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Approaches to optimize marine larvae production

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FACULTY OF BIOSCIENCES AND AQUACULTURE

NORD University

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Preface

This dissertation is submitted to fulfill the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA) at Nord University.

The ideas and concepts of the thesis developed gradually during my work with applied research on marine fry production from 2002 up until today. My engagement started in Nordland Research Institute, and continued from spring 2005 at Bodø University College. The latter institution received university status in 2011 under the name University of Nordland, and was in 2015 merged with other regional university colleges to form Nord University, which is the responsible institution for completion of my PhD. My researcher spanned aspects of cod larvae production like comparison of production protocols, benchmarking of commercial productions and testing of aspects of feeding in commercial production.

During my time in applied research, I grew increased overview of aspects of the establishing of successful fry production of novel species for aquaculture, and I gradually started to reflect upon what seemed to be a somewhat fragmented and inefficient overall approach taken by the research community as a whole. Combined with an increased interest in scientific methods in general and designs for applied research in particular, this lead to the studies that make up this thesis. In short, my work focuses on aspects of overall research structure of the process of establishing fry production of novel species, and on how experience from previous and ongoing projects may improve the efficiency of future projects to introduce novel species for aquaculture and aid fine-tuning of production regimes for established species.

The main concept of this thesis is my responsibility alone, but both design and execution of parts of the study has benefited strongly from valuable support from colleges and collaborators, Sylvie Bolla and Marion Cuny in particular. Supervisors during completion of the thesis were Sylvie Bolla (main supervisor) and Jarle Tryti Nordeide (co-supervisor).

Ove Nicolaisen, Bodø, October 8th 2018

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Of the staff at Mørkvedbukta Research Station, I particularly wish to thank Katrine Klippenberg, Tormod Skålsvik, Steinar Johnsen and Robert Eliassen for their valuable practical assistance during larval rearing experiments. I also thank Tor Ove, Katrine, Heidi, Dalia, Cecilia, Håkan, Grete, Ketil, Åsmund, Geir, Morten and more for pleasant times during the early years at the station, at both work and leisure. Also thanks to those colleges at the main campus that have contributed to make my academic life still giving and interesting.

At several occasions, senior academics at the Faculty encouraged and supported my enrolment into a PhD-program: Even though these early attempts did not succeed at the time, I would like to express my gratitude to professors Igor Babiak, Ketil Eiane and Kiron Viswanath for this support. In addition, I thank the Minister of Education and Research at the time, Torbjørn Røe Isaksen, the political responsible of the formation of Nord University by fusion. As this process led to a need to strengthen formal competence at the institutional level, funding was made available that enabled FBA to offer me the opportunity to finish the process towards a PhD. Thanks to FBA for finally offering me this opportunity.

I am also grateful to Tor Magne Jonassen and Cecilia Vargas in Cod Juveniles AS for cooperation and for providing insight into commercial production issues at the time. Thanks also to Cod Juveniles AS, Marine Breed AS and the Norwegian Cod Breeding Centre for providing eggs free of charge, and to the VRI Aquaculture program and the MARLIP program for funding experimental works.

Last but not least: Thanks to my supervisors, Sylvie and Jarle for bothering to supervise the completion of this PhD thesis.

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Definitions and abbreviations

Altricial fish: Fish that hatch as larvae at an incomplete developmental stage, often lacking much of the shape and functionality of a completed juvenile form, which first arrives after metamorphosis.

Aquaculture: The farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated, the planning, development and operation of aquaculture systems, sites, facilities and practices, and the production and transport (Source: FAO glossary of aquaculture).

Blinding: Procedures to hide the true experimental conditions from the participants (subjects, researchers, data analysists) to avoid bias.

DoE: Design of experiments.

Extensive larviculture: Low larval density and low intensity production in large, nature-like systems (ponds / lagoons. Low degree of control with production parameters. No externally added feed – based on naturally occurring feed (Olsen et al., 2004).

External validity: The extent to which results from an experiment are capable to conclude about (extrapolate to) the actual situation studied.

Precocious fish: Fish that hatch at an advanced stage, broadly resembling the shape and functionality of the completed juvenile form.

Propagation: Reproduction and / or spread of an organism.

Semi-intensive larviculture: Intermediate larval density and low intensity of production in suspended “mesocosms”, enclosures or large outdoor tanks. Added feed from external sources for first feeding - harvested or produced zooplankton (Olsen & al., 2004).

Randomization: The random allocation of treatment levels to a study unit to reduce effects from hidden variability and avoid bias.

Replication: Repetition of unique conditions (combinations of treatment levels) within a study to improve accuracy, estimate uncertainty and enable significance testing

Replicability (of studies): The ability for independent researchers to re-run reported studies and check the findings (Peng, 2009, 2015).

Reproducibility: The ability to re-compute analyses given access to data and access to details about performed statistical analyses (Peng, 2009, 2015).

OVAT: One-variable-at-the-time. Experiments examining the effects of several treatment variables on some response variable (s) by examining effects from each treatment variable separately from the others.

Abstract

Aquaculture has a long history in providing food for human consumption, an in its simplest form fish holding practices have been tracked 4500 years back. Later more advanced practices, mostly freshwater cultures, arrived and gradually spread around Asia and to Europe. The depletion of marine fisheries following the industrial revolution formed a basis for growth in modern marine fish aquaculture. Today marine fish culture is suggested as a solution to provide high-quality protein and essential fatty acids, particularly the polyunsaturated n-3 type, to a growing human population. It is also considered a means to support employment and prosperity at various geographic levels, nationally as well as regionally.

Successful production of marine fish demands the solving of multiple task from the larval stage to grow-out, and the hatchery stage is critical as it provides juveniles for later steps. Hatchery production is complex, species-specific and for many species its development and fine-tuning has been time consuming and costly. Successful production depends on broodstock holding, egg production, suitable larval feeds and technology including fixed (e.g. water treatment) and more readily exchangeable (tank designs and arrangements). Finally a whole range of production factors, both physical (e.g. temperature, light, water flow) and biological (fish density, feeding) should be under control. To complicate the situation, early stage marine fish experience rapid ontogenetic changes, which demand currently updated production settings during growth.

The overall aim of the study was to identify issues in larval fish production research and implementation in terms of overall effectiveness and suggest future improvements, with particular focus of in-tank factor control and effects on production outcome.

Literature examination of research practices at this area indicated limited cooperation and predominantly use of one-variable-at-the-time strategies (OVAT), mostly conducted by individual researchers or research groups, rather than structured research design strategies and truly collective overall research. The study shows potential to improve both standardization of conduct, analysis and reporting of studies.

The present study also included early stage screening experiments to examine effects from visual environment on Atlantic cod larvae behaviour and foraging. Small-scale early stage designs used feed ingestion and behavioural aspects as proximate response variables. To cope with rapid ontogenetic changes in larvae and address stage-specific issues, the trials were run as discrete short-term experiments at different larval stages. The experiments were suited to examine mechanisms at experimental scale to support later fine-tuning at larger scale: Light intensity, prey density and algal concentration all affected foraging success and relative importance of factors changed with larval stage. One categorical variable (bottom colour) could be fixed at its best level, and potential interactions between factors were identified. Trials also identified behavioural patterns of relevance to build appropriate rearing environment. The contribution from light intensity, micro algae and wall colour on larval phototaxis also changed with stage. White tank walls strongly attracted larvae, which may affect larval distribution in tanks.

In summary, there is room to improve the quality and standardization of conduct, methods and reporting of research, which demands improved collaboration within the research community. Additionally, small-scale controlled experiments at discrete developmental stages are useful to reveal characteristics of well-functioning rearing environments to guide up scaling to commercial scale production. Furthermore, the frequent use of one-factor-at-the-time studies indicates a predominance of pilot stage studies and a lack of coordinated efforts within the research community. Established methods for process optimization known from other sectors, using screening and optimization designs, should be adapted to this particular area to improve and speed up the research process, both during experimental and commercial scale stages. It is an open question how such research processes fit into an often fragmented, highly competitive and pure science oriented environment of academic research or whether it is better suited for dedicated research organizations provided with sufficient staff, funding and organization.

1. Introduction

1.1. The rationale of marine fish aquaculture

There is presently a strong rise in human populations, which if continued may affect access to, demands for and prices of food resources. According to the United Nations Population Division, the world population has increased from 2.5 billion in 1950 to around 7.4 billion in 2015, and is in 2050 predicted to reach 8.7 - 10.8 billion (Anon., 2017a), an increase of 18 – 46 % from the 2015 population. From the 1950s, there was a rise in marine fishery landings, which leveled off in the late 80s (FAO, 2016) due to overfishing during most of the 20th Century (Kennelly and Broadhurst, 2002; Thurstan and Roberts, 2014) which put many marine fish species and populations at danger (Anon., 2017b; FAO, 2016; Nieto et al., 2015). Today, about 90 % of the fish stocks are fully or over exploited (FAO, 2018). Despite these unfortunate developments of fisheries, the increase in supply of fish protein for human consumption still exceeds the increase in demands from a growing population, much due to an impressive growth in fish aquaculture (FAO, 2016; Thurstan and Roberts, 2014). Thus, further aquaculture growth may support increasing needs for high quality protein for future generations, due to a higher protein retention in fish than in terrestrial animals (Béné et al., 2015). Ideally, though, for such idealistic purposes, production of animal protein should base on transforming plant protein unsuited for human consumption into human food. This implies that in a "feed the world" setting, fish aquaculture should probably focus on species at low trophic levels for human food, not on expensive carnivorous species for high-end markets, which themselves depend on using limited protein sources suited for human consumption.

Besides from being a high-quality source of protein, marine fish contain essential fats, vitamins and minerals, and may even in small quantities significantly improve the nutritional impact from plant diets (FAO, 2016). Marine fish – particularly oily fish – are often high in polyunsaturated n-3 fatty acids (PUFAs), which show beneficial effects on human cardiovascular health and are important constituents of brain development and the nervous system (Calder, 2004; Kitessa et al., 2014; Thurstan and Roberts, 2014).

Aquaculture also has positive implications on employment. It employs more than 18 million people worldwide, most of which (94 %) in Asia (FAO, 2016). In EU, it employed 39000, in Norway 4600 people in 2015, 0.02 and 0.18 % respectively of the total employment (Anon., 2018a). Many nations thus consider marine aquaculture an up-and coming commercial industry and a future source of national export income, prosperity and employment, so strong incentives exist to improve established production as well as to diversify the industry in terms of introducing new candidate fish species.

In addition to nutritional, commercial and societal arguments for diversifying marine fish production, enhanced knowledge of biology and production of the different species may in the future show critical in the processes of conservation of endangered species.

1.2 The history of aquaculture

1.2.1. The early days

There is great uncertainty about the origin of fish aquaculture, but evidence suggests very early attempts to transplant fertilized eggs, entrap fish for holding, growth and harvest and to enhance rearing environment. Fishponds appeared in the Sumerian culture around 2500 BC (Nash, 2011). Around 2000 BC tilapia ponds (species unknown) were present in Egypt (Beveridge and Little, 2007), and around 500 – 200 BC pond cultures appeared both among Etruscans, Assyrians and Romans (Beveridge and Little, 2007; Drawbridge, 2007; Nash, 2011). Many hold Asia, particularly China, as the birthplace of the more advanced aquaculture practices: Sources from the Zhou Dynasty (Fan Li, 475 BC) describe semi-intensive pond cultures, propagation and spread of fry, mainly of carp species (Nash, 2011). Later reports of Chinese developments include rice-fish cultures (43 AD), fish polycultures (≈600 AD), increasingly advanced and intensive fish farming methods in combination with agriculture practices and improved techniques of fry production and propagation, and by the mid-1600s, detailed written descriptions of fry production and transport existed (Beveridge and Little, 2007; Nash, 2011). It is speculated that Chinese practices and species later spread around Asia.

1.2.2. Early Western Aquaculture

In Europe, the Romans probably introduced common carp (*Cyprinus carpio*) to Rome from the Danube river around 100-200 AD (Balon, 1995). Following Roman practices of keeping fish for storage, pond cultures of various freshwater fish spread throughout Europe up until the middle ages, particularly among the prosperous, priests and in monasteries, who held fish for consumption during religious feasts. During the early 15th century, construction and management of more elaborate pond systems appeared, and in the 16th century, literature addressing fish culture appeared (Beveridge and Little, 2007; Nash, 2011).

The Industrial revolution (mid-18th – mid 19th century) induced wide-reaching societal and technological changes, and formed the basis for developments to come, first in fisheries and later in European aquaculture. Industrialization led to urbanization and population increase, compact markets and improved transport, which both laid pressure on the freshwater resources and encouraged an increased commercial exploitation of marine fish stocks, supported by innovations in fisheries technology (Kennelly and Broadhurst, 2002; Nash, 2011). Due to this, both stocking and restocking of depleted freshwater resources and marine fish stock enhancement started to receive increased focus.

For freshwater fish, hatcheries for stock enhancement appeared already in the mid 1700s, and Stephan Ludwig Jacobi from Hanover reported this artificial fertilization and hatching of trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) in 1763 (Richardson, 2012). In the mid-1800s, freshwater hatchery practices spread over Europe and North America, e.g. in France in 1853, Canada in 1856 and US in 1870 (Beveridge and Little, 2007; Nash, 2011). Already in 1863 it was proposed (Anon., 1863) that culturing Atlantic salmon could be important in "capitalist food production, in line with bread and turkey", based on novel knowledge about its life history (Brown, 1862) and culturing practices (Francis, 1865). However, despite early achievements, commercial salmonid production in freshwater ponds appeared first in the 1890s in Denmark, using rainbow trout (*Onchorhyncus mykiss*), which was introduced from the US to Europe in 1879 (Nash, 2011). In Norway, grow-out of rainbow trout in the marine environment succeeded in the

early 1960s, and thus cleared the way for future marine production of rainbow trout and Atlantic salmon in Norway (Braaten and Sætre, 1973).

1.2.3. Environmental worries and the rise of marine sciences

For marine fish, development of new technology following the Industrial Revolution led to increased catches but also worries about marine fish stock deteriorations, and thus increased the interest for marine sciences during the late 1800s. Marine research stations and hatcheries appeared in both Europe and the US, focusing on basic research and propagation/ re-stocking of eggs or newly hatched larvae from wild-caught brood fish, and in 1886, G.M. Dannevig produced the first juvenile Atlantic cod (*Gadus morhua*) from larvae fed naturally occurring zooplankton in large concrete basins. In the early 1900s though, these re-stocking programs for marine fish were gradually shut down due to uncertainty about their effects (Bengtson, 2007; Drawbridge, 2007; Moksness et al., 2007; Nash, 2011; Schwach, 2014). Some of these approaches reappeared later though, for several reasons: General worry about negative effects from fisheries prevailed and were strengthened during the 20th Century. Technological advances, like more efficient trawls, echo-sound tracking and large high-seas factory trawlers increased pressure on fish stocks, resulting in major declines in marine fisheries and an end to the continuous growth in fish landings during the 1950s to 80s (Eigaard et al., 2014; FAO, 2016; Kennelly and Broadhurst, 2002). Thus, some interest in propagation and restocking still remained (Drawbridge, 2007), e.g. in Norway, where extensive and semi-intensive production of several commercially important species took place between the mid-1970s and -80s (Øiestad et al., 1985). This work was later continued within the PUSH sea ranching program (1990-1997). In Japan, the establishment of exclusive economic zones during the 1970s reduced access to the traditional fishing areas, which induced a national research and hatchery building campaign for stocking of national coastal waters (Bengtson, 2007).

1.2.4. The rise of modern marine fish aquaculture

Up to the Second World War, marine fish aquaculture research predominantly focused on restocking and conservational issues, but some important aquaculture related

discoveries took place, which led the way towards present intensive methods of marine juvenile production. From the early 1900s on, methods of purification and mass culture of marine microalgae developed (Nash, 2011; Preisig and Andersen, 2005), and in the 1930s, *Artemia* nauplii were identified as potential food for fish larvae, e.g. anglerfish (*Lophius piscatorius*), plaice *(Pleuronectes platessa)* and cod (Bengtson, 2007; Gross, 2009; Moksness et al., 2007; Rollefsen, 1939; Seale, 1933). In 1960, the smaller rotifer *Brachionus plicatilis* was introduces as live feed by the Japanese scientist Ito, which enabled feeding of even the smallest fish larvae (Moksness et al., 2007).

Due to these fundamental developments, the 1960s saw the first attempts to establish intensive juvenile production methods for e.g. plaice, sole *(Solea solea)* and turbot *(Scophthalmus maximus)* in Europe (Alderson and Howell, 1973; Jones et al., 1974; Shelbourne, 1964) and red sea bream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*) and pufferfish (Tiger puffer, *Takifugu ribripes*) in Japan (Bengtson, 2007; Drawbridge, 2007; Kikuchi, 2006).

During the 1970s, live feeding methods were fine-tuned with regard to production, enrichment and practical application of live feeds, and Japanese scientists identified important nutritional issues of live feeds, particularly concerning their fatty acid profiles (Bengtson, 2007; Moksness et al., 2007). In this period, intensive production of marine fish juveniles in Europe was developed, including turbot, European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), and functional protocols for these species were established by the late 1980s (Bengtson, 2007; Chatain, 1997; Drawbridge, 2007; Shields, 2001). In China, artificial breeding techniques for mullets (family Mugilidae), red sea bream, Japanese flounder, black porgy (*Sparus macrocephalus*) and large yellow croaker (*Pseudosciaena crocea*) also developed during the 1970s and 80s, which led to production of fry for both culture and ranching (Hong and Zhang, 2003). Throughout the 20th century a growing body of research on the early stages of many freshwater fish species, for basic research or conservational reasons, also added to the knowledge of basic biology and ecology of early life stages of fish in general (Kamler, 1992).

In Norway, research on marine cold-water fish aquaculture accelerated in the 1980s. In an attempt to establish Atlantic halibut (*Hippoglossus hippoglossus*) for aquaculture, comprehensive national research started in the mid-1980s, including efforts from both publicly funded research institutes, universities and commercial pioneers. Research covered most aspects of life and production cycles: ecology, ontogeny, brood stock and reproduction, hatchery techniques and larval rearing protocols - from extensive to intensive production methods. Despite intensive R&D (Mangor-Jensen et al., 1998; Olsen et al., 1999) and established commercial productions in Norway, Iceland, Scotland and Canada during the 1990s, production of juveniles for on-growth stayed at low and unstable levels for long, and is still today not satisfactory solved. From the mid-1980s, there were also attempts to produce Atlantic cod commercially by extensive and semi-intensive methods, while intensive techniques using rotifers and *Artemia* appeared at experimental scale from 1987 to 93. Around 1999, Atlantic cod was appointed the novel candidate for marine finfish aquaculture around the North Atlantic, among others due to a decline in commercial fisheries and resulting increased market prices (Anon., 2000; 2003).

To conclude, modern marine juvenile fish production developed over the last 5-6 decades, based on achieved knowledge about microalgae and live feed. Supported by comprehensive, long-lasting, expensive research programs, this has led to today´s intensive production of species like sea bream, seabass, turbot, halibut and cod in Europe (Anon., 2000; 2003; Chatain, 1997; Mangor-Jensen et al., 1998; Shields, 2001).

1.3 The current state of marine fish production

Marine fisheries capture increased from the 1950s but then stabilized from the late 80s. However, global per capita supply of fish for human consumption has increased up to present despite the world population increase. This is mainly due to aquaculture, which in 2014 at the first time exceeded fisheries in volume. Furthermore, the state of the world marine fish stocks is poor, with 31 % at unsustainable levels and 58 % being fully fished (FAO, 2016; Thurstan and Roberts, 2014). Given a continued population

increase and increase in demands for fish meat, growth in marine aquaculture is one potential source of marine fish supply.

While farmed aquatic animals today constitute 75% of total aquaculture production, aquatic plants and microalgae make up the remaining 25 %. Furthermore, by 2014, half the world´s total aquaculture production was freshwater based: Fishes, bivalve mollusks and other filter feeders. Worldwide, 87 % of aquaculture-produced fish were freshwater species - mainly produced in pond culture – while the remaining 13 % was marine production (FAO, 2016). Marine finfish production approximated 63 million tons, with Asia (53%), Europe (28.9 %) and the Americas (16%) as main producing continents, while Oceania produced 1% and Africa 0.2 %. The 25 top producing countries had 92.6% of the marine production (FAO, 2016).

China - the world´s leading aquaculture producer - had in 2014 more than half the world´s finfish aquaculture production, of which 4 % was marine species. By year 2000, 52 marine fish species had been successfully bred in China, although control with the entire life cycle was achieved for only 11 species (Hong and Zhang, 2003), so the potential both to increase the overall share of marine aquaculture and to optimize juvenile production of novel fish species is considerable.

In Europe, marine and diadromous fish species dominate production (79%), predominately intensively produced species for high-end markets (FAO, 2016). Today the top five finfish species produced for human consumption in the European Union (EU) are Atlantic salmon, rainbow trout, European Seabass, gilthead seabream and Atlantic Bluefin tuna (*Thunnus thynnus*) (Anon., 2018a). In Norway Atlantic salmon and rainbow trout dominate, added some halibut, cod and minor volumes of other species (Anon., 2018b).

In EU as a whole, growth of the aquaculture industry has stagnated and production has remained stable over the last couple of decades (FAO, 2016). The EU addresses this issue politically, and aims to strengthen and innovate the sector to support regional employment, growth and sustainability through e.g. the “Strategic guidelines for the sustainable development of EU aquaculture” and the “Blue Growth” concept. Large publicly funded R&D programs focus on both fine-tuning of existing production and introduction of novel species to marine aquaculture. The FINEFISH project (2005-2009)

targeted juvenile quality / malformations in marine fish hatcheries, while the DIVERSIFY program (2013-2018) explores biological and socio-economic potential of novel candidate species with an aim to expand and diversify European aquaculture. The presently running MedAID (2017-2021) and PerformFISH (2017-2022) projects aim to improve technical, social and economic performance of Mediterranean fish aquaculture to support regional industry and economic growth.

1.4. Rationales for future developments and species diversification

The arguments to improve existing production or to introduce novel species into marine aquaculture vary, dependent upon the initiator´s intentions, e.g.:

1) The humanitarian view to provide human nutrition and subsistence for the global population, as e.g. emphasized by the FAO.

2) A commercial view focusing on economic return to individual commercial enterprises and investors.

3) A geographically delimited view, focusing on the prosperity and employment of nations or regions, as often emphasized by e.g. the EU and Norway.

4) A conservational view appreciating the value of production of knowledge to assist stock enhancement, which may show important in restoration of threatened species or stocks in the future (Bengtson, 2007).

1.5. Societal and economic considerations in marine fish aquaculture

Establishing new fish species for commercial aquaculture is a complex process, involving a wide range of non-biological and biological considerations. A full depth evaluation of novel fish species for intensive commercial production should consider societal pros and cons, economical and market considerations, environmental effects as well as the biological basis and the zootechnical aspects of production throughout the life cycle (Paquotte, 1998; Quéméner et al., 2002; Ross and Beveridge, 1995).

1.5.1. Production economy and market considerations

From a strictly commercial view, economic considerations (e.g. production costs, competition, available knowledge and skills, technologies, present and potential markets, investment funding and a species´ potential as food and commodity) affect production profitability and thus commercial success and longevity (Bostock et al., 2010; Kjesbu et al., 2006; Paquotte, 1998; Quéméner et al., 2002; Ross and Beveridge, 1995). The perfect commercial aquaculture species is produced free of charge, grows rapidly on minimal amounts of cheap feed, produces high quality meat liked by everyone, is highly priced, and is flexible to manufacturing differentiation processes that expand customer demands and markets.

As a rule, high market value allows more R&D effort, higher production costs (labor, technology) and lower growth and survival before profitability is lost (Ross and Beveridge, 1995). Ethical considerations, like sustainability of production, impact on nature and native populations and the use of sustainable feed sources, are increasingly important in itself as well as in economic considerations due to possible effects on consumer behaviour and ultimately on profit.

1.5.2. Societal costs and benefits

For public authorities at the levels of regions or national states, cost-benefit analyses of development costs relative to societal returns in terms of employment, trade surplus and citizens´ prosperity are important when considering supporting R&D or commercial innovations, as costs are high and time perspectives may be long. During the time span 1988-2014, the public funding of Newfoundland and Labrador aquaculture was around 75 million $ (Anon., 2015). A public funding at a magnitude of around 300 million € to develop European sea bream and sea bass production has been suggested, while for Atlantic halibut, Norwegian public funding was estimated to around 50 million € over ten years (1988 – 1998), without achieving economically sustainable production (Ytreberg and Isaksen, 2001). For Atlantic cod a need for public funding of minimum 30-50 million NOK (3-5 million €) annually over at least 10 years was proposed – in pure R&D costs only - to establish cod as a novel species (Anon., 2003). For the above examples, private

investments of a similar magnitude - probably much higher – add to the total development budget for any given species, pinpointing the general rule that development of novel fish species for aquaculture is expensive and resource demanding.

At a macroeconomic / socioeconomic scale aquaculture competes with alternative beneficial use of marine and coastal areas like fisheries, tourism, port development, sea transport, boating, navigation and recreation, and may e.g. cause privatization of public lands and waterways, loss of fisheries and deterioration of recreational areas (Bostock et al., 2010; Primavera, 2006).

Aquaculture also causes a range of environmental effects, which should be included in the overall assessment of societal value. Effects from introductions and escapees on ecosystems (genetic influence on natural populations, habitat deterioration, spread of diseases and parasites, competition with native species, the use of wild fish stocks or land areas for fish feed production, and effluents from production (eutrophication, chemical pollution) are all potentially important environmental issues which may arise from marine aquaculture (Bostock et al., 2010; Kjesbu et al., 2006; Martinez-Porchas and Martinez-Cordova, 2012; Primavera, 2006; Ross and Beveridge, 1995).

Finally, in a global capitalistic economy, company structure and ethics, geographic belongings of owners, recruitment policies and prevailing regulatory rules and tax levels may affect geographic localization of production and employees, and subsequently the fraction of added value that stays locally, regionally or nationally and thus benefits the community.

1.6. Production technology in marine larval rearing

In the present context, basic technology includes technical structures kept unchanged over long periods due to large investment costs, excessive amount of work and time needed to change technology, like water intakes, water treatment systems, heating systems and production tanks (fixed volume, shape, colour and coatings). Some of these structures like water source, pumps, water treatment and heating systems do not decide production environment per se, but delimit the available water capacity, water quality

and temperature. Tanks are more easily exchangeable, and their size, shape and colours affect water flow, water exchange, water quality (Oca et al., 2004; Timmons et al., 1998) and light conditions (Naas et al., 1996). Piping systems can readily be changed. Tank piping (Timmons et al., 1998) and aeration (Sakakura et al., 2006; Shiotani et al., 2005), directly affect water exchange and water flow. Surface skimmers and tank cleaning devices (Chatain and Ounais-Guschemann, 1990; van der Meeren et al., 1998), help preserve water quality in tanks, while lighting technology decides intensity, frequency and spatial and temporal distribution of light through their positioning and fabric.

1.7. Aspects of biology and tank environment in larval rearing

For many candidate marine fish species, the establishment of sufficient juvenile production of acceptable quality is a major bottleneck for introduction into marine aquaculture (Dhert et al., 2001; Olsen et al., 2004). For any given species, biological production depends on multiple abiotic and biotic factors, varying in optimal factor settings between the different developmental or production stages, from brood fish through egg development, larval stage and juvenile stage to the adult stage and subsequently slaughter and processing for sale (Figure 1).

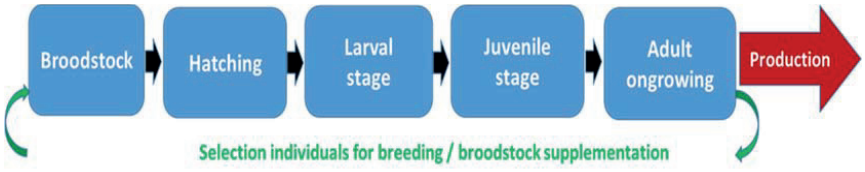

Figure 1: Production flow in intensive marine fish production.

Failure to control biological production at any given life stage may ultimately cause failure to introduce a species to commercial scale aquaculture. Abiotic considerations typically include environmental conditions like temperature, light, salinity and chemical properties of water as well as technological choices, while biological considerations include e.g. nutrition, feeding, tolerance to crowding, health/ disease issues and

hardiness in aquaculture (Kjesbu et al., 2006; Moksness et al., 2007; Quéméner et al., 2002; Rosenlund and Skretting, 2006; Ross and Beveridge, 1995). Governed by the constraints of the organism, these factors determine success at any given stage, in terms of survival, quality, production volume and thus ultimately commercial success. Both the improvement of present culture methods for established species and the implementation of these methods on novel species depend on the application and fine-tuning of already established principles (Bengtson, 2007).

Despite the established knowledge and achievements in larval rearing, and the successful juvenile production of sea bream, sea bass, turbot and sole (Chatain, 1997; Shields, 2001), challenges were still experienced for species like haddock, hake, cod halibut and wrasses (Brooker et al., 2018; Moksness et al., 2007; Shields, 2001). Development of commercial production of Atlantic halibut was hampered through the 1980s and 90s due to low fry production (Brown, 2002), which is still an issue for this species. Cod larval production prior to the so-called "cod-boom" was extensive or semi-intensive, but from year 2000, intensive technology and methods used for Mediterranean species were introduced (Karlsen and Adoff, 2003; Rosenlund and Skretting, 2006; Skiftesvik et al., 2003). Despite of the achievements with other species though, build-up of intensive production of cod took considerable research effort, time and money over many years (Anon, 2009; Kjesbu et al., 2006; Rosenlund and Skretting, 2006) and major issues like variable production and low juvenile quality were still present at the time of collapse of the cod aquaculture industry around 2010 (Tønnesen Busch, 2010; Vargas, 2015). Thus, despite earlier achievements, the complexity of larviculture necessitates extensive, time consuming and expensive species-specific research to master a wide range of biological and technical issues, including knowledge of behaviour, nutrition, feeding, health and rearing environment throughout ontogeny (Figure 2).

Figure 2: Factors affecting larval performance in intensive rearing

1.7.1. Broodstock, egg production and breeding

Broodstock provides eggs and larvae of good quality for research and commercial production. Short supply of eggs restricts access to larvae and juveniles, while sufficient year-through access enables up scaling and speeding up the R&D and benefits commercial production, by allowing efficient use of hatcheries, year-through fry production and thus flexible strategies for juvenile and adult on-growth.

The research strategy has initially been to produce quantity year-around, but little is done for quality. Even though there is some ambiguity about egg quality, studies show that egg quality and size may correlate with the viability and size of newly hatched fish larvae (Kjørsvik et al., 1990; Marteinsdottir and Steinarsson, 1998; Zhao et al., 2002); which may further affect the fate of larvae during both early and later stages.

Fishes show a great variability of reproductive styles (Balon, 1975; Chambers, 1997), spanning spawning and guarding behaviour, the preferred environment and the mode of fertilization, which have implications both in the wild and in aquaculture. Knowledge about reproductive biology, preferred environment, nutrition, suitable technological solutions, tending and reproductive aspects of individual variability of fish (size, age, genetics etc.) is needed for broodstock production (Izquierdo et al., 2001; Kjørsvik et al., 1990; Migaud et al., 2013; Pavlov et al., 2007; Tandler et al., 1995) (Figure 3).

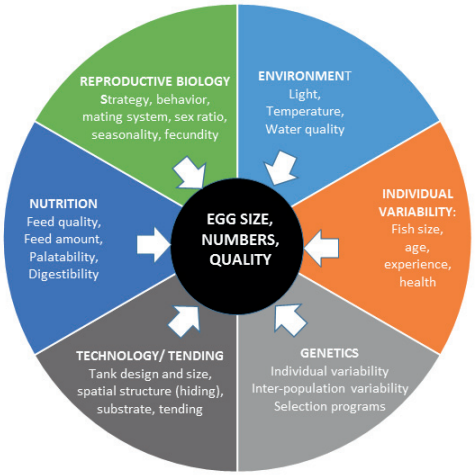

Figure 3: Factors affecting production and quality of fish eggs.

Selective breeding has gained the production of species like Common carp (*Cyprinus carpio*), rainbow trout, tilapia (mainly Nile tilapia *Oreochromis aureus*), channel catfish (*Ictalurus punctatus*) and Atlantic salmon, and lately breeding programs for marine fish like European seabass, gilthead seabream, turbot and Atlantic cod have been established (Lozano, 2011; McAndrew and Napier, 2011). However, typically, attributes of interest include growth, age at maturation, environmental tolerance and disease resistance at later life stages, (McAndrew and Napier, 2011; Nævdal et al., 2003) and breeding does not focus specifically on the larval stage. Furthermore, for breeding to be an option, production throughout the life cycle must already be mastered at sufficient volume to justify costly breeding programs (Nævdal et al., 2003), which means that selective breeding is an option first when larval production is reasonably well established.

1.7.2. Nutrition and feed in marine fish larvae

In nature, there is great variability in the nutritional needs of fishes, dependent upon ontogenetic stage and species. In aquaculture, the role of nutrition is to provide necessary components to the organism at focus to fulfill its requirements for development, health and acceptable growth, and ultimately to convert the feed into a desired product for human consumption. For marine fish larvae like other cultured

organisms, nutrition includes a sequence of activities: 1) to identify the necessary nutritional components, 2) to make feeds to distribute these components at sufficient amounts, which are accepted, digested and assimilated by larvae, and lastly 3) to provide a production environment and feeding regime that ensures efficient and sufficient feed ingestion.

A complicating issue in larval nutrition is the use of both live feeds and manufactured diets in a time sequence, dependent on the ontogeny of the species (Dhert et al., 2001).

Despite the achievements in larval nutrition over the years, much work remains to find optimum composition of macronutrients, micronutrients, and even of the already much studied polyunsaturated fatty acids (PUFA) (Hamre et al., 2013). During the latest decades, there have been considerable developments in enrichment products and production protocols for live feeds as well as of commercial dry feeds for marine larvae. However, gaps in knowledge of nutritional needs, feed technology and feeding practices are still considerable, and future improvements are to be expected. Developments in nutritional composition of feed often co-occur with improvements in feed technology and rearing practices, with a strong interdependence between these areas in terms of the final success of larvae.

Rotifers of the genus *Brachionus* have been the most used live feed for first feeding of marine fish over the last 4-5 decades, and have contributed to produce more than 60 marine fish species. Still, information on their needs of micronutrients is scarce (Yoshimatsu and Hossain, 2014), and there are particular issues associated with using rotifers for larval nutrition, both in terms of their production methods, growth diets and enrichment (Dhert et al., 2001). First, rotifer diets for both culture and enrichment may vary considerably in nutritional content, a variability that affects the contents of rotifers fed to larvae (Hamre, 2016). Furthermore, different production methods – batch, semi continuous and continuous cultures - are used (Dhert et al., 2001; Yoshimatsu and Hossain, 2014), which also affects the nutritional profile of feed organisms differently (Yoshimatsu and Hossain, 2014). Generally, protein levels and amino acid profiles in rotifers are genetically determined and independent from food quality, but not from culture conditions (Dhert et al., 2001; Hamre, 2016; Yoshimatsu and Hossain, 2014), while

their lipid levels and fatty acid profiles are determined both from culture diets and enrichments (Hamre, 2016; Hamre et al., 2013). Both rotifer diets and culture conditions vary between producers, which may partly explain the low predictability often seen in commercial larval production (Hamre, 2016). Other possible reasons for the variability include the duration of enrichment after cultivation as well as the human factor in terms of staff skills and care.

Artemia, on the other hand, receives lipids and essential PUFAs directly from the enrichment. The exact control of contents is difficult, as they selectively reconvert DHA to EPA (Hamre et al., 2013), and strain differences in DHA retention have also been observed (Evjemo et al., 1997).

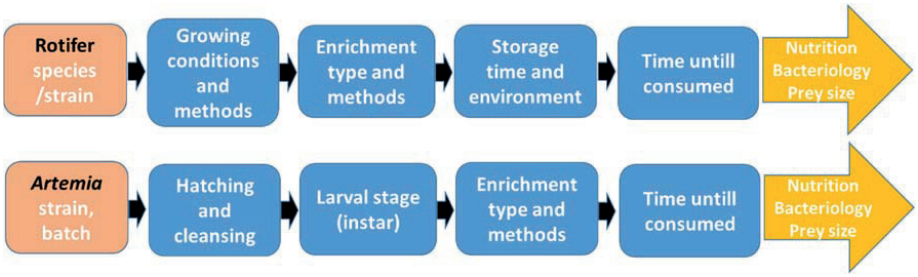

Figure 4: Live feed production factors for rotifers and Artemia

A main goal for nutritional research in marine fish larvae is to develop formulated micro diets to replace live feeds, due to their costs, workload and challenges with nutritional fine-tuning (Dhert et al., 2001; Hamre et al., 2013; Yoshimatsu and Hossain, 2014). Despite huge efforts over the last 30 years, such diets are still far from optimal, and for most species cannot entirely replace live feed (Kolkovski et al., 2009; Yoshimatsu and Hossain, 2014).

A good micro diet fulfils multiple aspects of larval nutrition: It contains sufficient energy and is nutritionally balanced in terms of protein and lipid profile, minerals and vitamins (Hamre et al., 2013). Next, larvae must find it, which depends on both the feed itself (colour, size, odor, sinking rate, movement) and the production environment (amount and frequency of feeding, feed dispersal, visual environment, larval density, water

circulation). Larvae must approach feed, which depends on movements, e.g. sinking. Further, ingestion depends upon taste, size, shape and texture. The actual value of ingested feed as a nutrient source also depends heavily upon leaking rate and residence time in tanks. A tradeoff will exist between leaking and feed permeability: Permeable feeds lose nutritional value more easily, while too impermeable feeds reduce spread of attractants, which may reduce feed uptake (Figure 5).

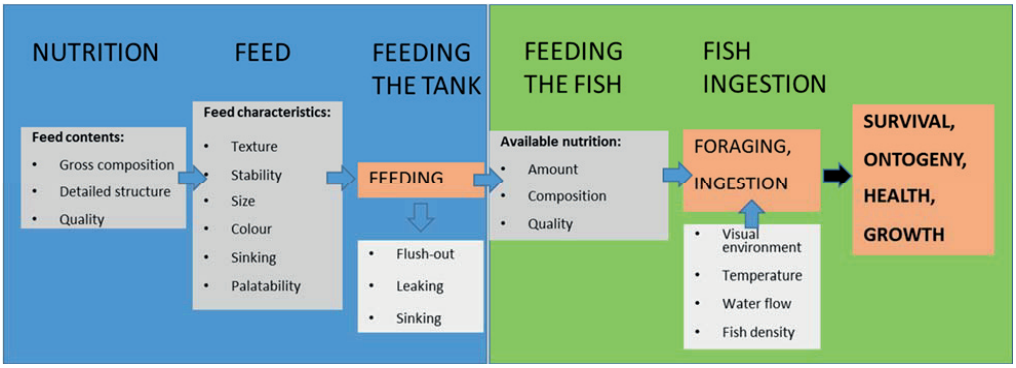

Figure 5: Aspects of nutrition, feeding and foraging

Finally, ingested feed must be digested and assimilated, which depends on feed type, technology and the nutritional components used, and feeding must pollute minimally in tanks, dependent on feeding regimes and tank cleaning routines (Dhert et al., 2001; Hamre et al., 2013; Kolkovski et al., 2009). Common micro diet technologies are micro binding, micro-encapsulation, and micro coating, which differ in characteristics like stability, leaching, digestibility and sinking (Hamre et al., 2013; Kolkovski et al., 2009; Watanabe and Kiron, 1994).

1.7.3. The tank environment

In addition to the basic, relatively fixed premises set by technology, tank designs, broodstock management, egg quality and the quality of live and dry feeds, a range of production factors are readily adjustable in-tank, and thus more easily available to producers to optimize ongoing production. These include temperature, water circulation

patterns and exchange rates, larval stocking density, feeding practices, tank tending and visual environment.

Temperature affects O_2 contents and fluid characteristics of water. Together with O_2 levels temperature sets the limits for metabolism at any given ontogenetic stage, and generally it affects growth and ontogeny of the organism. It modifies activity, with implications for hunting, ingestion and metabolism. Optimal temperature is species and stage specific and depends on the levels of feeding (Fry, 1947; Hunt von Herbing, 2002; Kamler, 1992; Priede, 1985). Temperature also affects bacterial growth, which has been suggested a main factor to threaten larval health in aquaculture (Vadstein et al., 2013). A strong relationship with growth is evident, but interactions with other production factors are less well studied.

Water flow affects distribution of fish larvae and feed, both live and inert (Baskerville-Bridges, 1999; Moretti et al., 1999; Sakakura et al., 2006), which further affects foraging success (MacKenzie and Kjørboe, 1995; Mahjoub et al., 2012) and ultimately survival (Barahona-Fernandes, 1978; Gaigon et al., 1998; Opstad et al., 1998). Flow dynamics also affect feed settlement rates (Appelbaum, 1989) and have, together with water exchange rates, a role in tank self-cleaning by transporting feed remains and dissolved matter out (Oca et al., 2004) and preventing areas of stagnant water in tanks from forming (Timmons et al., 1998). In circular tanks, flow patterns depend on tank size and shape, water flow-through, inlet and outlet design and aeration (Sakakura et al., 2006; Shiotani et al., 2005; Timmons et al., 1998). Potential unfavorable effects from suboptimal water flow or aeration on fish larvae are deformations, variable swim bladder inflation, stress, mechanical damage, and modified survival or growth due to altered prey catch or energy expenditure (Appelbaum, 1989; Divanach et al., 1997; MacKenzie and Kjørboe, 1995; MacKenzie and Kiørboe, 2000; Moodie et al., 1992; Opstad et al., 1998; Shiotani et al., 2005).

The optimal larval density may depend on species-specific energy demands, ontogeny and behavioural issues like intraspecific competition, feed quality and feeding protocols, spatial distribution of larvae relative to feed, physical tank environment and effects from larvae and feed on water quality (e.g. Baskerville-Bridges and Kling, 2000;

Hatziathanasiou et al., 2002). Increased larval density or survival demands adjusting feeding to avoid reduced feed availability per individual, which may alter intraspecific competition.

Feeding practices differ between species and producers in terms of feed types, sizes, the sequence of feeds applied throughout ontogeny, feeding frequencies, feed concentrations and distribution in space and time. Feeding lean on aspects of optimal foraging theory (Aksnes and Giske, 1993; Townsend and Winfield, 1985). Feed concentration and distribution affect detection and ingestion by larvae. Feed settlement rate and washout affect residence time of feed, and thus both feed concentration and tank pollution due to nutrient leaking. High rates of sinking or washout of rotifers have been observed in cod rearing, both experimentally (Busch et al., 2011) and in full-scale commercial production (Nicolaisen, O. unpublished), resulting in inadequate feed densities for survival and growth. Spatial distribution of rotifers is affected by salinity, temperature, oxygen and ammonia levels (Fielder et al., 2000; Lubzens et al., 1989; Øie and Olsen, 1993) while light may affect *Artemia* distribution (Gulbrandsen, 1996; 2003). Generally, an efficient feeding regime depends on the species and its developmental stage, feed characteristics as well as the settings of environment factors in tanks.

Tank tending methods (removal of settled feed and mortalities) may vary between tank sizes and producers, and the execution of tending may vary among staff members. Common cleaning methods are cleansing arms and manual siphoning. Efficiency of the methods depends on their quality of execution, and they may introduce bias into both research and commercial production control unless sufficient standardization and randomization is assured between tanks and participating staff members.

Fundamental components of visual environment in larval tanks are light source characteristics (intensity, spectrum), tank design (size, shape, colour) and properties of the rearing water (absorption, scattering). The visual environment interacts with prey characteristics (size, contrast, mobility, availability) and changing larval vision to govern feeding and behaviour in visually hunting fish larvae, and may affect distribution of both larvae and live feed organisms (Aksnes and Giske, 1993; Blaxter, 1975b; 1986; Downing

and Litvak, 2000; Fiksen et al., 1998; Gulbrandsen, 1996; Job and Bellwood, 2000; Monk et al., 2006; Naas et al., 1996; Tamazouzt et al., 2000; Villamizar et al., 2011b).

A general trend among many marine fish larvae is a poorly developed vision at hatch, which then develops during the larval period. Cones are found in the retina at first feeding and develop further in numbers and complexity, while rods, which support detection of movements, often appear first at metamorphosis (Blaxter, 1975a; 1986).

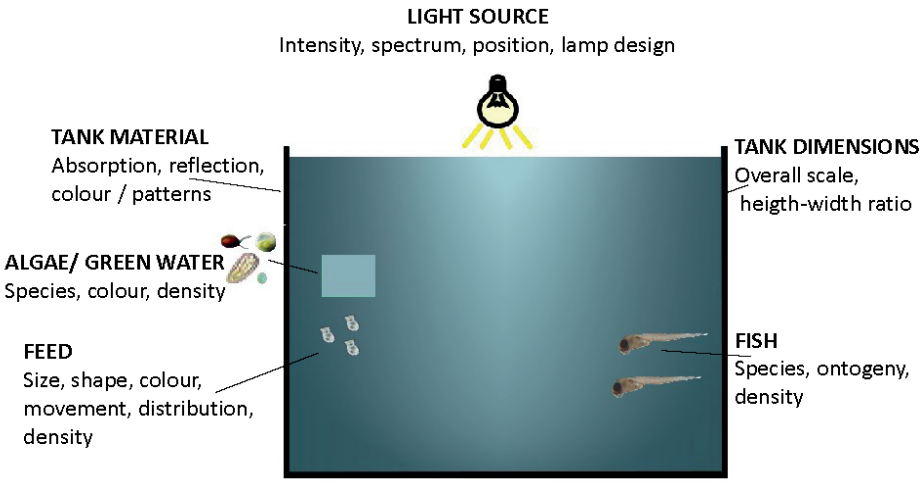

Figure 6: Factors affecting visual feed acquisition in fish larvae production

1.8. Outline of principles and issues of larval research

1.8.1. Organization of research

Effective research requires conduct in accordance with actual scope and complexity. Dependent on the motivation, aims, scale and stage of a research process, a broad range of organizational models apply, from individual, interest driven, basic "Small Science" to very large scale, cooperative, targeted, highly structured and strictly applied "Big Science" (Esparza and Yamada, 2007; Georghiou, 1998). Between these extremes, all different combinations of geographical scale, participants (individuals, enterprises, universities and institutes), size and composition of research groups, strength of the collaborations, hierarchical structure and motivation for the research occur.

Optimizing production of marine fish larvae is applied research, multifactorial, of high complexity, and generally work demanding. The solving of such situations of technical complexity generally calls for collaborative efforts and a holistic view, dealing not with discrete "bits and pieces" but with complete systems, an approach common in engineering (Bennett and Gadlin, 2012; Kell and Oliver, 2004). The collaboration structure (Paper I) was examined primarily to examine how organization and conduct support needs for structure of overall research at the area.

1.8.2. Basic principles of applied experimental research

Solid experimental research requires both sound formal and practical execution and the use of well-suited experimental design schemes. The basis of efficient design of experiments (DoE) as we know it arose with R.A. Fisher in the 1920s, who articulated core principles of experimental design; randomization, replication, blocking and factorial experiments (Fisher, 1926; Hall, 2007; Preece, 1990). While randomization serves to reduce bias, replication improves precision of parameter estimates, and enables estimates of experimental variability and performance of statistical significance tests. Blocking reduces systematic variability in experiments and improves sensitivity of statistical tests, while factorial experiments improve efficiency, reduce costs, reduce the need for replication relative to one-factor-at the time (OVAT), identify interactions (Box et al., 2005; Czitrom, 1999; Fisher, 1926; Hicks and Turner, 1999; Shaw et al., 2002), and may improve research ethics, due to effective use of experimental animals (Shaw et al., 2002).

Applied production optimization aims to move systematically within a multivariate "landscape" from a given starting point towards a set of conditions at desirable levels. Under such circumstances, different starting points complicate synthesis of collective results, and different technology, biological material (fish, live feed), nutritional issues, rearing practices and measurement methods additionally complicate interpretation. Consequently, the research process as a whole would benefit from standardizing conduct. If not so, as an absolute minimum, reporting should be clear and thorough, to identify deviations in conduct and to ensure replicability and reproducibility (Peng, 2015; Peng, 2009).

For the purpose of encouraging future improvements, attention was given both to the application of basic principles of experimentation and to standardization and reporting of conduct of studies (Paper I).

1.8.3. The sequence of applied research.

A complete working sequence for applied research optimization studies involves both initial basic descriptive / explorative research and later, systematic approaches. Early stage applied production studies may directly benefit from pure research at related fields. For larval research, this involves e.g. marine biology, ecology and descriptive morphology: Species distribution and habitat descriptions indicate the environment experienced in the wild in terms of e.g. temperature ranges and visual conditions to help set initial experimental domains for optimization. Studies of gape morphology and prey choice in the wild provide cues about size and types of food items at the various larval stages to guide constructing feeding protocols, and nutritional analysis of natural feed items may help develop live feed enrichments and commercial feeds. Once potential production issues are identified from basic research, pilot studies or work on other species, next steps involve structured studies using designed experiments at experimental scale, to closely study effects of factors in terms of larval performance and to aid later up scaling. One option at this point is the OVAT method, which focuses on one production factor at the time. A more efficient scheme for complex situations includes fractional and full factorial screening designs to select among factors for further study, sequential experimentation to find areas of improved performance (Box and Wilson, 1951; Box et al., 2005; Fisher, 1926; Mee, 2009), and ultimately more elaborated designs to reach optimal conditions (Box and Wilson, 1951; Box et al., 2005) (Figure 7).

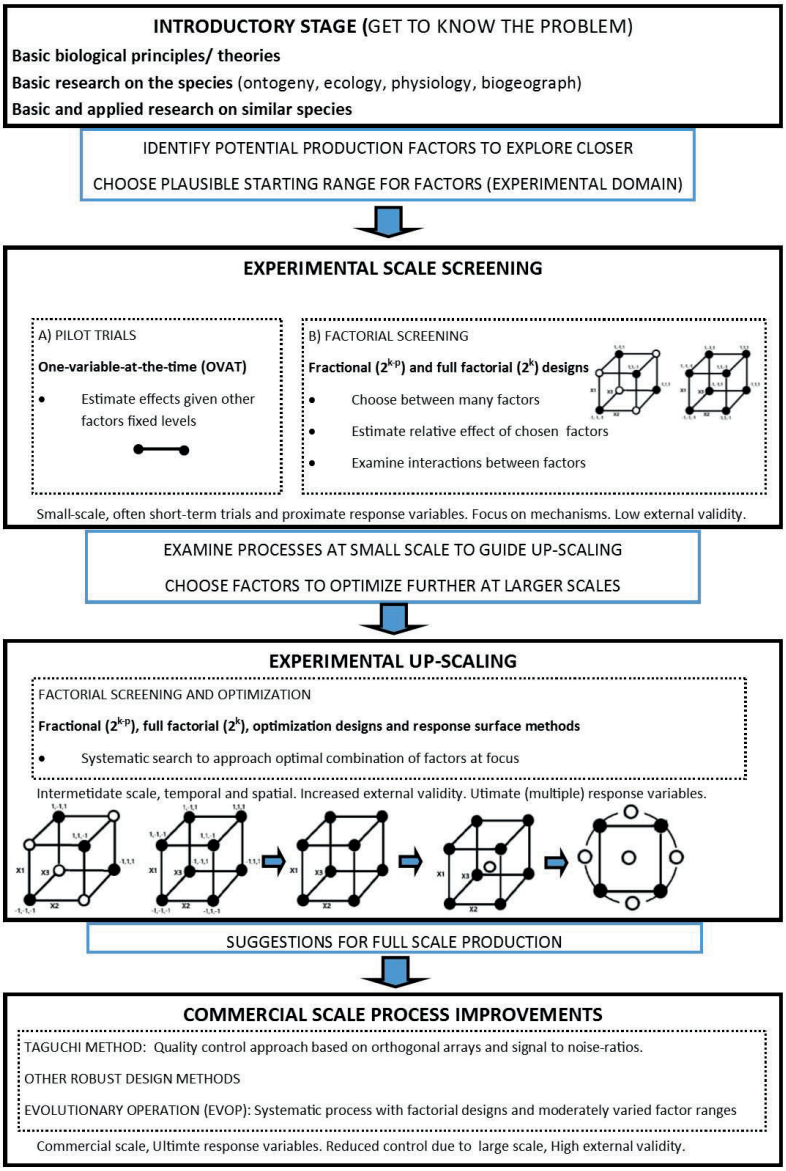

Figure 7: Tentative workflow for process optimization of marine larvae production

Based on results from such experimental procedures, a next step is upscaling towards commercially relevant duration and scale of trials. Larger scale affects heterogeneity of the tank environment and may alter optimal settings and introduce new influential factors to consider, e.g. tank design, water exchange rates and circulation patterns. The methods sketched for the purely experimental stage still apply, but studies increasingly focus directly on ultimate production outcome: growth, survival and quality.

A later development, put to use at various areas of the manufacturing industry, is the Taguchi method and related robust design methods for quality control (Antony, 2002; Montgomery, 1999; Park et al., 2006). These aim to find the levels of a set of controllable design variables (control factors) that make outcome of a process less variable - or more robust to negative effects from other, less controllable (noise) factors.

Finally, R&D is needed post commercialization to continuously improve production outcome, keep up with competitors and improve product quality and profits. Due to the obvious disadvantages of unfavorable conditions, the commercial stage places clear constraints on possible interventions with on-going production. One approach to deal with commercial productions is evolutionary operation (EVOP) (Box, 1957; Hunter and Kittrell, 1966). In short, the method makes presumed harmless planned alterations of the settings of few selected variables between production runs, which accumulate to form simple experimental designs, often factorials, for analysis.

Through the examination of research designs, focus was set on how the published research fitted into a general sequence of applied research (Article I).

1.8.4 The complicating role of larval ontogeny

To master intensive tank production from hatch to metamorphosis is often particularly challenging in fish larvae, as larval success depends on ontogeny of behaviour, size, metabolism, energy acquisition and a wide range of related structures and organs (Table 1) that develop rapidly and affect species-specific needs throughout the larval period (Blaxter, 1986; Hunt von Herbing, 2001; Job and Bellwood, 2000; Kamler, 1992; Kjørsvik et al., 2004; Leis, 2010; Zambonino Infante and Cahu, 2001).

Table 1: Ontogenetic changes in fish larvae: Functions, components and effects

FUNCTION	COMPONENTS	AFFECT	ULTIMATE EFFECTS FEED AQUISATION
LOCOMOTION	Body size, shape	Swimming performance, mode	Search range, capture
	Fins	Swimming performance	Search range, capture
	Muscles	Swimming performance	Search range, capture
	Skeleton	Swimming performance	Search range, capture
SENSING			
Vision	Cones	Colour vision, high light intensity	Search range, prey detection
	Rods	Low light intensity	Search range, prey detection
	Eye size	Visual angle and range	Search range, prey detection
Olfaction	Nasal epithelum	Detection water soluble substances	Search range, prey detection
Gustation	Taste buds	Assessment nutritional contents	Prey acceptance
Mechanoreception	Lateral line	Detection mechanical waves, movement	Search range, prey detection
	Mouth cavity	Acceptance of food texture	Ingestion
INGESTION	Jaws	Prey handling	Capture, ingestion
	Mouth	Prey handling	Capture, ingestion, prey size
	Gill rakers	Prey handling	Ingestion, prey size
	Teeth	Prey handling, pre-digestive treatment	Capture, ingestion, digestion
DIGESTION			
(Digestive system)	Gross structure	Efficiency of nutrition breakdown	Digestion, assimilation
	Volume	Food intake	Ingestion, digestion
	Digestive enzymes	Efficiency of nutrition breakdown	Digestion
	Mucosal epithelum	Transport of nutritional components	Digestion, assimilation
METABOLISM			
(Respiratory system)	Skin	Respiration	Delimits metabolism, sets O2 demand
	Gills	Respiration	Delimits metabolism, sets O2 demand
ENERGY STORAGE			
Yolk-sac	Endogenous	Access to storage energy at hatching	Timing of onset exogenous foraging
Liver	Exogenous	Access to energy accumulated post hatch	Buffer against food / nutrient deficiency

The main methodological issue arising from the complex ontogeny is that production factors rapidly change in relative importance and optimal settings over time, and should be adjusted accordingly to provide optimal rearing at any stage: Settings that work well at one stage may be suboptimal at others, which suggests optimization at different developmental stages separately. Due to its effects on behaviour, larval distribution, feeding and fish welfare, visual environment is important to fish larval rearing, so knowledge about optimal visual conditions is needed. Different species inhabit widely different environments and larvae change habitat choice and visual capacity over ontogeny. Thus, building both species- and age-specific knowledge is critical.

The works of papers II and III address the issue of ontogeny and serve as examples of factorial experimental studies of marine larvae as first steps to jointly study effects from multiple factors at different ontogenetic stages, to support further fine-tuning of production environment.

2. Aim of the study

The overall aim of this thesis was to identify, systemize and evaluate common issues experienced in larval fish production research to suggest improvements relevant to develop novel marine fish species for aquaculture or to optimize established productions. Due to the overall complexity of larval production, main focus was confined to effects from controllable in-tank production factors on production success, in terms of responses indirectly (behaviour, foraging) or directly (growth, survival) related to production. The study spanned from the examination of general issues of published larval production research to the testing of factorial experimental designs for multifactor studies and their application to examine effects from environmental factors on visual environment in tanks.

Specific sub goals of the study were:

1. To examine overall structural and methodological challenges of research on intensive marine larval production, and suggest improved strategies for such processes (Paper I).

2. To examine an alternative research design for production optimization that takes into account the complexity of factors present in larval tanks (Paper II).

3. To examine effects from multiple factors on visual environment in tanks, in terms of their influence on behavioural patterns and feed ingestion (Paper II and III).

3. Summary of papers and abstracts

Paper I

Issues of aquatic experimentation in research on marine finfish larval production in Europe and North America. https://doi.org/10.1111/raq.12204

This study focused on the suitability of established academic research practices to improve production of marine fish larvae, viewed in light of the high complexity at this research area. Focus spanned from overall research structure to in-detail investigation of study designs, methods, analyses and reporting.

Investigation of 74 studies showed limited collaboration, both nationally (22 %) and internationally (18 %), and about half were PhD or master studies. 32 institutions contributed, and studies were spread over 31 geographical locations.

Studies addressing feeding (26), light (17), temperature (11) and tank design (11) dominated. Despite many possibly interacting factors, 66 % of studies used one-variable-at-the-time (OVAT) designs, which are often inefficient in multi-factor situations.

Most studies used continuous trials, with treatments applied over an extended time. This approach mimics complete production runs but is less suited to examine in detail stage-dependent environmental demands of the larvae.

Of basic design requisites, replication was generally low and blinding rarely described. Furthermore, the many contributors and variable practices of execution, the variable levels of controlled design factors chosen, and the variability of tank-tending practices and methods for sampling and measurements, make between study comparisons and research syntheses demanding, which calls for better standardization among researchers participating at the field. Last, data analyses revealed some confusion about the choice between OVAT and factorial designs, some examples of dubious analysis of repeated measurements and variable practices in reporting both the execution and the results from analyses.

In conclusion, co-operation, standardization and more structured research should be an aim, including more efficient research designs, sound analyses and thorough reporting of designs, methods and analyses, to support replicability and reproducibility and to aid efficient peer-review of larval rearing studies.

Paper II

Nicolaisen O., Cuny, M., Bolla, S. (2014). Factorial experimental designs as tools to optimize rearing conditions of fish larvae. Aquaculture 422–423, pp. 253–260. https://doi.org/10.1016/j.aquaculture.2013.12.018

The study examined the use of factorial designs to aid optimizing Atlantic cod larval production in multifactor situations, focusing on effects from factors shaping the visual environment. We ran a short-term 2^4 factorial designs with added center points, at each of four larval ages and with the experimental factors light intensity, tank bottom colour, algae concentration and rotifer density. Foraging success was the short-term response variable.

Both light intensity, bottom colour and added algae affected illumination in tanks. Analysis pooled over all ages indicated improved effect on foraging from high feed density with age, and lowered foraging in white bottomed compared to grey and black bottomed tanks. Separate analyses at different larval ages showed changed importance of the different factors with age, but a consistent trend of increased foraging from low to higher values of light intensity, algae concentration and feed. Responses at the design midpoint exceeded expectations from a simple linear response, indicating interactions or curved effects, and there were significant interactions between bottom colour and both light and feed density at 15 DPH and between algal density and light at 20 DPH, indicating factors that jointly affect foraging and should thus be optimized together. Overall, the short-term factorial screening approach allowed identifying possible influential factors and their interactions at different ages, which will be helpful to guide further age specific optimization studies of tank environment in larval rearing.

Paper III

Nicolaisen, O, Bolla, S. (2016). Behavioural responses to visual environment in early stage Atlantic cod *Gadus morhua* L. larvae. Aquaculture research, 47 (1), 189-198. https://doi.org/10.1111/are.12481

Visual environment affects foraging and thus growth and survival in marine fish larvae. Key factors that shape visual environment in tanks were examined for their effects on

phototaxis and spatial distribution of Atlantic cod larvae. The study included two different small-scale factorial designs, each of which carried out as a series of complete factorial experiments at different larval ages between 5 and 35 DPH, to examine stage specific effects and changes over time.

In the first experiment, a 2^3 duplicated factorial design focused on effects from the intensity of incoming light, added algae and tank wall colour on phototaxis, using measures of average larval position at different factor combinations as the response variable. The positive phototaxis was weak in the youngest larvae, increased up to 27 DPH and then decreased. Stage specific differences in effects from various factors on phototaxis appeared, as well as interactions between factors.

In the second experiment, a 2^2 duplicated factorial design assessed effects from light intensity and wall colour at ages 6-28 DPH on the horizontal distribution of larvae. White tank walls induced aggregation of larva towards tank walls at all ages. No added effect due to light intensity appeared in this experiment at neither 6, 11 nor 28 DPH, and there were no interactions between light intensity and wall colour at any age. At 16 DPH, increased light intensity reduced affinity to tank walls, while at 21 DPH elevated light increased tank wall affinity. In conclusion, visual environment depends on joint effects from various aspects of rearing environment, which affect larval behaviour in different ways. Effects vary due to larval stage and may ultimately affect production outcome.

4. General discussion

4.1 Main findings and methodological issues

4.1.1. Paper I

The review of aquaculture studies addressing marine fish larvae production (Paper I) showed cooperation, both nationally and internationally, comparable to levels found in internationally coauthored studies in the Science Citation Index from year 2000 to 2011 (Wagner et al., 2015). This indicates that the observed levels of collaboration of this study are in accordance with levels of cooperation in science in general rather than being specific to this particular area of research.

Research collaboration has long been practiced and considered to be a pre in science, first in astronomy and later in biological sciences, including early collaborative projects and actions like the Challenger expedition, international congresses and committees on zoological and botanical nomenclature (Davenport, 1907) and the International Council for the Exploration of the Sea (http://www.ices.dk/explore-us/who-we-are/Pages/Our-history.aspx). Research collaboration appears at many levels, from strategic agreements between regions, nations or institutions to specific projects and informal contact among individual researchers, and includes activities like researcher exchange, workshops and networks. Dependent on the level of cooperation, its motivation spans global challenges, regional or national strategies for knowledge accumulation, competitive edge, growth and prosperity, purely commercial reasons, efficient use of public research funding, improved access to manpower and research infrastructures, and individual motivation among researchers: Idealism, interest, career, profits (Georghiou, 1998; Katz and Martin, 1997; Wagner et al., 2015).

Methodically, co-authorship is a well-established measure, used in bibliometric studies as a criterion for academic research cooperation over decades (Georghiou, 1998; Wagner et al., 2015). Weaknesses relate to unclear definitions and ranking of cooperation and co-authorships, ethics of co-authorships and blurred institutional belonging of contributors (Katz and Martin, 1997; Laudel, 2002; Marušić et al., 2011), and consequently the

disability of the method to reveal the full extent of collaboration (Laudel, 2002). Advantages include replicability and thus verifiability, relatively low costs, traceability over years and possible large sample size from database extractions (Katz and Martin, 1997; Wagner et al., 2015). In sum, considering the methodical issues, the method is a valid but partial indicator of academic research cooperation (Georghiou, 1998; Wagner et al., 2015). It is assumed to underestimate the total amount of collaborative efforts of the scientific community (Laudel, 2002), but quite accurately reveals the collaborative structure of concrete research efforts reported in experimental sciences.

The high share of PhD studies indicates that institution-internal cooperation to a great extent followed the classical hierarchic labour division between PI´s and their PhD students in academia (Freeman et al., 2001; Larivière, 2012; Laudel, 2002; Martinson, 2011), which raises questions about the actual extent of reciprocal internal collaboration taking place, despite the observed no. of internally shared publications. The high fraction of PhD projects additionally gives rise to some other issues. First, limited experience of postgraduate students makes the quality and scope of work depend on their individual supervisor's quality, aims and commitment. Second, short time available till degree completion, coupled with funding practices and economic incentives to maximize student throughput, delimits the extent and contents of work feasible during a PhD project, which may affect its quality (Cyranoski et al., 2011; Mervis, 2009) and may hinder the candidate´s participation in the systematic, long-term research processes that often characterize systematic optimization studies.

The fragmented organization with many contributing institutions, each often with a limited scope, complicates systematic optimization. Individual studies addressed one or very few factors, and their methods, experimental settings, physical arrangements, and probably technical staff competence, varied highly. Such variability complicates research synthesis, and thus ultimately affects the effectiveness of collective research carried out at the specific field.

About 23 % of examined studies were factorial designs while two thirds were OVAT designs. Furthermore, factorial designs were isolated studies, not explicitly claimed to be part of any systematic optimization processes. The predominance of OVAT designs is striking considering their general inefficiency compared to factorials.

Some claim that such exaggerated use of OVAT may be due to a lack of competence and training (Czitrom, 1999). In support of this view, recent works report of generally weak research, designs and analyses in science (Begley and Ioannidis, 2015; Ioannidis, 2005; 2014), as well as lack of competence at the graduate level (Harraway and Barker, 2005).

Additionally, today´s publishing incentive structure at governmental and institutional levels and the resulting strong competition for grants, positions, recognition, wages and careers encourage faculty to publish as many articles as possible based on relatively limited research (Binswanger, 2014; Broad, 1981; Freeman et al., 2001; Parnas, 2007; Smaldino and McElreath, 2016) over more infrequent publication summing up e.g. long-term optimization research. Early discovery studies using factorial screening are unlikely to be published (Gilmour, 2006), and may thus be less suited for academic research, with its strong incentives to maximize publication rates (Broad, 1981).

The choice of designs and project structure may also be influenced by academic funding, which increasingly includes individual PhD position grants to faculty members (Mervis, 2009). Combined with strong incentives for high throughput of students, this practice may favor simple short-term goals over systematic research.

The timeframe of this review should also be noted: Studies with focus on fundamental issues and single factor effects are often at their highest value during early day´s pilot research, to identify potentially important mechanisms and factors and suggest further direction for fine-tuning. However, data showed no signs of temporal development from initially simple designs towards more elaborate designs over the years. OVAT is often not an effective tool at late stage process optimization. At present, the comprehensive knowledge about various mechanisms of tank production, acquired over decades and for a number of species, should serve as a starting point for research on novel species as well as give direction for further work on established species. In such advanced stages of

research, it is questionable if the international collaboration reported in Paper I, based mostly on coauthoring of fragmented pieces of research, demonstrates a truly effective approach: More systematic, coordinated research should rather be aimed for.

The basic issues of formal experimental design reported were low replication of experimental units and incomplete or lacking reporting of randomization and blinding procedures. This situation is not specific to this particular area of research (e.g. Bailoo et al., 2014; Curtis et al., 2015; Holman et al., 2015). As these issues affect the quality of conclusions drawn from studies, steps should be taken to remedy the situation. In cases of flawed designs, the responsibility rests on the responsible researcher, and better training is an obvious means for improvements. Additionally, reviewers and journal editors have a part of the responsibility to remedy such issues. Low replication may also stem from reduced funding relative to originally planned in an application, which may cause reduced designs from purely economic reasons.

Practical execution varied strongly between examined studies, both between and within species. This included issues like basic production infrastructure, quality of experimental animals, settings of tank environment, tank tending and sampling, and measurement procedures. Such variability of conduct is a consequence of the high number of participants, with variable physical infrastructure, staff resources, economy, funding sources, ideas and practices. Some aspects, like tank environment settings, tending, sampling and measurement procedures are relatively easily dealt with by improved standardization within the research community. Physical design differences are more difficult to standardize, mainly due to high costs of changing technical arrangements like e.g. tank designs. Variability in research animals between studies is also more difficult to deal with, as it reflects many different sources: producers, batches, seasons, parents etc. Such variability may be important in itself, and may be estimated once designs are made comparable in other aspects. Standardizing of practical designs demands agreement upon common guidelines and baseline settings within the community. Practically, this must be a dynamic process, where baseline values are changed over time in accordance with up-dated levels of “best practices” for a given

species. Such an approach thus demands continuous commitment to and established formal instruments for standardization and cooperation.

One straight-forward approach to support increased standardization and improve designs and their replicability is thorough description of important aspects of both design and analysis, using guidelines for good reporting of all aspects of the study (see e.g. (Anon, 2013; Baker, 2016; Curran-Everett and Benos, 2004; Gerstner et al., 2017; Nosek et al., 2015; Udén et al., 2012). Another approach to standardize is establishing common "state-of-the-art" facilities for a whole research community to use. This ensures joint tank design, rearing practice and methods among studies, supports continuity of staff expertise and gives rise to effective use of expensive infrastructure. A recent attempt in this direction in European marine larvae research is the AQUAEXCEL initiative (http://aquaexcel2020.eu), which aims to provide "subsidized access to top-class aquaculture facilities and relevant services for researchers from academia and industry". Kept open for all interested parts at equal terms of participation, such an approach could be one possible way forwards.

Though, for optimization purposes, continuous focus and effort is crucial, while within the AQUAEXEL framework as it is described, each action would be just another project, competing with other academic and commercial projects for access. Thus, dedicated research infrastructure for optimization studies is to prefer, preferably also separated from everyday academic demands and control and sheltered from both the multitude of fractionate special interests, an ever-lasting nag for continuous grant collection and rigid demands for scientific reporting. Actions should of course follow a pre-set and highly structured program, flexible to adjustments within the limits of the methodical framework, and reporting of progress should be continuous. Even though not explicitly aiming for it, also this approach would provide spin-off projects for academic training and basic biology, and could offer valuable training for students in applied research.

The observed reporting issues found at all level of a study (experimental design, analysis, data description) might of course stem from all possible reasons, spanning from fraud and bad ethics via poor training and supervision to mere accidental errors. The

message here is anyhow that better focus and sufficient training, easily improve weak reporting in all cases except from fraud and poor ethics.

4.1.2. Paper II

The screening design explored effects from multiple production factors on visual rearing environment, using a short-term response measure (foraging success). It showed consistent patterns of main effects over time: Light intensity, prey density and algal concentration generally increased foraging success. It suggested time specific changes in the relative importance of factors, indicating that optimal settings relate to developmental stage. It identified one categorical variable (bottom colour) that could be fixed, and it identified potential interactions to consider in closer studies.

For this particular case, the response variable had its highest values at intermediate factor levels, indicating curved responses and presence of a "peak" of the response within the chosen experimental domain. This may occur because experimental domain settings were selected among levels applied in previous studies, which over the years have converged towards some optimum range of values – a response surface peak. Such a peak may be local or global, where a global peak represents the universal maximum over the full range of possible variable settings, while a local peak is a maximum appearing within some restricted fraction of the total possible domain, and thus may or may not be equivalent with the global peak.

The experimental approach separated experiments into discrete temporal intervals, where each complete experiment was initiated using experimental animals at a certain stage, taken from a common pool. This approach thus disentangled effects operating at particular stages from effects originating at earlier stages (temporal dependency). Such temporal effects, which typically may arise in long-term trials with repeated sampling over time, can be modelled and accounted for to still be able to compare overall treatment effects statistically, e.g. by methods like ANCOVA, MANOVA or mixed modelling. However, these approaches focus on overall effects, commonly spanning several developmental stages. They are thus not the most meaningful tools to optimize stage-specific conditions. Here, the aim was to provide empirical knowledge about

environmental effects at discrete ontogenetic stages, not to model overall effects over time. For this purpose, the relatively simple approach taken here worked well.

It should be emphasized that the foremost purpose of this study (Paper II) was to examine a novel approach for larval production environment studies, through an example focusing on how environmental factors affect foraging in rearing tanks.

The fact that this was a screening approach, run at limited spatial and temporal scale, places obvious restrictions on extrapolation of results to full scale production:

The first issue relates to external validity of controlled experimental studies in general, the extent to which settings, findings and effects from controlled studies can be generalized into broader contexts (Bracht and Glass, 1968; Campbell and Stanley, 1963). Reviews indicate that external validity as a methodological issue often has low priority in applied fields e.g. health research (Steckler and McLeroy, 2008), although for practical applications, external validity is of paramount importance. For aquaculture research, the issue typically includes generalizability from controlled experiments at experimental scale to commercial production scale settings. Tank size and shape are important factors that may directly affect visual environment, through their effects on light distribution (Naas et al., 1996) or indirectly through effects from light on the distribution of fish larvae or on their live prey (Gulbrandsen, 1996; Hee-Jin et al., 2014). Tank design solutions and scale also affect flow patterns (Moorhead, 2015; Ruttanapornvareesakul et al., 2007; Sakakura et al., 2006; Shiotani et al., 2005; Timmons et al., 1998), which may additionally affect fish larvae (Davis, 2001; Sakakura et al., 2006) and live prey distribution, foraging and ultimately production success. Thus, the closer a chosen experimental scale approaches a desired commercial scale, the higher the external validity of the study. Unlike in e.g. ecology, where external validity is generally assumed to be low in experiments, high external validity is more easily obtained in aquaculture, which *de facto* deals with a producer defined "artificial ecosystem". Thus, the closer a study is to the preferred system in all relevant aspects of scale and design, the higher its external validity *per se*. Of cause, drawbacks of full-scale studies are costs, increased work effort, and the often higher in-system variation / heterogeneity at large scales, which may blur inference about causal relationships. To conclude about the external validity of the present type of

studies, experimental scale studies foremost apply to understand mechanisms and give insight which can then be used to guide production optimization at commercially more relevant scales. At very small scales, they are not necessarily suited to establish estimates of production settings for direct transfer to commercial production scale.

The second issue relates to the inclusion of potentially influential factors: Ideally, all such factors should be included at the earliest screening stages, to examine which factors are the most influential and which might be omitted in further studies. In this particular case of visual environment, potentially important factors like e.g. wavelength (Villamizar et al., 2011b), positioning of the light source (Gulbrandsen et al., 1996) and differences in visual appearance of feed types, could be included. Failing to do so would of course delimit any conclusions about the left-out variables.

Third, expansion of study designs is a natural follow-up of screening studies, to improve reliability of parameter estimates and to approach optimal conditions. This includes a typical pattern scheduled in DoE literature, used with success for a wide range of applied commercial settings. improved replication, e.g. by blocking, fixing of categorical factors at their best levels, deleting of unimportant factors, moving the experimental domain systematically towards areas close to optimal solutions, and finally examining these areas more closely by optimization designs (Box et al., 2005; Hicks and Turner, 1999).

The response variable used (average no. of prey eaten at the various treatment combinations) reflects suitability of the visual environment to support foraging, and is thus a quantitative measure of successful feeding behaviour in a given environment. Studies that include quantification of various aspects of fish larvae behaviour, often in combination with measures of growth and survival, have a long history and give valuable contributions to guide feeding strategies and rearing protocols in larviculture (Brown et al., 1997). Studies include e.g. herring (Blaxter, 1968), European sea bass (Georgalas et al., 2007), Atlantic cod (Hunt von Herbing and Gallager, 2000; Monk et al., 2006; 2008; Munk, 1995; Puvanendran et al., 2002; Skiftesvik and Huse, 1987) and Atlantic halibut (Naas et al., 1992), and often use direct estimates of sub-components of behaviour obtained from observational techniques (Altmann, 1974), e.g. (Puvanendran et al., 2002; Villamizar et al., 2011a). The response variable of the present study can be viewed as

intermediate in a continuum from purely behavioural actions to ultimate measures like growth and survival. Foraging measured as actual feed intake may thus be regarded a more accurate measure of foraging success than e.g. “observed attacks”, which despite an observed attempt to feed in the end may eventually fail.

The short-term focus on feed intake in the present study foremost relates to the capability of larvae to forage visually in the given environment. As favorable visual conditions ease access to available food, this should increase feed intake or reduce energy needed for foraging activities, both of which may ultimately positively affect growth and overall production. Some researchers though hold short-term measures to be an oversimplification of the totality of mechanisms involved in larval foraging, and point out that short-term ingestion rates do not necessarily represent final outcome. First, optimal foraging theory assumes a response on prey consumption from changed prey density; the functional response (Holling, 1959; 1965). Planktivorous fish often display Holling type II type response, (Houde and Schekter, 1980), where ingestion rates first increase at low prey densities and then level out at elevated prey concentrations. It is generally recognized that fish larvae demand prey densities above some level to feed effectively, but it is also proposed that at high prey levels and continuous feeding, undigested feed may accumulate, gut evacuation speed up and digestive efficiency decrease (Rabe and Brown, 2000; Tilseth and Ellertsen, 1984). In consequence, optimizing of feeding protocols should include both prey density, frequency and possibly diurnal patterns of feeding, and pulse feeding has thus been suggested an alternative to continuous schemes (Lambert and Dutil, 2001; Rabe and Brown, 2000).

Another issue when moving from short-term to longer term studies is the increased need to control actual feed density. There are indications that long-term studies and commercial productions may suffer from low live prey densities and thus sub-optimal foraging, due to rapid washout or sedimentation of feed. One long-term feeding trial showed high rotifer sinking rates in 40 L tanks (Busch et al., 2011). Another trial, using 160 L tanks, showed up to 50 % prey density decrease in 3.5 hours, while at commercial scale, average density reduction of 36 % over four hrs. was seen (Nicolaisen, O., unpublished data). Additionally, the reporting of live feeding is sparse in many studies

(Paper I) in terms of both feed density and feeding frequencies. Viewed in this context, the short duration of the present study should give relatively stable prey density throughout the trials. In general in live feeding, in addition to focus on visual environment there should be more focus on feeding densities and frequencies, to explore their relationships with tank design/ size, water flow and live feed quality, and to examine how this ultimately affects larval growth and survival.

4.1.3. Paper III

The focus of this study was the behavioural consequences of the visual environment experienced by larvae at different developmental stages. The first experiment, focusing on phototaxis, showed increased attraction towards the light source with age. The contribution of the experimental factors light intensity, micro algae and wall colour changed with age, indicating that fine-tuning of visual environment is stage specific and should be carried out accordingly. The second experiment showed strong horizontal attraction of larvae towards white tank walls as compared to black, suggesting that care should be taken when choosing tank wall colours.

Phototaxis and walling behaviour may profoundly affect fish. Phototactic behaviour may change access to feed by concentrating larvae to certain areas. In e.g. Walleye Pollack (*Theragra chalcogramma*), starving larvae swam horizontally towards light from 4-6 dph (Olla and Davis, 1992), and such positive phototaxis is observed in other species as well (Blaxter, 1968; 1969; Naas and Mangor-Jensen, 1990; Olla and Davis, 1992), indicating the potential of light to generally affect larval fish distribution.

It has been suggested that walling behaviour physically damages larvae of several species. For both Atlantic halibut and striped trumpeter (*Latis lineata*) larvae it has been proposed that walling may cause deformities (Battaglene and Cobcroft, 2007; Cobcroft and Battaglene, 2009; Morrison and MacDonald, 1995). In sea bream (*Sparus aurata*) a "wall syndrome" is observed in the youngest larvae (Chatain et al., 1991), while in juvenile Pacific Bluefin tuna (*Thunnus orientalis*), serious effects from visual environment on survival under transport are reported (Okada et al., 2013).

Other factors that affect visual tank environment should be included in future studies, e.g. light/ dark cycles and wavelengths of light. Both European sea bass, sole and cod

larvae seem to perform better under blue bandwidth light (Villamizar et al., 2011a; Villamizar et al., 2011b). In sea bass blue light gave a homogenous distribution while red light reduced swimming and feeding and white light concentrated larvae at corners and tank walls (Villamizar et al., 2011a), as also seen in the second experiment of Paper III.

Also other environment factors, like prey distribution (Gulbrandsen, 2003), water flow (Davis, 2001; Gaignon et al., 1998; Timmons et al., 1998), aeration (Sakakura et al., 2006), larval density (Baskerville-Bridges and Kling, 2000) and temperature (Hunt von Herbing, 2002) may affect larval behaviour, distribution or ultimate outcome and should be considered relative to larval stage. E.g. in seven-band grouper (*Epinephelus septemfasciatus)* reared in large tanks, distribution of the smallest larvae seemed governed by water flow, while distribution of larger, actively moving larvae was associated with light intensity (Sakakura et al., 2006).

4.2 Implications for future research

4.2.1. General methodical issues

Observed issues of scientific conduct (Paper I) suggest a number of developments to improve larval research. These include improved quality and standardization of conduct, methods and reporting, and improved collaboration among the participating research communities and researchers at the specific area.

Improving skills in basic principles of good research, including design, analysis and reporting, would primarily be a task for the academic educational system.

The often species specific issues in larval research suggest standardization of conduct and methods to take place at the species level: A wide range of differences between species, like morphology, development, behaviour and ecological constraints, decide plausible tank solutions, feeding schemes, the duration of trials and tank environment for the different species. Some issues of conduct are rarely explicitly addressed and poorly standardized (Paper I), particularly the issues of representative sampling in tanks and the choice of suitable scales of conduct. Sampling issues can be expected to increase with scale and increased within-tank heterogeneity, and should be more thoroughly looked into. Tank size/ design should be chosen based on the aim of studies and the intended

levels of generalization one wants to draw from results. Few works explicitly address the design of tanks for research (Devauchelle et al., 1986; Harboe et al., 1998; Kolkovski et al., 2004; Moodie et al., 1992; Moorhead, 2015; Ruttanapornvareesakul et al., 2007) or mass production (Olesen and Minck, 1983; Opstad et al., 1998) of various species of fish larvae, and still the rule in aquaculture research seems to be that individual studies use what is available and practical. Both these two issues and other issues of practical conduct are best dealt with by binding agreements within the research community to ensure valid comparisons between studies.

4.2.2. Relationships between tank environment, foraging, nutrition and feed

It should be stressed that optimizing foraging behaviour is just one side of foraging success: Successful energy intake for growth in visual feeders relies on a combination of visual environment for foraging, balanced nutritional contents of feed, other feed properties (size and shape, acceptance, digestibility, hardness, sinking), feed availability (density, distribution, feeding frequency) as well as other factors that may affect overall energy budget or behaviour, e.g. temperature, water flow patterns and larval density.

A functional environment for foraging at any given larval stage is a prerequisite to study larval foraging capacity and behaviour, to test feed acceptance between different feed types or to carry out nutritional studies. It should be noted that feeds of different shapes, colours or physical properties likely require different foraging environments to perform optimally. (Kolkovski et al., 2009) stress the importance of standardized designs in nutritional studies, including optimal feeding conditions, close-to-commercial conditions and clearly defined controls diets/ feeding protocols. Specifically for live feed, rotifer diet and culture conditions vary between commercial hatcheries, which affects their quality and composition (Hamre, 2016). It is shown that production related variation in rotifer nutritional content can be large enough to affect larval rearing success during the first feeding stages (Dhert, 2001). Such variation in live feed quality may be one cause of the variable success and predictability often seen in fish larvae production.

Establishing a foraging regime is probably a system specific issue, dependent on combined effects from tank design, water inlet/ outlet settings, aeration, and feed properties. It has also hypothesized that different water temperatures may affect larval swimming due to temperature related changes in water viscosity (Hunt von Herbing, 2002). This implies that the fate of dry feed may also be affected by temperature in terms of changed feed residence time/ sinking rate. Additionally, there are possible effects observed in rotifers in response to both temperature and salinity (Fielder et al., 2000).

4.2.3. General study design issues

To ensure efficiency of the overall research process, a consistent, well-structured approach is needed. Here, such an approach, from basic research and pilot studies to production scale R&D, is suggested. Basic research is always needed to identify potential issues and factors of interest for a new candidate aquaculture species. The short term, small-scale screening studies of larval foraging and behaviour (Papers II and III) are such early stage studies, addressing how production factors affect short-term responses, aiming to build knowledge to guide fine-tuning before up-scaling to commercial facilities proceeds. Natural follow-up steps include closer examination of relationships between the identified important factors; determination of their relative importance, interactions and optimal settings, still at the experimental scale. At this stage, generally accepted principles of experimental design apply, including the use of fractional and full factorial screening designs, the fixing of categorical factors at their “best levels”, the sequential search for better solutions and finally the use of response surface designs to arrive at the best possible solutions.

Following such initial steps, a natural next step is to up-scale towards production scale R&D, which due to its increased volume and temporal scale increases costs and labour. Rearing tanks of larger scale result in more heterogeneous tank environment, which challenges sampling and adds uncontrolled variability and may thus demand increased replication to reach reliable conclusions. With larger scale, there will also be a change of response variables towards measures of ultimate production success: Growth, survival

and aspects of quality increasingly becomes the dominant response variables, which demands longer duration of trials to detect effects.

As experiments at intermediate to full scale are demanding in terms of both workload and access to sufficient no. of tanks and biological material (eggs, larvae), systematic research at this stage could benefit from replication in blocks (Box et al., 2005; Hicks and Turner, 1999) to fill requirements for sufficient replication. A typical block would thus constitute a partial or complete experimental design run during the same production cycle, which might then be expanded or repeated in subsequent blocks. In such cases, varying larval quality between repeated cycles due to variable broodstock or egg quality between suppliers, brood fish, batches, seasons or generations of fish in a breeding program, would add to the variability between blocks, which could be dealt with and analyzed statistically. Dependent on the chosen tank sizes one could still at this point carry out experiments addressing specific ontogenetic stages by applying larvae from common homogenous source populations, to avoid the generation of random variability prior to trials and the temporal dependency due to repeated measurements (Paper II). A great advantage of controlled intermediate to large-scale R&D approaches is that given sufficient available resources, follow-up and thoroughness of execution, it exceeds what is feasible in routine commercial productions.

As full commercial scale is approached, systematic engineering approaches from manufacturing and process industry optimization might come into use, e.g. evolutionary operation (EVOP) (Box, 1957; Hunter and Kittrell, 1966) and Taguchi designs or other variants of robust design (Hicks and Turner, 1999; Montgomery, 1999; Park et al., 2006; Rao et al., 2008). Such approaches still build on basic experimental design principles, but are adjusted to address production settings and goals: The Taguchi method is foremost a quality control approach aiming to find the best operating conditions to minimize variability of product quality around defined target values, given presence of uncontrolled “noise variables”. The EVOP approach is a strategy to optimize ongoing processes built on classical DoE principles. It methodically varies process parameters within acceptable limits for ongoing production, usually using factorial designs and repeating a complete design in cycles to achieve sufficient replication for subsequent

analysis (Box, 1957; Hicks and Turner, 1999; Hunter and Kittrell, 1966). Lately a derivative from Precision Livestock farming (PLF), a method to improve livestock production, was proposed for fish production in large sea cages, named Precision Fish Farming (PFF) (Føre et al., 2018). The method suggests real-time observations in cages using advanced censoring techniques to produce data from fish (observation phase) which is then interpreted and used as basis to make decisions and take actions. While the method was explicitly suggested for sea cage on-growth, aspects of this concept might also be interesting in larval rearing. There is still a lack of knowledge about tank dynamics in intermediate to large larval tanks in terms of e.g. water flow, prey/ feed density and light characteristics; how these factors interact with actual tank design and how they affect distribution, growth and behaviour of larvae of the specific species. Such issues could be examined by using new censoring technology. For manual measurements, sensors are available for underwater light measurement (intensity and wavelength), flow velocity (Sakakura et al., 2006) and various water quality parameters, and camera based sensors for rotifer density in tanks have been developed (Alver et al., 2007). Combined with sensors to assess larval size, density and behaviour throughout the water volume, such an approach could strongly contribute to increase knowledge about tank dynamics throughout a production cycle.

The application of these concepts of process optimization should be further explored for fish larvae production, separately or in combination, to evaluate their efficiency and eventually to adjust methods to closer fit with the specific challenges experienced in larviculture.

Continuous improvements are needed also at the purely commercial stage. From a commercial viewpoint in a competitive market, continuous improvements of production methods for established species, at both the enterprise and whole sector level, are necessary to stay competitive in business. At this stage, the methods outlined for large-scale R&D are still applicable, often in an even more conservative, less risk-taking way, with variable settings kept well within acceptable levels for production. Despite of their general usefulness, no examples of their use in commercial aquaculture have been found. Such limited use of both classical DoE techniques and robust designs is found also in e.g.

UK manufacturing, which has been suggested to rest upon a combination of weak training, management based and cultural barriers in the private industry, and communicational barriers between academia and industry (Antony, 2002).

5. Conclusions

Successful aquaculture production is based on established principles of basic biology; physiology (energy budgets), developmental biology and ecological theory (principles of visual predation/ optimal foraging/ prey selection). In aquaculture, the principles apply within a simplified, manipulated environment, with producer defined life history goals and in environments aiming to support growth and flesh production for human consumption.

It has been claimed that "In studies aimed to optimize egg incubation and larval rearing, morphometric, histological, physiological or bioenergetics studies may inform about causes of variable growth and survival, and may thus be a more fruitful and less expensive approach than blindly manipulating numerous variables in search of optimal combinations" (Kamler, 1992). Here I claim that despite of obvious advantages of basic, detailed knowledge about an organism at focus, there will still be a need to manipulate numerous variables in search of optimal combinations. The question is rather how to do these manipulations systematically to ensure that the "bits and pieces" of research end up as an effective research program as a whole. One way to achieve this is to apply existing experimental design and process optimization principles, adjusted for specific aquaculture related issues.

Better standardization of conduct among researchers is also necessary, to ease the synthesis of results. Due to the large inter-specific differences, such tasks should probably apply to groups of similar species or to individual species. The discussion includes the question about how to optimally organize research and ensure efficiency. Should it be left to individual academic researchers who define limited projects that support master and PhD theses at their universities? Are today´s large international joint projects initiated by many independent partners better? Or are large, highly

structured programs focusing on specific species and run at dedicated research institutes with sufficient technical infrastructure, staff and funding better suited to break the codes for successfully establishing juvenile production of novel candidate marine fish species for aquaculture?

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Paper II

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Factorial experimental designs as tools to optimize rearing conditions of fish larvae

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ABSTRACT

The objective of this study was to test short-term factorial designs to generate detailed knowledge about environmental demands of marine fish larvae, in order to optimize rearing conditions. The joint effects of the factors light intensity, tank bottom colour, microalgae addition and prey density were tested on foraging success in Atlantic cod (*Gadus morhua*) larvae at 5, 10, 15 and 20 days post hatch (dph), using independent 2^4 factorial short-term screening designs. The larval response to environmental factors changed with age. White tank bottoms negatively affected foraging at all ages, as compared to black and grey bottoms. Additional microalgae affected foraging at 5 dph, but then this effect vanished until day 20 dph. At 15 dph both light, bottom colour and prey density jointly affected foraging, and at 20 dph, an effect from prey density as well as an interaction between light intensity and algal density was observed. The results indicate that grey tank bottom colour is advantageous for cod larvae, and that microalgae addition may not be necessary beyond the first week of feeding. The factorial design approach was discussed in relation to the traditional one-variable-at-a-time (OVAT) approach commonly applied in studies of larval rearing. Our approach identified both interaction structure between experimental factors and stage-dependency of responses to rearing environment, not generally highlighted in OVAT designs. This suggests that short-term factorial designs are useful tools for future optimization of production of fish larvae.

1. Introduction

In marine aquaculture, juvenile production is a biological and economical bottleneck due to high mortality and frequent deformities at early stages related to suboptimal husbandry conditions (Chatain, 1997; Rosenlund and Skretting, 2006). In tanks, marine fish larvae face a complex range of physical, chemical and biotic factors as well as nutritional issues that may affect production (Kjesbu et al., 2006; Rosenlund and Halldorsson, 2007), so efficient ways to improve and optimize the multivariate tank environment are needed.

Studies of domestication of new species traditionally follow a one-variable-at-a-time (OVAT) approach (Fontaine et al., 2012), studying factors one by one, and keeping other factors controlled. In contrast, factorial designs consider effects from multiple factors simultaneously (Box et al., 2005; Fisher, 1926), and are capable to identify both interactions and optimal combinations of multiple factors. They also have an advantage over OVAT to reduce replication and costs needed for a certain level of precision in effect estimates.

Traditional experiments often last for several weeks from hatching to metamorphosis, a time period associated with rapid ontogenetic changes (Blaxter, 1986; Hunt von Herbing, 2001) and thus changed environmental demands. Treatment effects are typically assessed by repeated post-hoc tests at defined points of time. An issue arising from this approach is that once between-group differences are established, they accumulate and cause temporal dependency that may compromise validity of later comparisons. An alternative approach includes series of independent short-term experiments at chosen larval sizes, using appropriate short-term response variables. Each separate experiment then includes larvae at similar size and developmental stage, reared under identical conditions, and thus provides information unique to the specific larval size range studied.

We wanted to examine if factorial short-term experiments constitute a suitable alternative to the traditional long-term studies applied in larval fish rearing. A factorial approach was applied to examine simultaneously effects from four factors that shape the visual environment of tanks using larval cod *(Gadus morhua)* as a model species. As most marine larvae are obligate visual feeders/hunters (Blaxter, 1986) with poorly developed vision at hatch, visual environment is of key importance for prey visibility and foraging, and subsequently affects growth and survival. Factors affecting visual environment in tanks have been studied both in general (Naas et al., 1996) and specifically for various marine fish species (Downing and Litvak, 2000; Naas et al., 1992; Ostrowski, 1989; Rotllant et al., 2003). Effects from factors of visual environment on cod larvae in intensive aquaculture production have predominantly been studied by traditional OVAT long-term studies, e.g. light intensity (Monk et al., 2006; Puvanendran and Brown, 2002),

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tank colour (Monk et al., 2008), prey density (Puvanendran and Brown, 1999; Puvanendran et al., 2002) and added microalgae (van der Meeren, 1991; van der Meeren et al., 2007). Though, the latter was a 2-way factorial long-term study.

The objective of this study was to assess the potential of short-term factorial designs to generate detailed knowledge about environmental demands of marine fish larvae, in order to later optimize rearing conditions. We studied joint effects of the following factors: light intensity, tank bottom colour, microalgae addition and prey density, by applying factorial short-term screening experiments at larval ages 5, 10, 15 and 20 days post hatch (dph). Cod was the model species and rotifer ingestion was the short-term response variable.

2. Material and methods

2.1. Larval rearing

Fertilized eggs from batch spawning brood stock were collected at Norwegian Cod Breeding Centre, Tromsø, Norway and incubated at 4.4 ± 0.1 °C. At 35.2 °D, eggs were transported by plane to the University of Nordland, disinfected (400 ppm glutar aldehyde in seawater for 5 minutes) and incubated until hatch in black, conical bottom 270-L incubators at 6.3 ± 0.4 °C, with gentle aeration and 5 water exchanges per day. Hatching was defined as the day when 50% of larvae had hatched (0 dph). At 1 dph larvae were transferred to a 100-L cylindrical black holding tank at a density of 100/L. Sea water filtered to 5 μm was supplied at rates of 4.5 exchanges per day from 1 to 5 dph, and 8 exchanges from 6 to 20 DPH. Larvae were fed rotifers cultivated on Super fresh Chlorella SV12 (Pacific Trading- Aquaculture Ltd, Ireland) and enriched with 0.4 mg L^{-1} Multigain/PhosphoNorse at a 70:30 weight ratio. Larvae were fed three times a day at a density of 10 rotifers mL^{-1}. For green water, algal paste (Instant Algae Nanno 3600 ®, Reed Mariculture Inc.), was used at a density of 1 million cells mL^{-1}. Gentle aeration was applied centrally in the tank, and light intensity at the surface was set at 600 lx with photoperiod 24:0 L:D. Water temperature was raised from 6.7 to 10 °C over the first 5 days, and then maintained at 10.6 ± 0.6 °C over the remaining 14 days. The tank was daily cleaned.

2.2. Execution of larval trials

Independent short-term experiments were carried out at 5, 10, 15 and 20 dph to evenly span a rotifer feeding period commonly used for cod larvae. All four experiments were executed in a temperature controlled room (10 °C). Experimental units were black, approximately cylindrical shaped PVC tanks with total volume 12 L, depth 0.25 m, upper diameter 0.29 m and lower diameter 0.20 m. Tanks were arranged in two rows of ten, and shielded from light from neighbouring units by black partitions. Two days before trials, 10 L of aerated and filtered sea water was added to each of the gently aerated tanks, allowing water temperature to adjust to 10 °C. Distribution of treatment combinations was randomized, and bottom colour and intensity of light sources (lx at the surface) adjusted accordingly. The day before each trial, ≈800 larvae were sampled from the holding tank. 30 larvae were transferred 10 at a time to each of 20 seawater filled beakers, and then distributed at random to the tanks. Larvae were kept unfed in darkness over night (18–20 h) to empty the gut before onset of the trial on the next morning.

Preset light sources were turned on at onset of trials, and algal paste and rotifers distributed to tanks according to the experimental design. Each trial lasted for 5 h, based on a pilot study performed on the extreme settings of the experimental domain, indicating that this time span is suited to reveal short-term difference in foraging (Nicolaisen, unpublished). At termination light was turned off to prevent further foraging. Tanks were sampled one by one in a random sequence. All larvae from each tank were gently poured into a wide, light bottomed container, and 10 larvae transferred to a small beaker with a pipette. Excess water was removed and larvae killed by an overdose of MS 222, fixated in 4% buffered formalin and stored in 1.5 ml Eppendorf tubes at 4 °C for maximum four weeks. At examination, larvae were photographed and dissected under an Olympus SZX 12 stereo microscope equipped with Cell ^{A} software (Soft Imaging system GmbH). The guts were dissected and the number of rotifers counted. Standard length (SL) to the nearest 0.1 mm was obtained on fixated larvae from photographs using Cell A and was used for statistical analyses. Estimates of fresh standard length at the different ages was obtained by correcting for fixation effects based on SL measured on fresh larvae from three replicate tanks ($n = 45$), produced simultaneously and with identical protocol (Lanes et al., 2012).

2.3. Experimental design of larval trials

The general design principle compared to OVAT designs is illustrated in Fig. 1. All four experiments were identically designed as 2^4 factorial screening designs, replicated ($n_0 = 4$) in the added centre point (Table 1). In full factorial designs, all levels of each factor are combined with all levels of every other factor included in the experiment (Hicks and Turner, 1999). This allows assessing both main effects and interactions. Estimates of error variance are model dependent and achievable by assuming a model less than the full factorial model prior to analysis (Mee, 2009). As this was a screening study, searching for influential factors for more elaborate studies, 2nd Order and higher interactions were kept out from the model. This gave four main effects and six 1st Order interactions to be estimated, and left sufficient degrees of freedom to perform F-tests on model terms. The inclusion of centre points applies to cases where replication is costly or work demanding, and their main purpose is to increase overall replication and check for curvature (non-linearity) in responses (Esbensen, 2006). Our strategy left us with 20 tanks ($2^4 + 4$), as compared to 36 ($2\times(2^4) + 4$) if the whole 2^4 design was to be duplicated. The experimental domain (Table 1) was set to closely resemble factor levels as suggested from recent research (Brown et al., 2003; Puvanendran and Brown, 2002) and established production protocols. The commercial algae paste Instant Algae Nanno 3600 ®, Reed Mariculture Inc., USA, was used for green water. The tank bottom colour —originally dark —was adjusted to grey and white by circular plastic plates placed on the bottom. Grey bottom was estimated as the mean of averaged CMYK colour readings from black and white bottoms, obtained from 10 randomly chosen 5 × 5 pixel areas on photographs of tank bottoms using Adobe Photoshop CS4. Mean CMYK readings were 34, 35, 46 and 21, respectively.

2.4. Effects of surface light intensity, algae and bottom colour on illumination in tanks

As a follow-up experiment to the larval trials, the combined effects of light, bottom colour and algae on illumination in tanks were examined, using a duplicated 2^3 factorial design with $n_0 = 4$ additional centre points. The experiment was run under exactly the same conditions as in the larval trial. A LI-193 Spherical Quantum Sensor measuring photosynthetically active radiation (PAR) in the range 400–700 nm wavebands (μmol s^{-1} m^{-2}) was mounted through the tank bottom, and connected to a LI-1400 Data Logger (LI-COR Biosciences). Tanks were filled with filtered sea water, and experimental runs assigned at completely randomized order.

2.5. Data analysis

2.5.1. Larval trials

The response variable was the average number of prey eaten by feeding larvae in each tank, excluding non-feeding larvae. To assess overall effect from age and experimental factors, pooled data over all ages was analyzed by stepwise analysis of covariance (ANCOVA), allowing for 2-way interactions. At this point there was no a priori

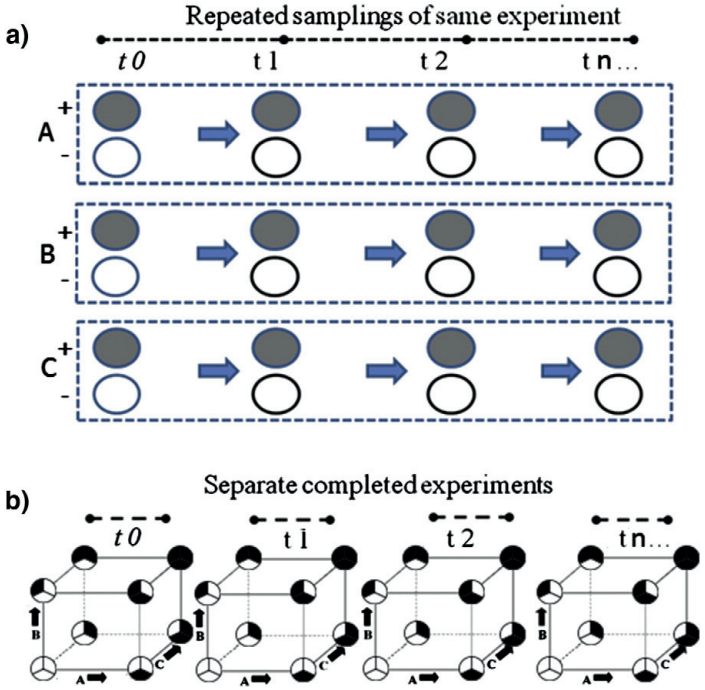

Fig. 1. Design principles of a) a traditional one-variable-at-a-time (OVAT) approach and b) short term factorial designs. a) Each experiment includes only one of the factors A-C, and experimental units are repeatedly sampled at defined time intervals. b) Shows separate complete 2^3 factorial experiments at different times, each including all factors A–C. Factor level combinations are indicated by the fill pattern of circles: The lower, left and right circle sectors correspond to levels of factors A, B and C respectively. Empty sectors indicate low factor level, while filled sectors indicate high level.

information about the quantitative aspects of bottom colour, so this factor was included as a categorical variable, with age, prey density, light and algal concentration as continuous covariates. Probabilities for entry and removal were set at $P = 0.2$ and $P = 0.25$, respectively. Model assumptions were checked by graphical examination of residuals. Effects at specific ages were analyzed by ANCOVA, allowing for two-way interactions. As this was a first screening, the usually accepted levels of significance ($\alpha = 0.05$) were relaxed, and an upper α-level of 0.15 accepted. ANCOVA was run with XLSTAT 2010, Addinsoft (Paris, France), data analysis and statistical software for Microsoft Excel.

Table 1
The structure of experimental design in the larval experiments, showing the design matrix coded in standard order and the factor levels in original units.

Coded standard order				Factor levels in original units			
Light	Algae	Prey	Bottom	Light (lx)	Algae (10^6 cells mL^{-1})	Prey mL^{-1}	Bottom
−	−	−	−	100	0.5	5	Black
+	−	−	−	1200	0.5	5	Black
−	+	−	−	100	2	5	Black
+	+	−	−	1200	2	5	Black
−	−	+	−	100	0.5	20	Black
+	−	+	−	1200	0.5	20	Black
−	+	+	−	100	2	20	Black
+	+	+	−	1200	2	20	Black
−	−	−	+	100	0.5	5	White
+	−	−	+	1200	0.5	5	White
−	+	−	+	100	2	5	White
+	+	−	+	1200	2	5	White
−	−	+	+	100	0.5	20	White
+	−	+	+	1200	0.5	20	White
−	+	+	+	100	2	20	White
+	+	+	+	1200	2	20	White
0	0	0	0	650	1.25	12.5	Grey

Plus and minus signs indicate the high and low factor levels respectively, following "Yates order" (Box et al., 2005). Additionally, the replicated center point ($n = 4$) is indicated by 0´es, indicating intermediate values of all factors. For analytical purposes in these particular experiments, bottom colour was considered a categorical variable, and thus does not strictly fit into the Yates coding order, but is included to visualize the design structure.

Table 2
Factorial analysis summary table: Effect from light intensity, bottom colour and algae on light illumination by the tank floor.

Source	Type III SS	df	MS	F	P	Parameter Estimates	
						Slope	SE
Intercept						4.00	0.11
Light	127.07	1	127.07	618.03	<0.001	2.81	0.11
Algae	1.04	1	1.04	5.04	0.046	−0.25	0.11
Bottom colour	5.12	1	5.12	24.90	<0.001	0.57	0.11
Light×Bottom colour	2.62	1	2.62	12.73	0.004	0.40	0.11
Light×Algae	0.551	1	0.55	2.68	0.130	−0.19	0.11
Bottom colour×Algae	1.11	1	1.11	5.39	0.040	−0.26	0.11
Bottom colour×Algae×Light	0.01	1	0.03	0.876	0.02	0.02	0.11
Curvature	2.625	1	2.625	12.76	0.004	0.91	0.25
Error	2.26	11	0.206				

Response variable: Light (μmol s^{-1} m^{-2}), $R^2 = 0.97$.

Shapiro-Wilks tests and Levene-tests for testing assumptions of normality and homogeneity of variance, as well as correlation analyses, were carried out using the statistical package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Figures were produced using Minitab 16 ©2010 Minitab Inc., Adobe Photoshop CS4 and the Microsoft software Paint and Office PowerPoint 2007.

2.5.2. Effect of surface light intensity, algae and bottom colour on illumination in tanks

Prior to this follow-up experiment, preliminary measurements (Hanna HI 97500 luxmeter) showed no significant difference in light reflected to the tank surface between the average value of white and black bottoms pooled (0.016 lx) and values from grey bottoms (0.015 lx, SD = 0.003, n = 15, one sample *t*-test, two-tailed, t = −1.769, df = 14, P = 0.099), indicating that grey could be considered a quantitative midpoint. Thus, effect of light, algae and bottom colour on light intensity at the tank bottom was analysed as a full factorial duplicated 2^3 design with centre points (n = 4). Analysis was performed on the coded design matrix, assuming continuous variables and equal spacing between colour values. Factors were coded in ascending order (−1, 0, 1) from low to high values of light and algae, and from dark to white bottom, respectively. The significance level was set at

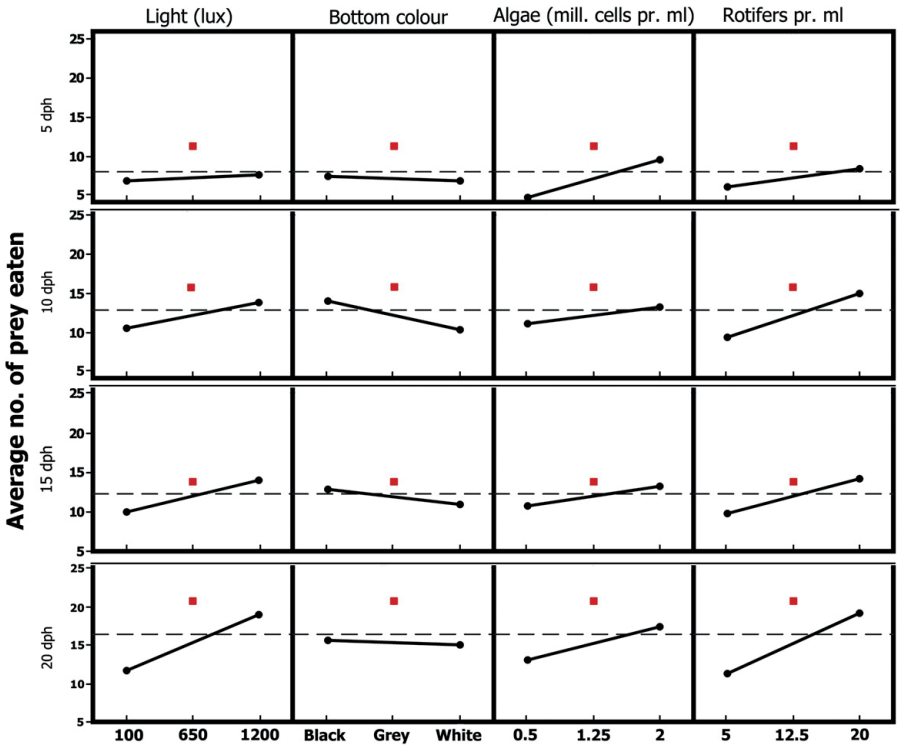

Fig. 2. Main effect plots for the experimental factors light, bottom colour, algal density and rotifer density at the larval ages 5, 10, 15 and 20 dph.

Table 3
Model summary from stepwise ANCOVA at the different larval ages.

			ANCOVA table			
Age	Model formula	Source	SS_{III}	df	F	p
5dph	$Y = 3.9 + 3.2\ A$					
		A	94.25	1, 18	2.75	**0.114**
10 dph	$Y = 10.9 + 0.0065\ LB$					
		LB	204.01	3, 18	1.23	0.327
15 dph	$Y = 3.8 - 3.4 \times 10^{-3}\ L + 9.9 \times 10^{-3}\ LB + 16.1\ AB + 1.2\ FB$					
		L	11.79	1, 15	0.66	0.482
		LB	160.02	1, 15	9.01	0.009*
		AB	28.04	2, 15	0.79	0.472
		FB	812.17	2, 15	22.87	<<0.001*
20 dph	$Y = 4.3 + 0.5\ F + 5.4 \times 10^{-3}\ LA + 4.4\ AB$					
		F	252.08	1, 16	5.64	0.030*
		LA	330.32	1, 16	7.40	0.015*
		AB	102.24		0.76	0.5311

Model formulas are coded as follows: Y = mean no. of prey eaten, A = algal concentration (million cells/ml), L = light intensity (lx), B = bottom colour (black/grey/white) and F = feed concentration (rotifers/ml). Significance at $P < 0.15$ is indicated in bold. Significance at the $P < 0.05$ is indicated by asterisk (*).

$\alpha = 0.05$ and analysis was carried out using the DoE module of the statistical package Minitab 16.0 (Minitab Inc.).

3. Results

3.1. Effects of surface light intensity, algae and bottom colour on illumination in tanks

Both increased light intensity ($P < 0.001$) and changed bottom colour from dark to lighter ($P < 0.001$) increased the illumination in the tanks, while increased amount of algae reduced light ($P = 0.046$) (Table 2). As analysis was performed on the design matrix, parameter estimates show change from one factor level to another, while the intercept estimates the overall mean. Thus, elevated light intensity from medium to high increased illumination in the water by around 70% (slope = 2.81), while changing the bottom colour from grey to white gave about one fifth of this effect (slope = 0.57). The interaction between bottom colour and light intensity ($P = 0.004$), showed that elevated light source intensity more strongly increased light levels by the tank bottom in white than in black bottomed tanks. In white bottomed tanks light increased from 1.34 ± 0.3 to 7.79 ± 0.5 μmol s^{-1} m^{-2} (mean ± se) while in black bottomed tanks the increase was 1.02 ± 0.2 to 5.85 ± 0.2. The interaction between algae and bottom colour ($P = 0.04$) indicated reduced bottom light due to elevated algal density in white bottomed tanks (-1.03 μmol s^{-1} m^{-2}), while in black bottomed tanks no such change was observed ($+0.02$ μmol s^{-1} m^{-2}). A positive curvature ($P = 0.004$) indicates that light measured at the design midpoint exceeds the value of the overall mean.

3.2. Larval trials —age groups pooled

Analysis of overall data revealed two significant interactions: A highly significant interaction between larval age and prey density (stepwise ANCOVA, $F_{1,75} = 18.27$, $P < 0.001$) indicated increasingly positive effect from high prey density on foraging with age. An interaction between prey density and bottom colour ($F_{1,75} = 5.04$, $P = 0.009$) was due to a generally lower beneficial effect from prey density on feeding in white bottomed tanks, as compared to black and grey bottomed tanks. There was also a less clear-cut interaction between light and algal density ($F_{1,75} = 3.31$, $P = 0.073$).

3.3. Larval trials —analysis by age

For all factors at all ages, the response at the design midpoint (intermediate values of all factors) exceeded expectations from a simple linear relationship, indicating presence of interactions or curved responses. A consistent pattern of increased foraging efficiency from low to higher levels of light, algal concentration and prey density was seen at all ages, while white tank bottoms generally lowered feeding compared to black ones (Fig. 2). At ages 5, 10, 15 and 20 average standard length (mm) ± SD was 5.0 ± 0.3, 5.7 ± 0.3, 6.1 ± 0.6 and 7.0 ± 0.6

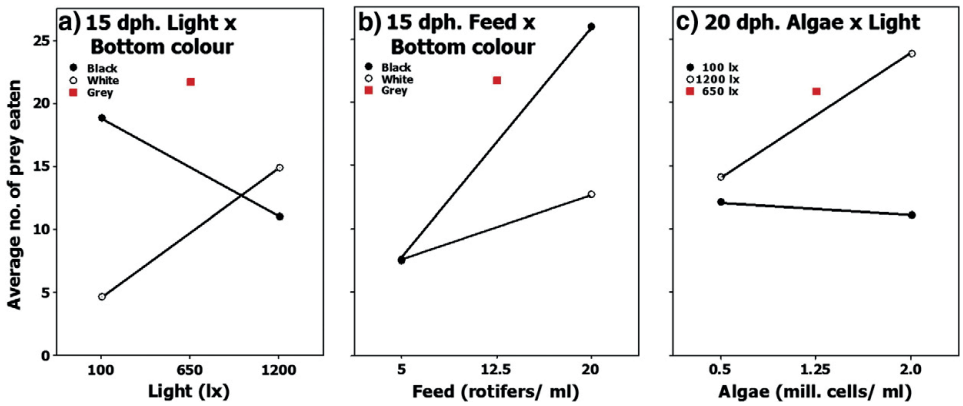

Fig. 3. Interaction plots showing significant interactions from the stepwise ANCOVA at different larval ages.

(n = 200), and overall fraction of larvae with feed in guts was 61%, 57%, 52% and 70%, respectively. The number of feed items (mean/max) in guts of feeding larvae at the respective ages was 8/40, 12/70, 16/110 and 18/101. The fraction of feeding larvae among tanks showed no important deviation from assumptions of normality or equal variance between factor levels at any of the ages. There was no correlation between standard length of feeding larvae and no. of prey in guts at any of the ages.

3.3.1. Five days post hatch

Enhanced algal density positively affected feeding ($P = 0.114$) (Table 3). All factors showed higher response at the design midpoint than at both the low and high factor settings (Fig. 2).

3.3.2. Ten days post hatch

Highly variable data resulted in uncertain interpretation at this age, and no significant effects from experimental factors appeared (Table 3). The main effect plots, however, still showed consistence with the overall pattern of effects; at the design midpoint the response was generally higher than values at low and high factor settings (Fig. 2).

3.3.3. Fifteen days post hatch

There were two significant 2-way interactions (Table 3). The light × bottom colour interaction ($P = 0.009$) indicates opposite effects from increased light on feeding depending on the bottom colour (Fig. 3a): Enhanced light intensity reduced feeding in black bottomed tanks, while in white bottomed tanks feeding increased. The design midpoint had the highest response. The feed × bottom colour interaction ($P < 0.001$) showed increased feeding as feed density increased, both for black and white bottoms, with the highest rate of change for black bottoms (Fig. 3b). Here, the overall highest response was at the combination black bottom – high feed. In sum, light intensity, feed density and bottom colour jointly affected feeding.

3.3.4. Twenty days post hatch

There was a significant interaction between algal density and light intensity (Table 3, Fig. 3c): Increased algal density had no effect at low light intensity, while at high intensity feeding was enhanced. There was a significant main effect from prey density on feeding, and the main effect plots indicate enhanced feeding due to increased light, algal density and feed density (Fig. 2). In sum, at 20 dph light, feed density and algae affected feeding.

4. Discussion

The present study demonstrates different influence of the environmental factors light intensity, microalgae density, bottom colour and prey density on larval feeding over the first 20 days post hatch in Atlantic cod. The short-term factorial screening approach allowed independent assessment of influence from the different factors at specific larval ages/sizes. The presence of interactions between experimental factors pinpoints the multi-factorial nature of tank environment on larval behaviour, and thus a need to simultaneously consider multiple factors when aiming to fine-tune or optimize environmental settings. When interactions are present, OVAT approaches fall short in identifying joint effects of co-acting factors, and are thus not well suited to identify optimal factor settings. The overall enhanced response at intermediate factor levels (the design center point) suggests that the present experimental domain represents suitable delimitation to carry out more thorough optimization studies.

The generally negative effect from white and positive effect from grey bottom colour on feeding suggests darker colours as better choices than white. Compared to black bottoms, lighter bottoms facilitate day-to-day visual control within tanks (Naas et al., 1996). Thus, for cod a grey bottom colour should be chosen, and further optimization studies should focus more thoroughly on the remaining factors; light, algae and prey density.

High variability in data was observed at 10 dph, past the point of no return in cod larvae (Ellertsen et al., 1980; Overton et al., 2010). At this age the population includes successfully feeding, growing larvae as well as larvae suffering from malnutrition, and discrepancies in nutritional status of larvae could thus subdue effects from environmental factors.

It is well known that fixation may significantly affect morphology of marine fish larvae (e.g. Hay, 1984). For cod larvae Yin and Blaxter (1986) estimated a formalin induced shrinkage of 8–12%. Though, within all four single experiments in this study, larvae were of similar size and fixated for the same time span prior to examination, so fixation effects would not bias experiment-wise results.

Larval density was kept low to reduce potential social interactions between larvae like aggression or competition for food. For instance, Puvanendran et al. (2008) demonstrated aggressive behaviour in cod already at 6 mm SL. In commercial situations larval densities are high, so optimization of light conditions for foraging in full scale production is one next step that has to be examined.

The response variable applied in this study measured short-term feed intake. It is well known that factors that affect feed intake consequentially will affect condition, growth and survival of fishes (Nunn et al., 2012). Similar measures of feed intake have proven capable to separate effects between levels of environmental predictor variables in cod, e.g. light intensity (Puvanendran and Brown, 2002; van der Meeren et al., 2007), light regime (Monk et al., 2006) prey concentration (Puvanendran and Brown, 1999) and microalgae (van der Meeren et al., 2007) in long-term experiments. Except for in the latter study, there was a correspondence between observed differences in foraging and growth, supporting the relevance of feeding related response measures for assessing overall larval success.

The improved foraging due to age, seen from analysis of overall data, is likely caused by improved feeding skills due to larval growth and development: Larvae generally undergo comprehensive ontogenetic changes, which enhance the ability to detect, capture and ingest prey through improved vision, locomotion and gap functionality, and ultimately improves foraging (Nunn et al., 2012). In a study of the foraging behaviour in cod larvae, both attack frequency, swimming speed, success of capture, search efficiency and total search area increased over the size range 5–9 mm SL, and total prey catch increased strongly while time spent for foraging decreased (Hunt von Herbing and Gallager, 2000). This dependency on ontogeny for larval performance is a strong argument for applying an age/size specific focus when seeking to fine-tune environmental conditions throughout the larval period.

4.1. Effects from tank bottom colour

Effect plots indicate negative effects from white tank bottom on feeding activity, as compared to grey and black, supporting the suggestions from Naas et al. (1996) that very light bottom colours should be avoided. Overall, the grey bottom had the strongest positive effect. Our results are in accordance with studies for species like herring *Clupea harengus* L (Blaxter, 1968), turbot *Scophthalmus maximus* L (Howell, 1979), dolphin fish *Coryphaena hippurus* (Ostrowski, 1989) and striped bass *Morone saxatilis* Walbaum (Martin-Robichaud and Peterson, 1998), where higher growth and survival was obtained in all-black than in all-white tanks. However, in haddock *Melanogrammus aeglefinus* larvae Downing and Litvak (2000) found improved survival in white tanks and lowered length growth in black tanks at low light intensity. In contrast to our study, cod larvae reared at commercial scale did not differ in foraging, growth or survival in black walled tanks between black and beige tank bottoms (Monk et al., 2008). This may be due to design differences between studies for factors like tank size and shape, added algae, lighting etc. which make direct comparisons difficult. Nevertheless, based on practical benefits from lighter bottoms for tending of tanks, the grey bottom is a practically good choice.

The interaction between bottom colour and light intensity at 15 dph reveals contradictory effects due to increased light between black and white coloured bottoms. Light is a potentially important cue for vertical position (Blaxter, 1975), and it may be that at this stage, the combinations of light intensity and bottom reflection changed spatial distribution in ways that affect larval prey acquisition. Vollset et al. (2009) found that at 10 dph cod larva stay close to the surface. Though, marine fish larvae also show a tendency to collect near tank walls, possibly due to phototaxis (Naas et al., 1996), and in striped trumpeter *Latris lineata* such a behaviour increases with larval age (Cobcroft and Battaglene, 2009). Regarding prey, some rotifer species also demonstrate positive phototaxis (Cornillac et al., 1983). Thus, white bottoms may introduce dual light sources in tanks due to reflection that affect access to prey, either directly by affecting larval spatial position or indirectly by affecting prey distribution.

4.2. Effects from microalgae density

Although there was a general trend that increased amount of algae increased feeding, significant main effect on gut fill due to increased algal concentration was seen only at 5 dph.

Many studies report beneficial effect from added microalgae (Cahu et al., 2003; Naas et al., 1996; van der Meeren et al., 2007), though reasons are not clear. It has been suggested that algae change turbidity and thus prey contrast (Naas et al., 1992), which may enhance feeding. Also, filtration of microalgae prior to first feeding has been seen both in nature and aquaculture (Reitan et al., 1994, 1998; van der Meeren, 1991), and in sea bass (*Dicentrarchus labrax*) Cahu et al. (1998) found positive effects on intestinal enzyme activity from algae at early feeding.

In cod, Van der Meeren et al. (2007) reported increased gut fill at 3 dph due to presence of algae, but no subsequent effects on growth. Skiftesvik et al. (2003) found no behavioral changes due to algae at 5 dph, while Overton et al. (2010) on the other hand, using the copepod *Acartia tonsa* as feed, found significant difference in gut fullness index at 5 to 10 dph between clear water tanks and tanks added algae. Again, design differences between studies (light intensity, tank designs, amount and species of algae and prey types) make generalizations difficult.

We conclude, due to the short-term nature and chosen response variable of this study, that observed effects relate to prey detection or behaviour rather than nutritional effects. This agrees with results from Attramadal et al. (2012), who found no negative effects on survival or growth in cod when exchanging algae paste with inorganic clay.

At 20 dph enhanced algal density improved feeding only at high light levels, indicating that optimal light at this age exceeds 100 lx while 1200 lx without added algae is too high. This is in accordance with Monk et al. (2006) who found that reducing light from 1200 to 650 lx at 28 dph improved capture success and growth in cod larvae.

Results for algae suggest a need for follow-up studies. Reducing or ending the use of algae in favor of fine-tuning of light or replacement with other substances would substantially reduce work demand, costs and the amount of organic load introduced to fish tanks.

4.3. Effects from light intensity

Effects from light were initially vague, but became more pronounced at 15 and 20 dph, indicating that optimal light intensity for cod larvae changes over time. Also, foraging success depends on the combinations of light and the factors bottom colour and algae, as discussed above for bottom colour and algae. The increased importance of light is probably due to the generally improved vision with age in marine fish larvae (Blaxter, 1986). In agreement with our results, Van der Meeren et al. (2007) found no difference in feeding incidence between different light intensities in 5 dph cod larvae, while Puvanendran and Brown (2002) observed improved prey capture from 9 dph, gut fullness from 14 dph and orientation towards prey from 16 dph at 1200 lx, as compared to lower intensities. Though, in the latter study, discrepancies in larval size between treatments evolved over time due to different growth rates, so repeated comparisons of foraging behaviour after size differences start to establish are debatable. In comparison, the present study compares effects between similarly sized larvae, exposed to identical rearing conditions prior to experiments.

4.4. Effects from prey density

Due to optimal foraging theory, increased prey density is expected to enhance prey encounter rate, and thus improve foraging success up to a certain point. The interaction between prey density and bottom colour at 15 dph suggests that larvae respond more strongly to increased feed density in the grey and black bottomed tanks. As bottom colour is a main factor determining illumination in tanks this could reflect improved prey acquisition at beneficial levels of light at this specific developmental stage. It could also be due to some mechanism affecting distribution of larva or prey, thus changing the experienced prey availability (see: Effects from tank bottom colour). The beneficial effects from increased prey density at 15 and 20 dph contradicts with results from Puvanendran and Brown (1999) who observed no improvement in foraging activity at rotifer densities above 4 prey mL^{-1}. However, Hamre et al. (2008) reported elevated survival at rotifer densities of 25–65 mL^{-1}, and Tønnesen Bush (2010) observed lowered prey sinking rates and high survival of cod larvae at 10 rotifers mL^{-1}, and suggested that prey density may have been a limiting factor for cod larval survival in previous studies. State-of-the-art aquaculture practices in general are in support of our results, as prey density is normally increased during the early larval period (Brown et al., 2003), and transition to more energetic feed types occurs as early as possible. Our results suggest that effects from prey density from 10 to 15 dph onwards should be closer examined in order to further optimize feeding protocols.

4.5. Practical implications and follow-up strategies

The main purpose of screening experiments is to identify influential factors and eventual non-additive effects (interactions) between factors, to suggest directions for further optimization. Natural next steps are to fix categorical factors at their optimal level, exclude non influential factors from future experiments and then to perform follow-up sequential experiments to narrow the experimental domain to an area of optimum response, using the method of "steepest ascent" (Box et al., 2005; Debye, 1909). Ultimately, higher order optimization models may be applied to accurately estimate optimum factor values.

In our particular case, tank colour should be fixed at its overall best level, grey, and remaining factors studied further using e.g. a Central Composite Design (Box and Wilson, 1951; Box et al., 2005). With *f* factors and n_0 replicates in the centre point, total number of design points (tanks) would be $n = 2^f + 2f, + n_0$ (Khuri and Mukhopadhyay, 2010), so e.g. $f = 3$ factors and $n_0 = 6$ centre replicates would demand the same work load and number of tanks (20) as the initial screening. When increased replication is needed, this may be achieved without more tanks, more personnel or drastically increased work load by repeating each single experiment in e.g. randomized blocks at successive days.

4.6. Conclusion

This study demonstrates the usefulness of short-term factorial experiments at discrete larval age/size as tools to track ontogenetically changed environmental demands and accumulate knowledge to support further fine-tuning and optimization of aquaculture conditions. It points out the importance to set levels of environmental factors in accordance with the changing developmental status of larvae, and offers knowledge at a micro scale to guide further fine-tuning of husbandry conditions. Specifically, grey tank bottoms enhanced feeding, and the modest effect from algae suggests that the period of adding algae could be shortened, or algae substituted with non-biotic substances or

improved overall light settings in tanks. The approach is cost effective and easily refined through the use of well established experimental design techniques.

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Over the preceding decades, there has been extensive efforts to introduce novel marine fish species for industrial aquaculture production. Establishing of reliable juvenile production has turned out to be a bottleneck in this process, for two main reasons. First, marine fish larvae are often fragile at hatch and go through rapid changes through-out the larval phase. Second, there is a complex interplay between available technologies, nutrition and feed types at the given point of time, and adjustable factors that make up the tank environment for a species at focus, e.g. water flow, temperature, light, feeding strategies, stocking density and tank tending.

Due to this complexity, coordinated, systematic research is critical to establish production in an effective, cost-efficient and timesaving manner. This thesis examines approaches to establish production carried out by the academic sector, discusses their suitability for the purpose and suggests improvements in both general conduct as well as overall strategies for future work at the area. The study includes two examples of early stage research and development actions, to aid improve the visual environment of larval rearing tanks, by using experimental designs dedicated to examine the contribution from multiple factors simultaneously.

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