a ration of 1.5% (of total tank biomass), e.g. 150 g of feed that will result in 100 g of feed wastage. Therefore, rations are often conserved accordingly to minimalize feed wastage whilst offering enough feed for the animal so that it is satisfied and performing optimum growth. Rations can often be split throughout a day depending on the husbandry protocol.

1.9.3 Feed size: size matters

Pellet size is clearly important from one critical morphological perspective – the individual's gape size. The way a fish or crustacean consumes a feed is also an important factor in considering what size pellet to offer. Gilthead sea bream, *Sparus aurata*, for example, "chew" large pellets before swallowing them. During chewing, some feed is lost into the water, and with it precious nutrients that could otherwise be used for growth. Ballester-Moltó *et al.,* (2016), demonstrated that feeding pellets smaller in size than manufacturer recommendations to gilthead sea bream reduced the need for chewing, reducing the loss of up to 42 grams of feed per kg of fish. However, Hossain *et al.,* (2000), demonstrated that fingerling African catfish, *Clarias gariepinus*, evacuate small feed particles from their body more rapidly than larger particles, reducing nutrient assimilation in the gut and in turn affecting growth rates. Other issues with small pellets lie in the fact that they contain relatively lower levels of nutrients compared to larger pellets. Give animal too small-sized pellets, and it has to spend more time finding and consuming enough pellets to meet its nutritional requirements. Overall, inefficient pellet size can increase energy expenditure during feeding, diverting it away from energy that would otherwise be used to increase growth. The implications for aquaculture operations are simple – use the pellet size and delivery rate that is most efficient for your operation and produces the least waste.

1.9.4 Stress and welfare

Considerable attention has been given in recent years to the possible detrimental effects of stress on animal populations resulting from various aquaculture practices. These practices typically include handling, sorting, grading, transport and even housing temperatures or densities (Boerrigter *et al.,* 2015). Such stimuli, or stressors, induce a reallocation of energy from growth to preserve homeostasis. This response is adaptive in the short term, but prolonged stress can result in adverse measurable effects (Barton and Iwama 1991; Wendelaar Bonga 1997; Brijs *et al.,* 2018). Stress is a subjective concept, which is often used to describe both the stressor and the stress response. Any factor that disrupts the homeostasis of an individual can be defined as a stressor; this includes environmental, bacterial and conspecific interactions (Barton and Iwama, 1991). The stress response can be grouped into primary, secondary and tertiary responses (Figure 9). The primary response encompasses the perception of the stress and brain neurotransmitter activity stimulating sympathetic activity and catecholamine release, and the hypothalamic-pituitary-interrenal axis (HPI) (Wendelaar Bonga, 1997). The hypothalamus releases corticotropin-releasing hormone (CRH), triggering the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH stimulates interrenal tissue to produce and glucocorticoids, mainly cortisol in fish, (Wendelaar Bonga, 1997; Figure 9). The rapid increase of cortisol assists in coping to stressors by increasing gluconeogenesis to provide immediate energy, altering behavior, and reducing the activity of physiological processes that are not necessary to respond to the stressor. Following the subsidence of the stressor, circulating cortisol quickly returns to basal levels, and normal physiological processes are resumed.

Most often, plasma cortisol is measured to assess stress in fish because of its responsiveness to acute stressors, its relative ease of measurement, and its functional significance in physiological processes affecting fish health (Wendelaar Bonga, 1997). Common features of the behavioral response to stress in fish are reduction in the feed intake levels and/or disruption of the feeding behavior (Bernier and Peter, 2001). A fundamental 'whole animal' response to stress, but one not so commonly studied in aquaculture is a change in the metabolic rate. A change in metabolism to cope for activity has been suggested as a possible method for evaluating stress in fish (Barton 2002), since stress imposes a metabolic load on fish which consists of two components, an energy demand required for coping and an energy cost to correct the accompanying imbalance. In his classic paper, (Fry, 1947), provided a basis for a description of factors affecting animal activity within its environment and defined the concept of scope for activity. Stress may therefore further limit a fish's bioenergetic capacity by reducing the energy available for other performance components within its scope for activity; therefore, measuring metabolism enables one to measure an individual's aerobic scope for activity.

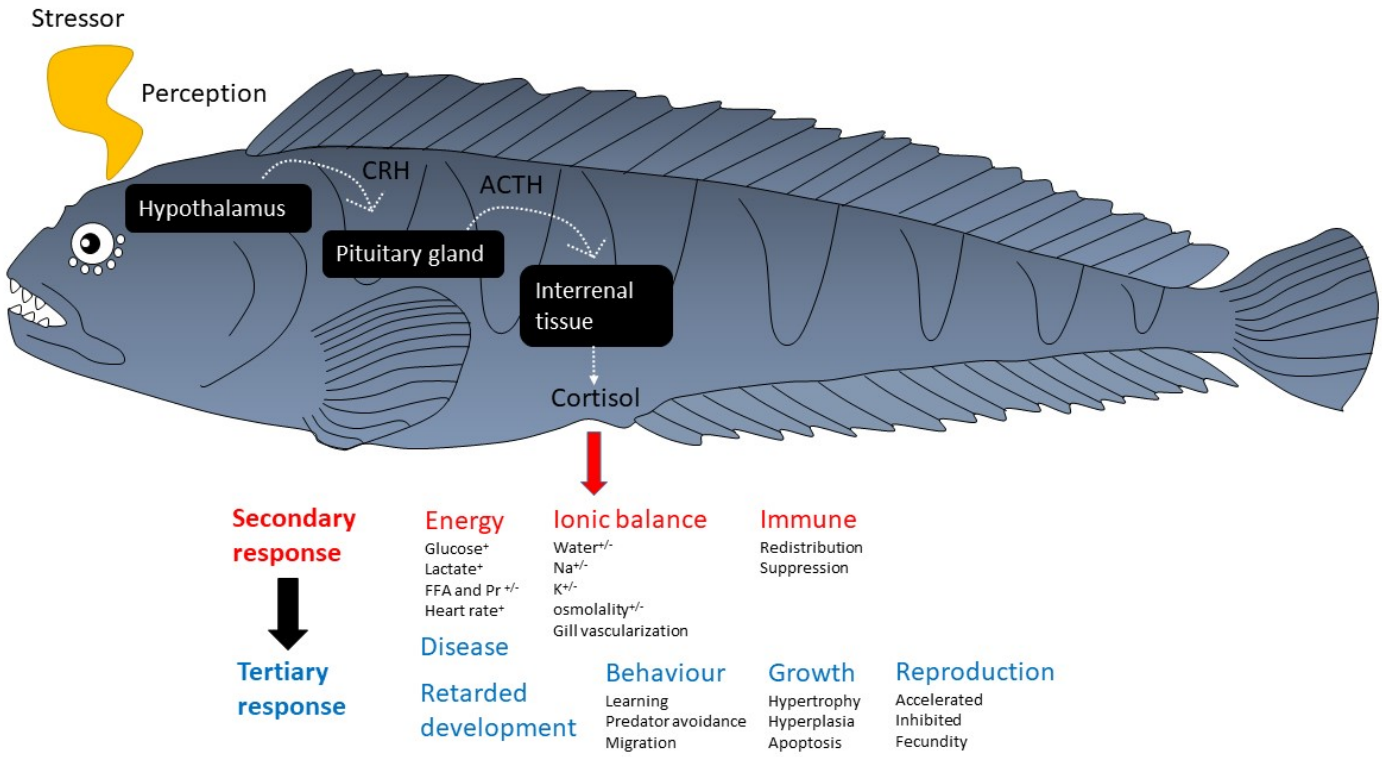

Figure 9: The stress response in fish is coordinated by many systems. One system is the hypothalamus-pituitary interrenal (HPI) axis that first releases adrenocorticotropic hormone (ACTH), which in turn stimulates the interrenal tissue to secrete cortisol. This primary stress response induces a secondary response that includes metabolic, hematological, hydromineral, and structural changes (Barton and Iwama 1991). Consequences on growth, health, and reproduction account for the tertiary stress response (Barton and Iwama 1991; Wendelaar Bonga 1997).

1.9.5 Temperature

Temperature tolerance is a key determinant of the resilience and acclimatization of animals in aquaculture environments, and it is well known that species-specific thermal tolerances are a primary driver establishing the environments in which animals live (Pörtner and Peck, 2010). As ectotherms, temperature influences the rate of all chemical reactions in the body of fish and crustaceans. In crustaceans, an increase in temperature universally increases growth rate, most of the time, without any evidence of an optimum temperature (Hartnoll, 2001). Growth rate may decline at the highest temperatures, but this is accompanied with severe mortality, therefore the decline has little biological significance temperature (Hartnoll, 2001 and references within). Increased temperature accelerates growth by shortening the intermoult period, increasing the moult increment, or both (see references within Hartnoll 2001). In fish, growth rate has an optimum temperature for growth (T_{opt}). Temperature variations diversely affect fish species, and while information is growing regarding the effects of thermal variation on aquatic systems, limited and conflicting knowledge is available on impacts to the metabolic and physiological traits of fish (Boltaña *et al.,* 2017).

In aquatic systems, animals are exposed to spatial and temporal variations in temperature that significantly affect individual physiological traits. The thermal tolerance range of ectotherms is often determined by the range of thermal variation in their natural habitat, an organism living in stable environments with little change is likely to be stenothermic, as opposed to eurythermal organisms which can function at a wide range of temperatures. Thermal tolerance temperatures are therefore most narrow for animals inhabiting high and low latitudes, whereas the tolerance range tends to be widest for fishes inhabiting mid-latitudes where seasonal differences in temperatures are most pronounced (Pörtner and Peck, 2010). This has large implications on husbandry environments in aquaculture for both fish and crustacean species. For instance, fish reproduction is likely to be affected by increasing water temperatures arising from climate change (Pankhurst and King, 2010). For aquaculture, the consequences are rather simple, temperature needs to be controlled and coordinated with the thermal window of the organism being farmed. For instance, T_{opt} for growth and survival for Atlantic wolffish is 12.1°C for juveniles 106-127 days after hatching (Árnason *et al.,* 2019), as mentioned earlier in this thesis. Therefore, in an aquaculture production system of this species temperature should not exceed 12.1°C for this life stage. Whereas in European lobster, larval period in 20^{o}C water is around 12 days (Drengstig and Bergheim, 2013) but can be severely longer if temperature is not controlled.

2.0 AIMS OF THE THESIS

This thesis addresses the challenges of reducing dependence on fishmeal for aquafeed and species diversification for a sustainable development of Swedish aquaculture. The goal was to contribute to a knowledge base for the farming biology and culture operations of two species deemed as promising, the Atlantic wolffish and European lobster, with a circular economy model and minimal environmental impact in mind in line with UNs agenda 2030.

The major aims of the thesis were to:

1. Investigate the potential of using various locally produced seafood by-products as resources for production of alternative protein ingredients to use in future diets, including a novel protein concentrate produced from herring by-products by the pH-shift process, and a meal from mussel by-products
2. To evaluate optimal dietary/nutritional requirements and farming conditions for growth and welfare of the two species at their larvae or juvenile stages, European lobster and Atlantic wolffish. This was done through a series of objectives in the form of papers II-V:

Paper I. To optimize of the pH-shift process for three combinations of herring by-products by characterizing differences in proximate composition and protein recovery

Paper II. 1) To evaluate a commercial dry feed for use in a pilot commercial scale lobster hatcheries, with the overarching goal to improve European lobster larviculture operations.
2) To investigate the importance of cannibalism on larval growth and survival, both in high-density communal rearing systems and individually in isolated cells.
3) To perform a proximate analysis of lobster larvae and establish a basis of a species-specific feed formulation.

Paper III. 1) To examine the suitability of feeds with novel protein ingredients from local sources on the growth of recently metamorphosed PL.
2) To optimize the form of feeds and suitability of certain protein sources by adding supplements found naturally in crustaceans.

Paper IV. To determine the protein requirement for juvenile Atlantic wolffish using isocalorific feeds.

Paper V. 1) To investigate how wolffish cope with an acute and chronic temperature challenge in terms of the effects on metabolic rate, stress and growth physiology.
2) To learn more about the stress response in Atlantic wolffish.

3.0 MATERIALS AND METHODS

3.1 Experiment designs

This thesis is based on five different studies, hereafter described as papers I-V. In Paper I, the pH shift process was optimized on lab scale, using herring by-products in order to produce a protein concentrate that could be tested in future feed trials, most notably in paper III. Paper I utilised a block design in which three by-product combinations in the pH shift process were subjected to different treatments. Paper II, III and IV, utilised a randomized design where individual larvae or juveniles were subjected to different treatments (paper II) or test diets (paper III and IV). In paper V, a randomized crossover design was utilised, with four treatments and two replicates. A summary of the experimental designs can be found in table 3.

Table 3: Overview of experimental designs in papers I-V

Paper	I	II	III	IV	V
Species	*Clupea harengus* (carcasses)	*Homarus gammarus*	*Homarus gammarus*	*Anarhichas lupus*	*Anarhichas lupus*
Total number of factors in study	*2*	*5*	*1*	*1*	*2*
Replicates per factor	*3*	*3** *50†*	*50*	*3*	*2*
Time exposure (days)	*na*	*14*	*30*	*90*	*50*
Factors	pH Centrifuge force (g)	[1]Feed* [2]Feed size* [3]Daily feed times* [4]Cannibalism† (mixed) Cannibalism † (separate)	Diet	Protein	Temperature Exposure time (days)

[1]Factors used in experiment 1 (of corresponding paper)
[2]Factors used in experiment 2 (of corresponding paper)
[3]Factors used in experiment 3 (of corresponding paper)
[4]Factors used in experiment 4 (of corresponding paper)

3.2 Species, facilities and husbandry conditions

The experiment that is reported in paper I was conducted at the Food Science and Nutrition Department at the University of Chalmers, Sweden, between April 2015 and May 2016. Wild caught herring used in this study were sourced from Skagerrak 5727/1126 on 08/04/15 from a commercial fishing vessel. The by-products were kindly donated by Sweden Pelagic AB (Ellos Sweden). At storage, samples were frozen at -80°C until further processing and measurements.

In paper II and III, gravid female lobsters were donated from commercial fishermen operating in Gullmarsfjorden, Sweden, and delivered directly to the Sven Lovén Centre-Kristineberg, Gothenburg University, Sweden during September- October 2015 (II) and September-October 2016 (III). During preparation for experimental periods, lobsters were individually maintained in opaque 40 L perforated boxes with a 8:16 L:D photoperiod, immersed in a flow-through system, incorporating "deep" water pumped to shore and originating from *ca.* 33 m depth. Temperature fluctuated with season but with a minimum and maximum 6-16°C. Lobsters were fed *ad libitum* with fresh frozen shrimp, *Pandalus borealis*. Egg samples were removed and the eye index protocol (Perkins, 1972) was used to approximate embryo development at periodic intervals. For hatching, up to three ripe adults were removed and placed in an experimental facility for preparation of the experiments in papers II and III. Hatching systems were matched to ambient water conditions, and temperature was gradually increased (<1°C per day) to 18.0 ± 0.5 °C by mixing "deep" water with increased fractions of the same water body passed through a counter current heat exchanger. Hatched larvae, of the same brood, were collected daily in aerated, circular 80 L tanks incorporating a 1 mm outflow filter. For paper II, hatching commenced in February, and ceased in August 2016 enabling 4 experiments to proceed of *ca.* 3 weeks duration each. Whereas in paper III hatching commenced February 2016- September 2017, enabling 3 progressive feeding trials *ca.* 3 weeks duration each.

For the experiments in paper II, larvae were removed from collection tanks, counted and then placed, in equal distribution, amongst 90L conical rearing hoppers (day zero, or T_0). Dry and wet feed rations (experimental details in section 3.3, according to the each specific experimental protocol (see section 3.3-3.4) were then delivered daily. After 14 days, surviving Z3 larvae were placed in Aquahive trays for <7 days to allow sufficient larvae to metamorphose to PL. For paper III the same larval rearing protocol was used to obtain PL as paper II. However, for each feed treatment, Orkney Cell matrices were labelled alphanumerically and placed inside circular 100 L tanks. Each Orkney cell was used to test an experimental diet throughout the experimental period (March 2017- September 2017).

For paper IV and V, the fish used, originates from a clutch of fertilized Atlantic wolffish eggs, which was caught in a bottom trawl off the coast of west Iceland in April 2017. The larvae were then hatched and reared at the Icelandic Marine and Freshwater Research Institute, Grindavík, Iceland, when fish reached 7 grams, 1200 individuals were transported to the experimental aquaria facilities at the Department of Biological and Environmental Sciences, Gothenburg University, Sweden. The fish were fed *ad libitum* on a daily basis using a dry marine feed (Skretting amber neptun, grade 1.0 for 7-20 g and Skretting amber neptun grade 2.0 for 20-40 g) up until the start of the experiments in November 2017 (IV) and March 2018 (V). The water system was a recirculating marine aquaculture system, with a stable temperature of 10±1.0°C and a salinity which was 32 ppt ± 2 ppt. In paper IV, 360 fish weighing *ca*. 40 g were tagged with passive trasponder tags (12mm size, Biomark, USA) and randomly distributed into eighteen, 20 L experimental tanks (n=20 fish per tank), with three replicates per treatment (n=60).

In paper V, fish weighing *ca.* 50 g were tagged with passive transponder tags and randomly distributed into eight 20 L experimental tanks (n=14 fish per tank), and assigned to four experimental groups; consisting of an acute exposure to 15°C, a chronic (50 day) exposure to 15°C, a control group for sampling after 24 h in 10°C, and a control group for sampling after 50 days in 10°C with 28 fish per treatment. At the start of the experiment, temperature was raised to 15°C in four of the eight experimental tanks. Two of the experimental treatments 10°C and 15°C, were sampled after 24 h of exposure to their respective treatments, whilst the four tanks groups were left for 50 days in their respective temperature treatments. During sampling from each experimental tank, eight fish were transferred to a holding tank for respirometry measurements, whilst six fish were sacrificed for physiology sampling.

3.3 Feed ingredients and production (paper III and IV)

In total, four protein test ingredients were used in paper III and IV. In paper III, the herring meal was produced by the pH shift process (Figure 10, optimized by paper I), followed by freeze-drying. The shrimp meal was produced on site at a shrimp boiling and peeling company by flocculation of shrimp boiling water with carrageenan according to the principle of (Forghani *et al.,* 2020). Flocs were separated by flotation and subsequently spray dried (Anhydro Lab S3 spray dryer, Forghani *et al.,* in manuscript). A novel mussel meal powder was also used in paper III, produced using a patented protein extraction process by the local, Swedish company Mussel feed AB. The meal originated from discards of the blue mussel, *Mytilus edulis,* deemed too damaged or too small for human consumption. The powder arrived in a 20 kg batch and was stored at 10°C until further use. For papers III and IV, a commercial fishmeal protein source was purchased, and stored at 10°C until further use. Table 4 shows the composition of all major protein ingredients used throughout this thesis.

In August 2018, the pH shift process was investigated for potential upscaling using an industrial sieve at Chalmers University (Figure 11). In total, 50 kg of protein concentrate was produced from 60 kg of herring by-products. There is no paper about this method included in this thesis; however a discussion will follow in section 4.1.

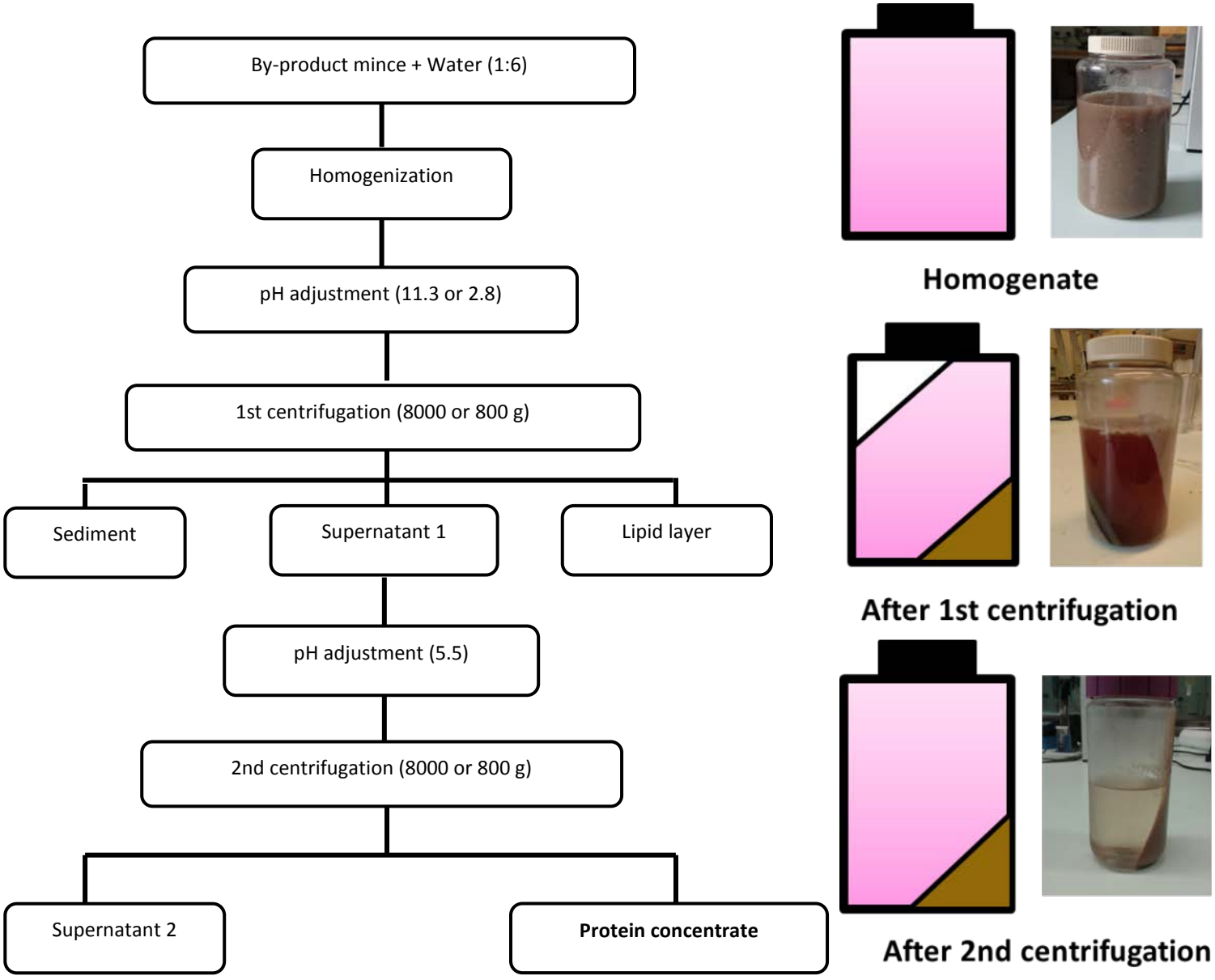

Figure 10: Overview of the pH-shift process modified from paper I.

Figure 11: Large-scale application of the pH shift process using commercial sieve technology. Instead of using gravity as the separation step (as done in paper I), a filter with mesh size 10 µm was used to separate different fractions is step 1 and step 2 of the pH shift process.

Table 4: Chemical composition of major feed ingredients utilized in paper III and IV.

	Feed ingredient			
Proximate composition %	**Herring meal[1]**	**Shrimp meal[2]**	**Mussel meal[3]**	**Fishmeal[4]**
Protein	76.09	66.48	60.84	72.50
Lipid	14.32	12.80	10.93	5.30
Ash	3.44	15.97	15.93	17.50
Gross energy (MJ/kg)	23.72	23.10	20.60	20.70
Water	3.77	7.26	4.78	6.70
Essential amino acids %				
Arginine	4.80	4.29	4.07	3.73
Histidine	2.04	1.55	1.10	1.53
Isoleucine	3.60	2.76	2.29	3.64
Leucine	6.49	4.87	3.62	4.69
Lysine	7.29	5.19	4.43	7.30
Methionine	2.72	1.69	1.39	2.03
Phenylalanine	3.24	3.05	2.09	2.68
Threonine	3.67	2.52	2.62	2.49
Valine	4.37	3.05	2.50	3.26

[1]Produced from the pH shift process (paper I), used in paper III

[2]Produced from protein flocculation according to Forghani et al. (2020) followed by flotation (Forghani et al, in manuscript), used in paper III

[3]Produced from patented technology from Mussel feed AB, used in paper III

[4]Used in paper III and Paper IV.

3.4 Experimental diets

In paper II, a total of four diets were utilized, a wet feed used in marine hatcheries, (Planktonic artemia supplement, “PAS”, 700–1,000 lm grade copepods, Trondheim, Norway) as an initial control feed. Followed by three commercially available dry formulated larval feeds, “C1,” “B1” and “B2” particle size grade Otohime feed, (Marubeni Nisshin Feed Company, Tokyo, Japan).

In paper III, ten experimental diets were used in order to test three differing objectives, formulated as three experiments. In experiment 1, raw shrimp, *Pandalus borealis,* was used as a reference diet, with four additional diets: isocalorific and isonitrogenous commercial fishmeal, experimental shrimp meal, herring meal, or mussel based feeds. In experiment 2, the reference diet was tested against a freeze dried form of the raw shrimp, an oven dried form of the shrimp and an oven dried form with an immune supplement added. In experiment 3, the reference diet was again compared with a herring meal based feed, a herring meal feed with an astaxanthin supplement, a herring meal based feed with a glucosamine supplement and a herring meal based feed with both supplements included. The formulations were loosely based on findings from paper II.

In paper IV, six experimental diets were formulated to be isocalorific but contain differing protein increments; 35%, 40%, 45%, 50%, 55% and 60%.

In paper V, a commercial feed (Skretting amber neptun, grade 2.0) was fed to fish in all treatments.

3.5 Sample collection

In paper I, samples of protein concentrates were collected during each step of the pH shift process. In total, three batches of protein concentrate were produced and analyzed.

In paper II, batches of Z1 larvae from five different broodstock (2–4,000 larvae, or *ca.* 10–20 g wet weight in total from each animal) were collected 10–12 hr post hatch, rinsed in distilled water, blotted, wet-weighed and frozen at -80°C until required with each sample measured in triplicate to quintuplicate to ensure accuracy.

In paper IV, four fish from each tank (N = 12 per diet) were sacrificed for analysis in week 12 of the experiment, all fish were euthanized with Tricaine methanesulfonate (MS-222) followed by destruction of the brain. Sampling for biometrics, biochemical and blood parameters took place as described in paper IV. Feed was collected daily in order to calculate feeding rate.

In paper V, 12 fish were sampled at 24h and 50 days of exposure to the respective temperature treatments. In addition to the biometric and blood sampling that was done as in paper IV, heart and spleen were also collected and weighed in order to calculate relative ventricular and relative spleen mass. The gut was removed in order to measure the intestinal barrier function with Ussing chambers.

3.6 Ussing chamber technology and respirometry

The Ussing chamber technique was used in paper V for assessment of intestinal barrier function according to the procedure described by Ussing and Zerahn, (1951), but modified by Sundell *et al.,* (2003) and Sundell and Sundh, (2012). In short, 16 fish that were exposed to either 10 or 15°C in the experiment were sacrificed by the same method as described in section 3.5. The intestine was removed from the fish from the stomach to the anus and cut open longitudinally. The intestine was divided in two different regions, an anterior, mid intestine region and a posterior mid intestine region. The segments were then mounted into the Ussing chamber for analysis of the intestinal barrier function.

Respirometer chambers were also utilized in paper V for assessment of resting metabolism and maximum metabolism. After exposure to 10 or 15°C for 24h or 50 days, eight fish from each temperature exposure and time point were randomly selected and removed from experimental tanks and placed into one of eight identical custom-made Perspex respirometers (volume = 1 L), which were submerged in a larger experimental tank (volume = 1000 L). More details on these procedures can be found in paper V.

3.7 Chemical analyses

3.7.1 Sample preparation paper I

In paper I, mixed herring by-products were received from Scandic Pelagic AB in Ellös Sweden. After manual sorting into different parts (frames + heads or frames + heads + viscera or viscera alone), by-products were stored in -80°C, before further usage. For subsequent analysis of proximate composition, and also as a first step in the pH-shift process, herring by-products were thawed in a tight plastic bag under running cold water and then mixed in a ratio of 1:6 with ice-cold deionized water followed by homogenization for 2×15s using an Ultra Turrax T18 Basic homogenizer (IKA, Taquara, RJ, Brazil).

3.7.2 Protein determination, paper I, II, IV and V

To determine protein in paper I and II, a modified method of the Lowry assay (Markwell *et al.,* 1978*)* was used. In principle, the sample gives rise to a measurable color change, when a reagent is added; in proportion to the amount of protein present. The reaction is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent. To solubilize protein, the muscle homogenate (10 mg /mL) was diluted in a sodium hydroxide solution (0.1 M NaOH) and agitated, more technical details can be found in paper I and II.

In papers III and IV, total protein was measured using a LECO nitrogen analyzer (TruMac-N, LECO Corp., USA), with a 5.58 conversion factor. Approximately 1 g of the different samples with 2 replicates, were subjected to nitrogen measurement.

3.7.3 Lipid content, papers I-IV

Total lipid content in papers I-IV was measured by the method from Lee, et al, (1996). In principle, the process extracts lipids by the non-polar solvent chloroform. Membrane-associated lipids, however, require more polar solvents such as methanol to disrupt the hydrogen bonding or electrostatic forces between the lipids and the protein. Hence, a chloroform-methanol mixture was used. Once the lipids are extracted by the solvent, they can be separated from the aqueous phase through addition of salt and subsequent centrifugation. The lipid containing chloroform phase end up as a lower phase which is collected. The lipids can then be isolated from the chloroform by evaporation under nitrogen gas and total lipid content calculated gravimetrically. In the case of papers I-III the lipid composition was also analyzed in terms of its fatty acid profile using gas chromatography-mass spectrometry (GC–MS), after a methylation step, to stabilize the fatty acids. More technical details can be found in papers I-IV.

3.7.4 Ash, papers I-IV

Ash content in papers I-III was measured by placement of 1g samples in a muffle furnace at 550°C until only ash remained. Samples and the crucibles were then weighed out to calculate ash content per gram dry sample.

3.7.5 Moisture, papers I-IV

Moisture content in papers I-IV was determined by drying 2g samples in an oven set 105°C in at until achieving constant weight (overnight).

3.7.6 Amino acids, paper I and III

For total amino acid analysis is papers I+III, a modified method was adopted from Özcan and Şenyuva, (2006) which is based on acid hydrolysis of the proteins followed by liquid chromatography-mass spectrometry (LC–MS) analysis. Samples were heated for 24 h at 110°C using a tube heater in order to digest amino acids, before reversed phase LC–MS analysis was performed. More details on this method can be found in paper I.

3.7.7 Plasma nutrients and hormones, paper IV and V

Blood plasma nutrients in paper IV, glucose (paper IV and V), protein content, free amino acids and free fatty acids were measured using commercially available colormetric kits (Sigma Aldrich) with protocols which were adapted for wolffish plasma, using a 96 well plate reader. Preliminary experiments were carried out to produce dilution curves for each respective measurement. Plasma osmolality in paper IV and V was measured using a cryoscopic osmometer advanced model 3320 micro-Osmometer 4, whereas hematocrit and hemoglobin in papers IV-V was analysed using a Hawksley reader and a handheld after centrifugation of blood.

In paper IV, plasma ghrelin was analyzed following the N-Ghrelin protocol established by by Hosoda *et al.,* (2000), slightly modified by Jönsson *et al.,* (2007) with the exception that plasma was not extracted before measurements and iodinated human ghrelin (NEX388010UC, PerkinElmer, USA) was applied as tracer. All samples were assayed in duplicate. The ghrelin RIA was validated for wolffish with a test of parallelism.

In papers IV-V, cortisol was measured by a modified method from Sundh *et al.,* (2011) using cortisol antibodies (code s020; lot:1014-180,182) purchased from Guilday Ltd.

3.7.8 Gill NA-K-ATPase, paper V

In paper V, gill Na^+/K^+ATPase (NKA) activity was analyzed using an NADH-linked kinetic assay in a 96-well microplate run at 25 °C for 10 min, as described in McCormick, (1993). In principle, the ATPase assay is a kinetic enzyme assay which measures the disappearance of NADH; this disappearance is coupled to NKA activity.

3.8 Calculations

In paper I, protein solubility in the solubilization step (i.e. at pH 2.8 or 11.3) was calculated by:

(Protein concentration of supernatant 1/ Protein concentration of homogenate)*100

Protein solubility of the precipitation step (after pH 5.5) was calculated using the following formula:
(Protein concentration of supernatant 2/ Protein concentration of supernatant 1)*100

Protein yields over the two centrifugation steps of the pH process were calculated as below.
Centrifugation step 1:

(Protein concentration of supernatant 1* Xml of supernatant 1)/ (protein concentration of homogenate* Xml of homogenate)*100

Centrifugation step 2:
100- (((Protein concentration of supernatant 2* Xml of supernatant 2)/ (protein concentration of supernatant 1* Xml of supernatant 1)*100)

Total protein yield (%) was calculated as: Protein yield in step 1 × protein yield in step 2

3.8.2 Growth and consumption parameters

All growth calculations were based on individually tagged fish, whereas consumption calculations and feed conversion ratios (FCRs) were calculated on a tank basis. Daily feeding rate (F, % biomass in tank) and FCR were calculated with the following formulas:

$$F = \frac{C_T}{W} \times 100$$

Where C_T is daily feed consumption (g consumed) and W is the mean daily biomass weight in that tank (g).

$$FCR = \frac{C_T}{(W_F - W_I)}$$

Where W_F was the final tank weight (g) and W_I was the initial tank weight (g).

Condition factor (K), specific growth rate (SGR) and weight gain (%) was calculated using the following formulas:

$$K= 100 / (W / L^3)$$

Where W was weight (g) and length was L (cm).

$$SGR= 100 \times Ln [(W_F / W_i)] / Days$$

Where W_F was the final individual weight (g) and W_i was the initial individual weight (g). SGR for length was also calculated with this formula, however length (cm) was used instead or weight parameters. Weight gain (WG) was calculated with the following equation:

$$WG= [(W_F – W_i) / W_i] \times 100$$

Where W_F was the final individual weight (g) and W_i was the initial individual weight (g) hepatosomatic index (HSI) and Mesenteric fat index (MSI) was calculated as shown below:

$$HSI = L_W / B_W$$

Where L_W was the individual liver weight (g) and B_W was the individual body weight (g)

$$MSI = MF_W / B_W$$

Where Where MF_W was the individual mesenteric fat weight (g) and B_W was the individual body weight (g)

3.8.3 Hematology calculations

The Mean corpuscular hemoglobin concentration, (MCHC), was calculated with the following equation:

$$MCHC = H_B / Hct$$

Where Hb was hemoglobin content and Hct was the hematocrit values.

3.8.4 Barrier function Calculations

P_{app} was calculated using the equation:

$P_{app} = dQ / dt \times 1/ AC_o$

Where dQ/dt is the appearance rate of mannitol in the serosal compartment of the Ussing chamber, A is the area of intestinal surface exposed in the chamber and C_o is the initial concentration on the mucosal side.

3.8.5 Metabolic calculations

$\dot{M}O_2$ was calculated using the following formula:

$$\dot{M}O_2 = [(V_r - V_f) \times \Delta C_{wO2}] / (\Delta t \times M_f),$$

where V_r is the volume of the respirometer, V_f is the volume of the fish (assuming that the overall density of the fish is 1 g per mL of tissue, thus V_f = mass of the fish, M_f), ΔC_{wO2} is the change in the oxygen concentration of the water within the respirometer (C_{wO2} is the product of the partial pressure and capacitance of oxygen in the water, the latter being dependent. Absolute aerobic scope (AAS) and factorial aerobic scope (FAS) was calculated by the following formulas:

$$AAS = \dot{M}O_{2max} - \dot{M}O_{2min} \qquad FAS = \dot{M}O_{2max} / \dot{M}O_{2min}$$

Where $\dot{M}O_{2max}$ was the maximum metabolic rate of an individual and $\dot{M}O_{2min}$ was the resting metabolic rate.

3.9 Statistical analyses

In papers I, IV, and V, statistical analysis was performed using SPSS software version 20 (IBM SPSS Statistics for windows, version 20.0, IBM Corp, Armonk, NY, USA). In paper II and paper III, data were analysed using GraphPad Prism (GraphPad Software, San Diego, USA). The significance levels for all studies were set at $P \leq 0.05$.

The effect of by-product origin, pH treatment and centrifuge force, in paper I, was evaluated using a three-way ANOVA followed by a Tukey post-hoc test significance amongst groups.

In paper II, percentage survival and development were arcsine-transformed, prior to analysis using Student's t test (unpaired). Whereas, survival and development for individually reared larvae used Fisher's exact test on actual numbers of live:dead individuals. Carapace length (CL) was analysed specifically within zoa stage between groups (Student's t test and with ANOVA for Z1, individually reared larvae. In paper III, all percentage data values were arcsin transformed prior to analysis (i.e. moult increment, the percentage CL increase and growth rate. In addition to moult increment and growth rate, intermoult, longevity and SGR were compared between feed treatments, using ANOVA and Tukey post-hoc test, or alternatively Kruskal Wallace and Dunns post-hoc test.

The effect of differing grades of protein increment in diet were investigated in paper IV using one way nested ANOVA, with a fixed factor or diet and a random factor of tank incorporated into the design. When a significant p-value was detected, multiple comparisons between groups were conducted with the Tukey post hoc method. When the assumptions for parametric analyses were not met, a Kruskal-Wallis test was utilized. Dunn's post hoc method was used to analyze medians of significant p-values from the Kruskal-Wallis.

In paper V, an independent paired t-test was carried out for a test of difference between the differing time points in the experiment of each respective temperature and also between each temperature at specific time points. For cortisol data, a Man Whitney U test was used due to unsuccessful data transformation with Log10. For the Ussing data, a two-way ANOVA was carried out to test for the effect of temperature and also gut region in the respective temperatures.

4.0 RESULTS AND DISCUSSION

4.1 Chemical compositions (I-IV)

4.1.1 Paper I: Chemical composition of protein isolates produced from the pH shift process

In paper I, the chemical composition of the protein concentrates by different versions of the pH process showed considerable variation. The findings revealed that the alkaline version of the process gave significantly higher protein recovery (60.4%) compared to the acid version (49.2%). In contrast, previous studies on gutted herring and herring light muscle found no significant differences between the acid and alkaline version of the pH-shift process, although slightly higher yields were found with acid processing in the latter study, with reported yields varying from 57-59% and 68-74%, respectively (Undeland *et al.,* 2002; Marmon and Undeland 2010). Also with rainbow trout by-products, there was no difference between the alkaline and acid version of the process, and yields as high as 80-90% were reported (Chen and Jaczynski, 2007). Using silver carp by-products, (Zhong *et al.,* 2016) reported a higher yield for the acid version of the process compared with the alkaline, but only with a very acidic solubilization pH (pH 2). It is thus clear that different species, and different morphological parts, respond differently to acid and alkaline solubilization in the pH-shift process, and also give different recoveries; therefore the process should be evaluated and optimized for each fish species. During subjugation to the pH shift process there was no significant effect of by-product combinations on the total protein recovery. This finding opens the pathway for all by-product parts to be utilized in future pH shift applications.

There were large differences found in the chemical characteristics of protein concentrates. In general, the pH shift caused a significant increase is moisture content, from the original raw material (*ca.*70%) to the corresponding protein concentrates (*ca.*90%). This was expected and reflects a higher water holding capacity of proteins after subjection to the pH-shift process. Disregarding this increase in moisture content, and expressing data on a dry matter basis, there was a significant increase in protein from the raw material (*ca.*36%) to the respective protein concentrates (*ca.*75%). Overall, this represents a *ca.* 110% increase in protein concentration from raw material to protein concentrate and confirms the main purpose of the pH shift process, to isolate protein. This finding was followed by a significant reduction in ash from all by-product minces to concentrates, from *ca.* 20% to *ca.* 5%. The fact that the ash values we obtained in concentrates (4-6% dw basis) are much lower than the average ash content reported for fishmeal, around 17-24%, (Miles and Chapman, 2005), reflects that the principle of the pH-shift process; to solubilize and separate proteins from e.g. bones prior to their precipitation is different from classic fish meal production, which utilizes steam heating followed by sedimentation of heat denatured proteins with bone carried over.

A significant increase in ash, water content and lipid content was found in protein concentrates when a lower centrifuge force tested as a way to increase scalability of the process. Overall, this suggests that lowering the separation force affects the nutritional composition of the final protein concentrates from the pH-shift process.

Based on the effect on nutritional profile from by-product combination, pH and centrifuge force used; concentrates based on frames + heads and frames + heads + viscera subjected to a pH of 11.3 and a centrifuge force of 8000 g were deemed to be most promising treatments for future studies. An amino acid analysis carried out on these two samples showed no significant differences between the treatments. When a comparison to fishmeal data from the literature was carried out, no lack of EAAs were observed in the herring protein concentrates. Essential dietary amino acids for fish and shrimp are threonine, valine, leucine, isoleucine, methionine, tryptophan, lysine, histidine, arginine and phenylalanine. The finding that inclusion of viscera had no influence on the amino acid profile is positive as it allows the use of the entire by-product from herring filleting. The fact that the protein concentrates produced in the present study contained sufficient amounts of EAA provides a major advantage for future nutritional studies, compared to other alternative protein sources such as SBM. More detailed analysis of the treatment effects on nutritional parameters of herring protein concentrates are provided in paper I. Overall the results were promising and support that the pH-process could be a future technique to produce highly concentrated protein ingredients from fishery by-products.

Table 5: Nutrient content of fish meal and targeted ranges for alternate ingredients, modified from Gatlin *et al.* (2007) and the nutrient data laboratory, US department of agriculture (2012), now showing data from pH shift produced protein concentrates.

Nutrient (%)	Fishmeal	Targeted ranges for alternative ingredients	Soybean meal	Protein concentrate produced from paper I
Protein	65-72	48-80	48	*Ca.* 75
Lipid	5-8	2-20	0.9	*Ca.* 9
Fibre	<2	<6	4.2	n.a
Ash	7-15	4-8	5.8	*Ca.* 5
Starch	<1	<20	N.D	n.a
Arginine	3.75	>3	3.153	*Ca.* 4.9
Methionine	1.75	>1.5	0.68	*Ca.* 1.8
Threonine	2.5	>2.2	1.766	*Ca.* 4.3
Lysine	4.72	>3.5	3.08	*Ca.* 7.8
Long chained omega-3 fatty acids	~2	*	3.2	*Ca.* 3.5

4.1.1.2 Results from large scale production using the pH shift process

During larger scale pH-shift processing, the method differed significantly compared to the methodology employed by paper I. Overall an industrial sieve with filer size 10 µm was used to replace the centrifuge in the separation step 1 and 2 of the process. Also a water ratio of 1:10 was used to as opposed to the water ratio of 1:6 used in paper I. The moisture content of protein concentrates was higher than reported for paper I with values of *ca.* 97%. This can however be further reduced by other physical means. The protein concentrate has recently been freeze-dried and is pending further analysis with plans to test inclusion in aquafeeds for fish species after this thesis. The change in separation method is likely to have affected the protein concentrate compared to paper I, highlighting the need for further characterization studies. The upscale trial gave valuable insights into upscaling the pH-shift process for herring by-products.

4.1.2 Paper II: Chemical composition of lobster larvae, a knowledge base for species specific feed production

In paper II, the proximate composition analyses carried out on *H. gammarus* Z1 larvae and *H. americanus* Z1 larvae revealed stark differences, compared to feeds (Table 6). *H. gammarus* larvae contained a protein content (53%), that was lower than the previous literature data for *H. americanus* (68%), whilst the ash content was much higher (28% and 18.4%, respectively). Further analysis of wet and dry feeds showed that protein (56.3%-60.0%) and lipid (12%-15.9%) composition exceeded that of *H. gammarus* Z1 larvae (protein, 52.1%; lipid, 3.0%; Table 6). The total ash content of both wet and dry feeds used in paper II was lower (Otohime) than that of the *H gammarus* Z1 larvae (table 6).

In the absence of sufficient macronutrients, cannibalism in *H. gammarus* may supplement exoskeletal development and mineralization. It appears from our results that nutritional requirements may be specific for both species and developmental stage (i.e. *H. gammarus* and *H. americanus*). The stark difference in protein found in larvae of European lobster and American lobster could also be down to measurement protocols utilised in paper II and by Conklin (1995). Paper II utilised a colorimetric assay with an underlying principle to measure solubilized protein (Lowry assay, Markwell *et al.,* 1978). The method quoted by Conklin (1995) appeared to be based on a Kjeldahl analysis (data was taken from Raymont *et al.,* 1971; Capuzzo and Lancaster, 1979), with an underlying method used to measure total nitrogen and apply a generic conversion factor to calculate protein. This has important implications for our comparisons, since conversion factors have been criticized by Moore *et al.,* (2010), which highlighted that this method lacks analytical selectivity for protein because protein is measured on the basis of sample nitrogen content. Adulteration incidents exploiting this analytical vulnerability (for example, melamine) using non-protein nitrogen have been recognized to give unreliable results (Moore *et al.,* 2010). Similar to melamine, crustacean exoskeleton is composed of chitan, another source of nitrogen which can interfere with this method and cause bias in calculated protein values based on non-protein bound nitrogen. Nonetheless, regardless of the differences in compositions reported by paper II and past studies, the findings present a platform for future feeds on *H. gammarus*. The findings of paper II provide a knowledge base for macro nutrients; however a more detailed analysis on micro nutrients is warranted. Crustaceans are a high source of phospholipids (Conklin *et al.,* 1980), n-3 PUFA (Haché *et al.,* 2015), astaxanthin (Lim *et al.,* 2018) and a source of essential amino acids (Barrento *et al.,* 2009), all of which could play a crucial part in the increased survival rates observed from cannibalism (see later).

4.1.3 Paper III: Chemical composition of alternative lobster feeds

In paper III, the proximate composition of the feeds used are shown in table 6. The dry feeds used in each experiment were designed to be isocalorific and isonitrogenous so no differences in protein or energy between the differing dry diets were found. However, when compared to the reference feed (raw shrimp), on dry basis, the protein of all the experimental diets dry diets were lower (60% vs 68%) except for those produced from the reference shrimp in experiment 2. The reference diet also contained 4% lipid, whereas the experimental dry diets used in experiment 1 and 3 were 9-14%. Ash content showed significant variability in the diets of paper III. All diets produced from herring meal contained reduced ash (3-4%) compared to the other dry diets (10-12%) and the reference feed (5.5% wet basis, 25% dry basis). The low ash content displayed by the experimental diet based on herring meal provided an interesting insight into the production principle utilized in the pH shift process (Paper I). As this thesis has already described, the pH shift process was used to produce high quality fishmeal from bone rich herring by-products by the removal of ash during a first separation step (paper I). There appeared to be no major differences in amino acid profiles of the experimental feeds utilised in paper III.

Table 6 shows a comparison between the chemical composition of larvae found in paper II and the chemical composition of feeds used in paper III. In paper III feeds were not directly based on larva data (from paper II), in order to allow for formulation flexibility and compare alternative protein sources more comparatively. Due to the paucity of information for the nutritional requirements of European lobster, larger doses of protein and lipid were introduced to the diets in order to guarantee no lack of essential macronutrients.

Table 6: Proximate composition of experimental diets used to feed European lobster z1 and PL in paper II and paper III. In paper III, F= fishmeal based, M= mussel meal based, S=Shrimp meal based and H= herring based feeds, Reference= raw shrimp diet. In experiment 2, FD=Freeze dried shrimp pellets, OD= Oven dried shrimp pellets, ODS = Oven dried shrimp pellets with immune supplement. In experiment 3**,** H= Herring without supplement, HA= Herring with astaxanthin, HG= Herring with glucosamine and HAG=Herring with astaxanthin and glucosamine.

	Composition data from paper II					**Composition data from paper III**											
Composition g/ 100 g $^{-1}$	**H. americanus Z1**	**H. gammarus Z1**	**Otohime C1**	**Otohime B1, B2**	**Planktonic AS**	**F**	**M**	**S**	**H**	**Reference**	**FD**	**OD**	**ODS**	**H**	**HA**	**HG**	**HAG**
DM- dry matter	10.00	11.00	93.70	93.70	12.00	92.69	93.61	92.12	94.27	22.00	99.00	99.00	99.00	91.95	92.32	91.73	92.47
Ash	18.40	28.42	15.00	13.50	6.00	10.47	12.45	11.35	3.88	5.50	25.00	29.40	28.85	3.56	3.72	3.56	3.44
GE MJ/kg						17.96	18.48	18.85	18.86	3.98	18.10	16.73	16.41	18.75	17.84	18.75	17.78
CP- crude protein	68.00	52.00	58.30	56.30	60.00	58.53	58.99	58.93	59.66	14.96	68.00	66.64	65.78	59.71	59.37	59.71	59.60
Lipid	5.00	2.95	12.90	15.90	12.00	10.09	9.97	13.67	11.56	0.88	4.00	6.99	6.88	11.51	14.48	11.51	14.40
Calcium		6.30	2.70	2.50		2.36	0.96	1.36	1.44	0.50	2.27	3.77	3.71	1.40	1.40	1.40	1.36

4.1.4 Paper IV: Chemical composition of diets and effects on Atlantic wolffish nutritional parameters

In paper IV, protein content ranged from 35-60% in the diets. For comparative purposes, the proximate composition of diets is included in table 7 along with the commercial dry diet that was used in paper V. There was considerable variation in carbohydrate content in all six diets that were produced; diet 1 contained the highest carbohydrates with 24%, compared to diet 6 that contained 0.03%.

At the end of the feeding experiment, there were no differences in crude protein content of whole fish or fillet among the different dietary groups. In contrast to this, there were several significant effects on fat and lipid parameters caused by the different diet treatments. Fish fed the higher protein diets (50-60%), had higher liver lipid content (70-75%) compared to fish which were fed the low protein experimental feeds (35-45%), which had liver lipid content ranging between 60-67% dw, whereas fillet fat content was not effected by diet. Our liver lipid values were consistent with Stefanussen *et al.,* (1993), who found liver fat to range between 60-65% in *A. lupus* fed a high protein dry diet (dw adjusted). The differences in liver lipid content between dietary groups may reflect a shift in lipid metabolism, where energy needs to sustain growth at lower protein levels are met essentially by ready mobilization of liver energy reserves. Whereas in the high protein levels, dietary energy was sufficient to maintain high growth rates and therefore energy could be saved. Similar results have been reported on European seabass, *Dicentrarchus labrax* (Peres *et al.,* 2014), and in sea bream, *Sparus aurata* (Peres *et al.,* 2011), but during fasting periods. In the case of *A. Lupus,* our liver lipid data suggest that the liver is very fatty compared to common aquaculture species such as salmonids (Kullgren *et al.,* 2010). More discussion on this is presented in paper IV, but overall it is clear from these results that more research is warranted with regards to lipid storage and lipid metabolism in wolffish sp.

Table 6: Proximate composition of experimental and commercial diets used in paper IV and V.

	Paper IV						Paper V
Composition g/ 100 g -1	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Skretting amber neptun 2.0**
DM- dry matter	95.31	94.94	94.63	94.44	94.38	94.03	93†
Ash	5.31	6.17	7.16	7.9	8.49	9.44	12
GE MJ/kg	**18.01**	**18.12**	**18.38**	**18.28**	**18.58**	**18.40**	20
CP- crude protein	**35.49**	**40.43**	**45.42**	**50.37**	**54.51**	**59.16**	55
Lipid	16.15	15.93	15.29	13.53	12.74	11.43	15
Carbohydrates	23.97	18.18	12.48	8.56	4.27	0.03	11*
Essential AA %							
Arginine%	1.44	1.73	2.04	2.3	2.53	2.81	
Histidine%	0.67	0.78	0.89	1.01	1.11	1.23	
Isoleucine%	1.47	1.72	1.94	2.2	2.4	2.67	
Leucine%	2.07	2.43	2.74	3.12	3.42	3.8	
Lysine%	2.61	2.97	3.31	3.75	4.09	4.59	
Methionine%	1.65	1.57	1.5	1.44	1.39	1.29	
Phenylalanine%	1.2	1.43	1.66	1.91	2.12	2.35	
Threonine%	1.01	1.16	1.28	1.42	1.52	1.69	
Valine%	1.47	1.7	1.88	2.13	2.31	2.57	

4.2 Growth performances (II-V)

4.2.1 Paper II: Growth performances of lobster larvae in differing husbandry protocols

Paper II was the first to demonstrate successful larviculture of *H. gammarus* larvae fed a diet of dry commercial food, under rearing conditions similar to a commercial hatchery (experiment 1). Larval performance in terms of survival and developmental rate between successive stages were comparable to a wet feed commonly used in lobster hatcheries and no significant differences were found in survival or development rate between the PAS and Otohime C1 feed.

In experiment 2, a wide feeding regime (six times per day over 24 hr), using a mechanical feeder, improved the performance of *H. gammarus* larvae compared with the same ration offered under a more typical regime (three times by hand over an 8-hr illuminated period). Development rate was significantly accelerated when larvae were fed six times a day as opposed to three times a day. Providing a gradual and regular introduction of feed every *ca.* 4 hr may promote larval performance by more closely matching larval feeding behavior. Under the six times per day feeding regime, a significant fraction of the feed ration was introduced during darkness. *H. gammarus* larvae hatch overnight and wild zoea perform diel vertical migration (DVM) and become negatively phototactic as they develop (Middlemiss *et al.,* 2015), whilst in captivity, young PL are more active during darkness (Schmalenbach and Buchholz, 2013). Together, this suggests that in terms of both yields and operating costs, lobster larviculture operations could be improved by adopting automated feeding protocols alongside reduced lighting.

Experiment 3 investigated the effects of differing feed particle sizes, and found small feed particles improved larval performance compared with a larger particle size. No significant differences were found in groups fed the b1 or b2 grade Otohime feed; however development rate was faster in individuals fed the smaller feed (b1), with 20% more larvae which had metamorphosed into z3 individuals at the end of the experiment. Future research investigating digestibility and the effects of food particle size on survival, development and growth at all larval stages is recommended.

Experiment 4 investigated the effects of cannibalism in communally and individually reared *H. gammarus* larvae. Communally reared larvae, fed dry feed, showed significantly improved survival and development rate (compared with experimentally unfed larvae). Whilst this is unsurprising, some individuals in unfed groups nevertheless survived, developed and successfully metamorphosed, demonstrating that nutrition from conspecifics was sufficient to foster larval development. Larvae were also reared individually in suspended Orkney cells to eliminate cannibalism between live larvae, and to allow full control of rearing diet. Larvae fed only dry feed showed the highest mortality rates and poorest development of any experimental group. In comparison, Kurmaly *et al.,* (1990), found that *H. gammarus* larvae reared in similar growing conditions, and fed on artificial diets, recorded poor growth and high mortalities during moulting. The group fed conspecifics only (cannibal treatment) showed significantly higher survival throughout the experiment, and was up to twice as high than the dry or mix treatments from day 12 (84% survival compared with 40%–52%, respectively). In the conspecific group, 80% reached PL stage within 16 days (2 days after T4; data not shown) compared with a more typical survival of at least 10%–15% in *H. gammarus* hatcheries (Ellis *et al.,* 2015). This finding represents the highest growth and survival of *H. gammarus* larvae in the literature. Individual rearing, using an appropriate feed and feeding protocol, could theoretically reduce larval losses due to cannibalism by over 60%. Although the group that was fed the mixed diet did have access to deceased larvae in addition to dry feed, survival and development rate was low. Kurmaly *et al.,* (1990) found that early-stage *H. gammarus* larvae were particularly discriminatory, and ingestion appeared to depend on chemical and textural cues, this can be supported by our personal observations where we observed that in the mix feed group, the deceased larvae feed was sometimes ignored or was not eaten. Evaluation of larval choice between conspecific and feed particles could also form part of future studies and should provide a basis for future feed designs. In the composition data present above showing differences between the chemical composition of Z1 larvae and the commercial feeds that were utilized, presumably, when conspecifics were removed from the diet of larvae, the nutritional content of dry food on its own was insufficient to support larval growth and survival.

4.2.2 Paper III: Growth performances of lobster postlarva fed novel, protein sources

Paper III was the first experiment to screen a wide variety of feeds that could potentially be used in *H. gammarus* larviculture operations. Principally, a dry feed was created that resulted in comparable larvae growth and survival to a typical wet feed utilised within lobster hatcheries (experiment 2).

Experiment 1 screened a variety of alternative protein-based feeds and compared growth performances to a candidate wet shrimp feed used in lobster hatcheries (ELCE 2016 *personal communication*). *H. gammarus* PL showed significantly lower survival when offered musselmeal-based feed, compared to all other treatments with the exception of fishmeal. The shrimp reference diet significantly reduced the intermoult duration which lasted 14 days compared to 16-17 days in the dry feeds. The carapace length of PLs was also significantly increased by the reference diet (17% increase) compared to the other experimental diets (13-15% increase). Therefore, the time taken to moult into the next stage was significantly shortened and the size of PLs was significantly increased by the reference diet. Overall, the results suggest that a shrimpmeal-based feed promotes an improved growth rate compared to feeds containing musselmeal, herringmeal and standard fishmeal and improved survival compared to fishmeal and musselmeal-based feeds. Experimental feeds that included a source of crustaceans or crustacean meal have also tended to improve performance in juvenile *H. americanus* PL reared on increasing proportions of *Artemia* (Tlusty *et al.,* 2005) krillmeal (Floreto *et al.,* 2001) and for adult animals, crab waste (Skonberg *et al.,* 2008). Tlusty *et al.,* (2005), suggested that poorer performing lobster feeds may be lacking in essential nutrients, compared to Artemia controls.

4.2.2.2 Experiment 2

Experiment 2, aimed to optimize the form of the wet shrimp reference diet and elucidate the effects of oven-drying on the growth performances with or without an immune supplement. Bio-Mos was chosen as the principle supplement as it has been used in *H. gammarus* hatcheries previously with reported benefits to larvae (Daniels *et al.,* 2013). PL survival was very high, over 90% of individuals successfully moulted to stage V in all experimental feed treatments. PL fed both the wet shrimp reference and experimental feeds containing freeze-dried shrimps showed improved performance, when compared to either of the oven-dried shrimp treatments. In short, the intermoult duration was significantly shorter whilst SGR was significantly higher. No MDS and low incidence of moulting complications were observed in this experiment.

The nature of processing an ingredient prior to formulation and subsequent incorporation into a commercial feed often has important consequences (Glencross *et al.,* 2007). It is well known that protein damage can occur during heat treatments, this in turn can lower digestibility and affect feed pellet palatability. Gabaudan *et al.,* (1980), found that protein digestibility and metabolizable energy of krill and brine shrimp was reduced in oven-dried, but not freeze-dried samples.

The novelty of experiment 2 is that we created a dry feed, in principle, that is comparable to growth and survival of a typical wet feed utilised within lobster hatcheries. Albeit this was only in the form of a freeze-dried version but this provides platform for future feed designs to emulate. Secondly, from the results we suggest that other feeds tested in paper II (i.e. oven-dried mussel meal supplied as an industrial by-product used in experiment 1) could be improved if an alternative drying technique was used, meaning that they should not be simply dismissed as protein sources for this species (due to the negative results in experiment 1). Digestibility or feed intake studies with small PL which eat tiny feed particles intermittently are technically challenging, and potentially studies with adult lobsters could be performed to determine feed digestibility and palatability.

4.2.2.3 Experiment 3

Experiment 3 was designed to "bridge the gap" between the shrimpbased-protein sources and other experimental protein sources, by supplementation with astaxanthin and/or glucosamine. Herring meal produced from the pH shift process was chosen as the test ingredient for this experiment due to its drying method (as illustrated by experiment 2, was important for PL). Overall, PL offered raw shrimp reference feed, and herring (H) meal feeds containing one additive only (HA, HG), showed the highest survival, PL offered the herring based feeds suffered most from moulting complications compared to the shrimp control. Crustacean diets are a source of astaxanthin (Lim *et al.,* 2018) and chitin (Niu *et al.,* 2013) which have both been shown to enhance growth, survival and stress tolerance in crustacean diets (Niu *et al.,* 2013; Lim *et al.,* 2017). Whilst survival of PL in HA and HG diets was significantly increased compared to those fed herring alone, in general all four herring based feeds were inferior to the shrimp reference diet. Of particular importance, the incidence of both MDS and moulting complications at stage V were not eliminated by any of the supplements. Furthermore, PL fed HAG feed, which contained both supplements, was one of the lowest performing diets in terms of survival and development, indicating that a combination of both supplements at the high doses created an antagonistic effect on PL performance.

All in all, experiment 3 shows that the shrimp reference still outperformed the herring based diets in all parameters measured other than survival and emphasizes the need to establish the nutritional requirements for this species. Experiment 3 also shows that moulting problems cannot be simply alleviated by adding high doses of a supplement. In a recent review, one of the strategies highlighted for future research was the need to understand how alternative proteins can interact with other raw materials in feeds, *"It is very helpful to know that a new raw material interacts positively or negatively with others"* (Turchini et al, 2019). The approach we took with experiment 3 falls well in line with this statement especially and shows the importance of testing interactions between raw materials in feed this can be illustrated by the negative results from the HAG treatment, which we originally hypothesized to be a stronger performer than the HA and HG based feeds.

4.2.3 Paper IV: Growth performances of Atlantic wolffish fed graded increments of protein

Paper IV is the most detailed protein requirement study performed to date on a wolffish species in terms of experiment design, growth parameters and physiology parameters (see below). Fish fed the high protein diets (50-60%) showed significantly increased weight gain, SGR_w and increased condition factor, compared to fish fed the lower protein diets (35-45%). In general, fish fed high protein diets also consumed more feed, compared to low protein diets. Overall, this study shows that juvenile, wild-caught Atlantic wolffish require relatively high protein diets (>50%) for an optimal growth in a laboratory setting. The benefit of a high dietary protein inclusion was evident although there was individual variability in growth rate in all dietary groups (Figure 12), which seemed to depend on the fact that some individuals probably were weaned on to the experimental feeds with differing success, independent of diet. The results of paper IV are in accordance with previous observations (but not thoroughly tested), which suggest that, for wolffish of similar size, the nutritional requirement for protein is above 50% (Stefanussen et al. 1993; Strand et al 1995).

The values for SGR_w (*ca.* 0.3%/day for high protein diets) and weight gain (25-35% for high protein diets), obtained in paper IV are the lowest growth rates that have been observed for *A. lupus*, when compared to the expansive literature, where figures as high as 1% have been reported (Stefanussen *et al.,* 1993; McCarthy *et al.,* 1998; Árnason *et al.,* 2019). Paper IV contributes to this data further through the use of implanted microchips with unique numbers, PIT tags, the advantage with PIT tags is that it allows an individual's growth rate to be tracked throughout the experimental period. With the use of PIT tags in this study, it revealed individual fish in all diets that showed no weight gain throughout the experiment. Consequently, growth rate was highly variable (Figure 12) across all diets and this caused the mean values to be dragged downwards by a combination of data variability and net weight loss for individuals who showed no history of weight gain.

Figure 12 also shows the high growth rate from some individuals which grew through the experiment and well within the range of the literature, in all diets (Árnason *et al.,* 2019; Stefanussen *et al.,* 1993). However, in the low protein diets, fewer individual fish showed these high growth rates but were still comparable to the highest growth performance observed from high protein diets (Figure 12). Growth in fish is mainly dependent on feed consumption; the results of high individual growth rates, coupled with the consumption data from the diets suggest that individual fish were able to compensate for lower protein by increasing their consumption rate and perhaps the establishment of a feeding hierarchy in the experimental tanks. Finally, the results of paper IV show the challenges of weaning wild fish onto experimental diets, but with the use of PIT tagging, this problem can be alleviated with the use of individual growth tracking and thorough analysis. Further and more detailed studies are required to determine the optimum protein levels in feed for *A.lupus* at different sizes classes.

4.2.4 Paper V: Growth performances of Atlantic wolffish "living on the edge"

Paper V supports the previous studies which showed reductions in growth at elevated temperatures in *A. lupus* and *A. minor* and the hypothesis that at 15°C the high allostatic load leaves no scope for increased growth (McCarthy *et al.,* 1998; Hansen & Falk-Petersen 2002; Magnussen *et al.,* 2008; Árnason *et al.,* 2019). Overall, the weight growth data (*ca.* 0.68%/day for 10°C) obtained in paper V is in accordance with previous growth observations for *A. lupus* (Stefanussen *et al.,* 1993; Árnason *et al.* 2019). Fish maintained in the 10°C treatment showed significantly increased SGR_w and weight gain compared to fish exposed to 15°C. On the other hand, the condition factor of fish exposed to 10°C was lower than that of fish exposed to 15°C, which was caused by a reduced SGR_L in the latter. Overall our findings suggest that the growth of fish in 15°C was stunted. This finding was also recently confirmed by Árnason *et al.,* (2019), who showed that Atlantic wolffish exposed to 15°C were exerting spinal deformities via a non-symmetric and compressed structure of vertebrae. The stark contrast in weight growth rates compared to paper IV also support that there was a challenge of weaning fish onto experimental feeds in paper IV, since paper V utilised a commercial marine diet (Table 6).

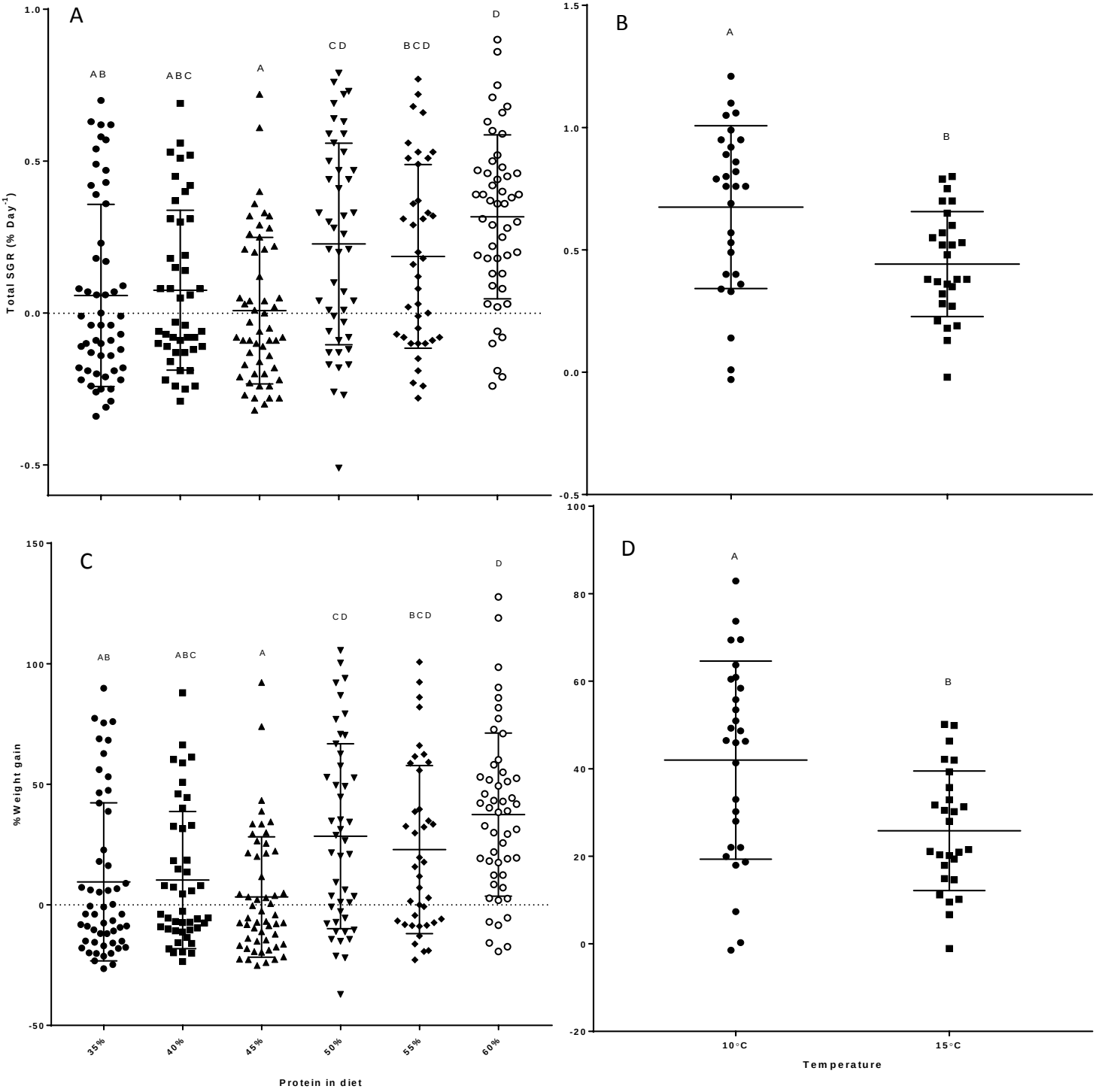

Figure 12: A) Total specific growth rate for weight (%/day), for paper IV, horizontal bar showing mean ± st d. B) Total specific growth rate for weight (%/day), for paper V, horizontal bar showing mean ± st d. C) Total weight gain (%), for paper IV, horizontal bar showing mean ± st d. D) Total weight gain (%), for paper V, horizontal bar showing mean ± st d.

4.3 Stress physiology (IV+V)

4.3.1 Paper IV: stress parameters of Atlantic wolffish fed graded increments of protein

A number of standard measurements for stress were applied in paper IV (cortisol, glucose, hematocrit, hemoglobin, osmolality, MCHC), but none of them were affected by dietary protein content and they were within the range previously found for *A. minor* (Foss *et al.,* 2001; Le François *et al.,* 2013; Knutsen *et al.,* 2019). Paper IV also became the first study to measure the cortisol response in *A. lupus,* with mean levels *ca.* 15 nmol/L^{-1}. Compared to other marine fish species, wolffish seem to have a milder stress response when exposed to a variety of abiotic stressors. Factors such as life style can cause considerable variation in cortisol responses. For instance, the basal resting levels of plasma cortisol reported in Atlantic salmon are less than 15 nmol/ L^{-1} but when stressed, cortisol can rise up to 220 nmol/L^{-1} (Fast *et al.,* 2008). Whereas in lump fish (*Cyclopterus lumpus*), a semi pelagic teleost, the response is lower - levels typically rise from 15 nmol/L^{-1} to 151 nmol/L^{-1} (Hvas *et al.,* 2018). For comparison, the levels of cortisol found in paper IV, and in acute stress situations in *A. minor* (Lays *et al.,* (2009) and Le François *et al.,* (2013) were all within the lower range, <30 nmol/L^{-1} concentrations. The stress response of the *A. lupus* and *A. minor* species warrants more research for future aquaculture purposes (paper V), however the results obtained in paper IV, suggest that dietary treatments and general husbandry conditions did not cause a stress response.

4.3.2 Paper V: Stress parameters of Atlantic wolffish "living on the edge"

Paper V explored the physiological responses to elevated temperature in juvenile Atlantic wolffish and successfully elucidated the effect of an acute (24 hours) and chronic (50 days) temperature stress challenge on the metabolic response. Fish exposed to the 15°C treatment had an increase in SMR and MMR when compared to fish reared in the 10°C treatment, both after 24 hours and 50 days. We observed a significant reduction in MMR and SMR from day 1 to day 50 at 15°C (Figure 13), suggesting a potential acclimation in a longer term context, this was in accordance with a previous study on Atlantic halibut (Gräns *et al.,* 2014). Nonetheless, we have shown that at day 50, fish exposed at 15°C still had a significantly higher metabolism than fish at 10°C. We measured aerobic scope via the AAS and FAS formulas to assess scope for activity of the fish as postulated by (Fry, 1947). When we applied AAS to calculate the aerobic scope for activity, we could not detect any difference between 10°C and 15°C. In contrast to this, a significant reduction in aerobic scope occurred at 15°C treatment at both time points, when FAS was calculated. A larger discussion on this contradiction is presented in paper V but overall, using the two metrics provided comparability and paper V was one of the first studies to do this. In the review by Clark *et al.,* (2013), the authors highlighted that the two metrics can run counter to each other when using the same data, we observed results in conjunction with this. These results complement the growth data that we observed (shown in section 4.2.4), coupled with a trend that feed intake was reduced at 15°C, showing that at 15°C, *A. lupus* is thermally challenged. The increased metabolism implies a higher energy cost at elevated temperatures which might be one explanation for the impaired growth. Overall, paper V adds to the body of work that has allowed for any potential thermal acclimation of metabolism by examining the effects of chronic thermal exposure (weeks) on aerobic scope, (Healy and Schulte, 2012; McArley *et al.,* 2017) and expanded our knowledge of the stress response in Atlantic wolffish.

Paper V was the first study to characterize gut barrier function (GBF) in the Atlantic wolffish. GBF was significantly reduced in fish exposed to 15°C as shown by a reduction in trans-epithelial potential (TER), indicating a higher leakiness induced by the temperature stress. Sundh *et al.,* (2010), showed that that adverse environmental conditions (low water flow, low DO levels at low and high temperature), that can occur in sea cages, elicits primary and secondary stress responses in Atlantic salmon post smolts. The intestinal barrier function was significantly affected by prolonged hypoxic stress even when no primary stress response was observed, indicating that GBF is a good marker for evaluation of chronic stress and that it can be a valuable tool to study the impact of various husbandry conditions on health and welfare in teleosts. Paper V adds to this earlier work and shows that the Ussing chamber technique is an important method for measuring in vitro stress in teleosts.

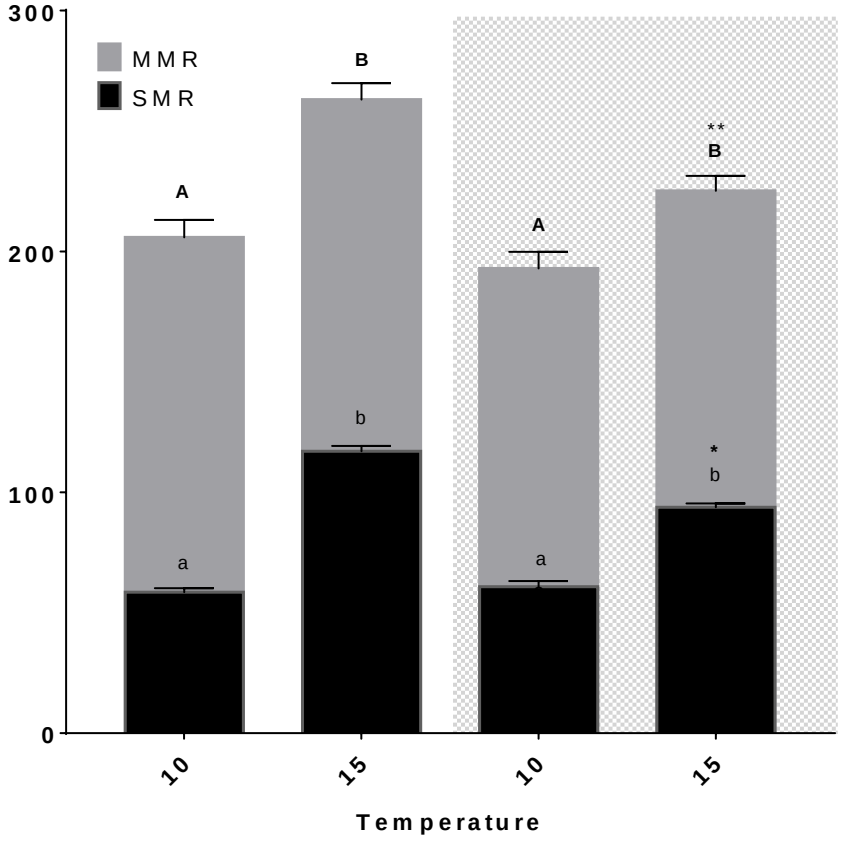

Figure 13: Standard metabolic rate (SMR) and Maximum metabolic rate (MMR) in the two temperature treatments following a 24 h exposure (clear background) and a 50 days exposure (grey background). Bars represent mean with standard error. Letters denote significance groupings between the two temperatures for the specific time exposure (as indicated by upper case for MMR values and lower case for SMR values. Asterisks denote a significant change from 24 hours exposure to the 50 days exposure in the respective temperature (as indicated by 2 asterisks for MMR values and 1 asterisk for SMR values).

We also found that the anterior region of the intestine was "tighter" than the posterior region, (shown by the TER). The result was somewhat unexpected, since the proximal intestine is considered the region of active nutrient uptake in teleosts and therefore, the area where TER should be at its lowest (Sundell and Sundh, 2012). This finding indicates that the posterior intestine is the region for most nutrient uptake in Atlantic wolffish, which is supported by our measurements of lysine transport as it increased from the anterior to the posterior regions of the intestine. Also, the major morphological differences between the anterior and posterior intestine of *A. lupus* that were previously reported, lends further support to our findings (Hellberg and Bjerkås, 2000). More discussion on GBF is presented in paper V but from the main findings, GBF was negatively impacted in the 15°C treatment. Also, the results highlight that an in-depth study of the gut physiology of *A.lupus* is warranted, as the unique characteristics of the GBF *of A.lupus* can impact feeding (e.g. diet) and husbandry protocols (e.g. temperature) in future aquaculture operations.

Along with paper IV, paper V became the first study measure baseline cortisol but also as a response to temperature elevation in *A. lupus.* Plasma cortisol levels were within range of the reported values of < 30 ng/L^{-1} concentrations, observed by Lays *et al.,* (2009) and Le François *et al.,* (2013) in spotted wolffish*, A. minor,* and with paper IV. We hypothesized that the move from 10 to 15°C would induce a primary stress response, however no significant response in cortisol values were observed in either of the temperature treatments at any of the exposure times, which were contrary to the growth, metabolic data and GBF results, suggesting that no primary stress response took place. In contrast, *A minor* showed elevated levels during stress (70 nmol/L^{-1}; Lays *et al*., 2009), which is still low compared with other species (see earlier values for Salmon and lumpfish) but clearly shows a cortisol response to stress.

In paper V, considering that the sampling was not continuous, it is not possible to determine the exact time for a potential cortisol peak that might have occurred. It is also possible that cortisol is not the best indicator for stress in *A. lupus,* due to the lack of response observed in the acute and chronic treatments which were well within the duration (24 h) of peak cortisol values observed by Le François *et al,* (2013, 0.5h-37h), on *A. minor*. Cortisol is well known to act as a stimulant of the gluconeogenesis pathway, and promotes increased glucose levels though the secondary stress response (Wendelaar Bonga, 1997). For glucose however, no significant differences were found between temperature groups at day 1 or day 50. However, we observed increased blood glucose levels in the chronic 15°C treatment when compared to the acute 15°C group when mobilization of glucose reached a maximum level of 1.1 mM after 50 days. This suggests that *A. lupus* has a low capacity for energy mobilization during stress. The findings of paper V, emphasize the need continuous and simultaneous sampling for cortisol and glucose and more discussion of this is presented in paper V. Our experimental results have provided information for the establishment of best welfare practices of wolffish cultivation with focus on both growth and health performance, which are equally important in fish farming, whilst contributing to an intense ongoing debate in the field of the OCLTT hypothesis (see paper V). Paper V, will hopefully encourage other scientists to publish absolute and factorial aerobic scope values and help clear up misinterpretations in data sets. The stress data complements the growth data and will also contribute to knowledge on how this species may cope with increased ocean temperatures in the wild.

5.0 CONCLUDING REMARKS

5.1 Chemical compositions

One of the aims of this thesis was to address the challenges of reducing dependence on fishmeal for aquafeeds. The evaluation of feed ingredients is critical to nutritional studies and feed development for aquaculture species. Work included in this thesis includes the utilization of three novel protein sources (pH-shift produced protein concentrate from herring by-products, protein concentrate from shrimp boiling water and meal from mussel by-products), and a comparison of performance with fishmeal in feeding trials on novel marine candidate aquaculture species. The total protein of the new ingredients varied from 60.8%-74.9% and was highest for the herring meal that was produced from the results of paper I. The mussel meal, that was used in paper III showed the lowest value which was 60.8%. The shrimp meal, which was produced in a collaborating project, was ranked 3rd with its content of 66% protein. All protein sources that were tested showed promising compositions that could be used to alleviate the use of fishmeal in aquafeeds. However, "*a feed is only as good as its ingredients*" (Glencross *et al.,* 2007), the results of paper III indicate that certain considerations need to be taken into account when replacing fishmeal for novel species. Such considerations include the nutritional requirements of the species under investigation, the nature of processing of the ingredient (e.g the drying step), digestibility and palatability. The ability of a species to use the test ingredient (e.g the ability of lobsters to digest glucosamine or chitin and the ability of wolffish to increase feed intake to make up for low levels of protein) and defining factors that interfere with that process (e.g antagonizing interactions between astaxanthin and glucosamine sources) are perplexing. This is perhaps the most complex and challenging part of the ingredient evaluation process.

5.2 David vs Goliath, The pH shift process and fishmeal from by-products

Due to higher raw material prices and a larger focus on minimizing waste in seafood processing, by-products are increasingly used in fish meal (Jackson, 2012; FAO 2018). Consequently, due to the high bone and low meat content of these products, the yield of protein obtained during fishmeal production decreases when using by-products (FAO 2018). This has far-reaching consequences on the environmental emissions of fish farms as the introduction of fishmeal with low bone content in aqua feeds resulted in reduced nutrient effluents in fish farms (Kiessling, 2009). Whereas Sevgili *et al.,* (2015) showed that differing sources of fishmeal, had substantial differences in environmental effluents in turbot farming (including a by-product fishmeal which showed poor phosphorous retention). There are thus strong environmental incentives to produce protein concentrates with low ash levels from fish by-products for aquafeed production. The protein levels reached in the concentrates of paper I (75–80% dw basis) were generally higher than those reported for fish meal (55–70%, FAO, 2018). The pH-shift process is also carried out under cold conditions which are thought to preserve the proteins and maintain a higher bioavailability than proteins produced with heat. Treatments such as excessive or intensive heat steps of protein ingredients can reduce protein digestibility (FAO 2018) and are caused e.g. by changes in sulfhydryl groups and disulfide bonds in the protein (Opstvedt *et al.,* 1984). There are plans to test pH-shift produced protein sources in digestibility trials in fish species (see section 6).

To date, it is not possible to fully compare the pH-shift process with classic fish meal production from an economic point of view, since the data from this thesis was based on lab scale processing. While the pH-shift process does not require heating, which is fundamental in fish meal production, it consumes water and acid/base. Depending on the separation step required to then separate non-solubles from solubles in the pH-shift process; separation equipment in some form is indeed required, something which is, however, also needed in fish meal/fish oil production. Our promising data on protein yield when applying a centrifugation force as low as 800 *g* in paper I imply that relatively simple equipment is enough, however more work is needed in order to test potential upscaling. It has also been shown, through previous studies and this thesis, that pH-shift processing can be done with filtration in each of the separation steps instead of centrifugation, which could make it even more simplistic and scalable (Nolsøe, 2011). However, this would require another characterization study, similar to paper I, to fully unravel the composition of the protein concentrate. We believe, however, that it would be easier to set up pH-shift processing in small scale at local fish processing plants where daily amounts of by-products might be relatively small compared to setting up fishmeal/oil processing technology. Trials in larger scale are however needed to verify this.

5.3 Mussel meal; a promising source that needs more research

Mussel meal performed poorly for lobster larvae (paper III), but its potential as a promising protein source should not be written off. Paper III highlighted the large gap in our knowledge for the nutritional requirements of European lobster and this is likely to have had an antagonistic consequence on the success of the mussel meal based diet due to its harsher method of production compared to the freeze dried protein sources that were used. There are limited studies that have tested the inclusion of mussel meal in diets but recent work in Sweden has shown successful growth of Arctic Charr fed on de-shelled mussel meal (Langeland *et al.,* 2016; Vidakovic *et al., 2016)*. Berge and Austreng, (1989), used whole, milled mussel in the diets of rainbow trout but saw reduced digestibility presumably due to the inclusion of shell. Studies incorporating this organism in the pH shift process have also been performed (Vareltzis and Undeland, 2008, 2012), but not in an aquafeed perspective, therefore this could provide a promising method for shell removal without the need for mechanical separation. The high nitrogen removal capacity of this organism (the blue mussel) and high quality protein (Lindahl, 2013), makes it a highly valuable resource for sustainable aquaculture systems (Lindahl *et al.,* 2005). It is unfortunate that this thesis was unable to demonstrate its potential for lobster but nonetheless future studies testing its potential for IMTA and ecological carrying capacities, life cycle assessments, potential in innovative processes such as the pH-shift process and inclusion in feeding trials are all warranted (see section 6). Recently (September 2019), a three month feeding trial was concluded on mussel meal based aquafeeds in the diets of Atlantic wolffish (data not included in this thesis). Preliminary results showed no differences in growth parameters between the mussel feed and a conventional fishmeal based feed. The potential usage of mussel meal goes back to chemical compositions and interactions (5.1), and the suitability may depend on a variety of factors such as species and life stage.

5.4 European lobster aquaculture, a rose surrounded by thorns

This thesis was performed at Sweden's first pilot-scale lobster hatchery and shows that satisfactory *H. gammarus* larval performance in the zoa stages and PL stages (survival development and growth rates) can be attained with formulated dry feed. However, development of a species-specific diet is challenging and was out of reach of this thesis. Further studies, investigating varying nutrients in dry feeds and how they could further improve larval performance are needed if the commercial culture of this species is to ever become a reality. Furthermore, regardless of dietary considerations, this thesis has demonstrated that cannibalism still remains a significant challenge to productivity in crustacean rearing facilities. The importance of cannibalism for the survival and growth of larvae indicate the benchmark that formulators should aim for when designing a novel feed (as paper III tried to emulate in the form of a reference shrimp diet to compare with experimental diets). There still remains a paucity of research testing various protein levels in diets for *H. gammarus* and *H. americanus* and future consideration should also be paid to the interaction between phospholipid requirements and the protein source in aquaculture feeds as pointed out in paper III (See Conklin *et al.,* 1980; Coutteau *et al,* 1997). For example, juvenile *H. americanus,* diets based on casein, showed high levels of mortality due to MDS, which were alleviated by supplementation with dietary soybean lecithin (Conklin *et al.,* 1980). However, no phospholipid requirement was found for lobsters when purified crab protein rather than casein, was used as the primary protein source (Kean *et al.,* 1985). Therefore, the interaction between protein source and phospholipid levels may have important implications for formulation of practical diets.

Despite the challenges highlighted by this thesis (and previous papers) in cultivating European lobster, interest has remained high. Whilst current hatchery practices yield survival many orders of magnitude higher than in the wild, there is still room for improvement on the typical survival to PL and this may be achieved by moving away from live or "wet" feeds, and embracing a dry formulated feed designed for the species according to life stage. Advances in ongrowing technology and engineering aim to reduce operating costs and improve the production of lobsters. For example, the practice of placing PL individually across cells reduces cannibalism, but demands cleaning and feed of potentially thousands of cells, this is extremely labour intensive. To solve this problem, the Aquahive system was invented, a system of circular, stackable trays containing a honeycomb of adjacent cells acting as "apartments" for individual lobsters (UK, http://www.aquahive.co.uk/). Continuous upwelling water delivers food particles regularly, allowing feeding and cleaning of PL within minutes. The overall footprint is also reduced by about 100-fold, compared to original raceway-based cell systems (Powell and ELCE 2016). This has led to significant reductions in hatchery size and will make lobster culture more economic in the long term context.

In conclusion, this thesis confirms the usefulness of the method of Tlusty *et al.,* (2005), to screen a wide variety of candidate feeds on juvenile lobsters. However longer term trials, greater than a few months, are needed in order to proceed using the best performing feeds (see section 6). This thesis also provides a breakdown of lobster feed composition, and a method to make satisfactory dry feed (e.g. freeze-dried shrimp, experiment 2 in paper III) which gave identical performance to raw shrimp feed, and may assist home aquarists and the restocking subsector. Although it is challenging to understand the ecological and nutritional needs of juvenile *H. gammarus,* the results of this thesis show that a diet containing a proportion of shrimp protein concentrate, recovered from local shrimp industry boiling waters, was the best source of a sustainable lobster feed for the emerging lobster aquaculture sector. Hopefully we will see lobsters playing an increasing role in aquaculture in the years to come.

5.5 Atlantic wolffish aquaculture, a stillborn concept?

From an aquaculture perspective, the growth performance of the Atlantic wolffish in this thesis is poor. A low growth rate along with high diet protein requirement will demand high investment costs from a farming operation; however as described in the introduction, wolffish have several important advantages for farming such as the apparent ability to consume dry feed after hatching, a large economic short cut which would allow a farmer to avoid using commercially expensive live feed. Furthermore, product price is the single most important feasibility factor and a high price can easily outweigh the negative effects of slow growth performance and make a farm operation commercially viable. The *A. minor* species supposedly has faster growth rates as demonstrated by Moksness *et al.,* (1994). However, *A. lupus* should not be written off as a candidate species for cold water aquaculture due solely to this. Recently, Árnason *et al.,* (2019) were able to demonstrate a growth potential for *A. lupus,* with growth hormone implants, which increased by weight gain 30% throughout the experimental period in a 3-11°C temperature range. The species clearly shows potential for growth promotion through domestication and selective breeding and coupled with this, previous work has indicated that growth rates and growth efficiency in *A. lupus* are significantly influenced by feed composition and dietary protein (Stefanussen *et al.,* 1993; Moksness *et al.,* 1995; Strand *et al.,* 1995). This was confirmed by paper IV in this thesis, the results suggest that both increased growth and higher growth efficiency will be possible at 10–11°C as the optimal dietary formulations for wolffish become more known and as rearing conditions and domestication are optimized.

The protein requirements of *A. lupus* appear to be similar as for other marine species, with minimum protein levels for optimum growth being 50% protein. The cost of feed is between 40% and 60% of the total production cost in fish farming and protein is the most expensive macro-nutrient in the feed. Therefore, lowering the protein levels in the feed will significantly reduce the production cost. The results of this thesis show the challenges of weaning wild fish onto experimental diets. Further and more detailed studies are required to determine the optimum protein levels in feed for *A.lupus* at different sizes classes. In terms of welfare, the results from this thesis suggest that cortisol may not be a good parameter to measure stress in Atlantic wolffish, or that the species is very tolerant to stress which is advantageous from a farming point of view. The increased energy consumption we observed at 15°C leaves no scope for increased growth in this species, thereby confirming the hypothesis made by previous studies that 15°C is the upper critical temperature for this species (Árnason *et al.,* 2019), but no acclimation takes place after 50 days.

6.0 FUTURE PERSPECTIVES

The challenge of fishmeal replacement is likely to be addressed with a strategy rather than a single raw material that can eclipse the "golden standard" set by fishmeal. These strategies will likely form a combination of technological and nutritional plans of action. One such strategy, proposed by Turchini *et al.,* (2019), was to supplement ingredients which are limited. For example, in the case of this thesis, combining the herring meal produced by paper I, with ingredients which are more economic such as SBM but contain a nutritional shortcoming such as a deficiency in EAA's. Supplementation with prebiotics, palatents and dietary supplements and testing the interaction of protein sources with other raw materials was also proposed as strategies to take into account for future studies (FAO 2018; Turchini *et al.,* 2019); these plans of action were all taken into account by paper III. Finally, further development of by-product processing technologies and feed manufacturing technologies were the other strategies that were proposed (FAO 2018; Turchini *et al.,* 2019) and this was also covered in this thesis by paper I.

Limiting factors of all the protein sources used in this thesis were their production methods, since large scale production was not possible to carry out we had to rely on lab-scale or pilot scale trials. Further efforts should focus towards a life cycle assessments based on optimized and up-scaled production system for each protein source. Furthermore, a digestibility study utilizing both the pH-shift produced protein concentrates and mussel meal is warranted.

The decreased performances resulting from the different feeds used in paper II, III and IV highlight the need to optimize the nutritional requirements for novel species, before testing new protein sources. For European lobster, the works performed by this thesis has led to Sweden's first pilot scale lobster hatchery and work is now underway to rear eggs to PL stage IV before release and seeding wild populations. For future aquaculture to more advanced life stages, the optimization of husbandry protocols for adult stages needs to be performed, as with what was established by paper II, for the zoa stages.

Feeds produced for Atlantic wolffish in paper IV reveal that a high protein diet is needed in future aquafeeds and the work of paper V reveals that the stress response is likely to be slow, but will have significant consequences on growth. These papers provide a platform for future studies to test both protein requirements and alternative protein sources at different life stages.

Finally, the use of an inter-disciplinary approach, undertaken by this thesis, highlights its importance in testing novel protein sources and offers insights into the challenges and limitations of working with novel proteins and novel aquaculture species; it provides a local case study, which could be incorporated into a global context for promoting sustainable aquaculture and diversifying our food production. More inter-disciplinary works should be performed in order to contribute towards eventual optimization of novel feeds and novel candidate species in a local and global perspective and help solve the food production crisis.

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