Larval growth and condition of eastern Baltic cod (*Gadus morhua* L.) in the laboratory – Effects of key environmental factors

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Summary

The early life stages of marine fish are susceptible to the effects of abiotic environmental and intrinsic and extrinsic biotic factors. Water temperature and feeding levels are among the most important of these. The interaction of all these factors determines the individual fate of an egg or a larva as much as it does affect a cohort or a population. Describing the effects of these environmental factors on larval growth, condition and survival under controlled laboratory conditions is essential to understand the mechanisms of recruitment in the field. The Eastern Baltic cod population has attracted a lot of scientific interest, but fundamental knowledge on the vital rates of the larval stages under controlled conditions in the laboratory has been lacking.

In the framework of this thesis, I reared Eastern Baltic cod larvae from hatching until the late pre-metamorphosis stage and performed experiments on the effects of water temperature and feeding levels on growth and condition, the effect of temperature, body size and species on starvation-induced changes in condition, the effect of fluorescence marking on vital rates of eggs and larvae and the effect of different “green water” types on early larval feeding activity and survival.

As a result of these experiments, I described the somatic and otolith growth, morphological and biochemical condition of Eastern Baltic cod larvae and parameterized growth models for these parameters. This knowledge can be used to compare the vital rates of larvae from different cod populations and to estimate recent growth rates from biochemical condition. I also compared rates of change in biochemical condition, biochemical thresholds and time to death as proxies for physiological processes under food-deprivation and found species-specific adaptations to withstand the effects of starvation in eight different species. Further, I found sublethal chronic effects on vital rates of Eastern Baltic cod eggs and larvae that were marked with Alizarin Complexone and I formulated recommendations to future stock enhancement activities, how these effects should be assessed. I observed improved feeding incidence and precocious feeding in yolk sac larvae that were reared in the presence of a unicellular autotrophic algae and a heterotrophic protist.

This thesis addresses fisheries scientists and aquaculturists working with cod, as well as readers with an interest in the characteristics of early life stages of marine fish and principals of growth and condition in general.

Zusammenfassung

Die frühen Lebensstadien von marinen Fischen sind den Auswirkungen der sie umgebenden belebten und unbelebten Natur ausgesetzt. Wassertemperatur und Nahrungsangebot sind mithin die wichtigsten dieser Umweltfaktoren. Die Wechselwirkung all dieser Faktoren bestimmt das Schicksal einer einzelnen Larve ebenso wie das einer Kohorte oder Population. Um die Ursachen für Rekrutierungsschwankung und –erfolg verstehen zu können ist es wichtig den Einfluss der Umweltfaktoren auf das Wachstum, die Kondition und das Überleben von Larven unter kontrollierten Umweltbedingungen zu untersuchen. Der in der östlichen Ostsee beheimatete Dorschbestand wurde bereits durch viele wissenschaftliche Arbeiten beschrieben, jedoch fehlen bis zum heutigen Tage wichtige Kennzahlen über die Vitalparameter seiner frühen Lebensstadien unter kontrollierten Laborbedingungen.

Im Rahmen dieser Arbeit habe ich Ostseedorschlarven der östlichen Population in ihrem somatischen Wachstum, ihrem Otolithenwachstum und ihrer morphologischen und biochemischen Kondition beschrieben und Wachstumsmodelle für diese Parameter aufgestellt. Dieses Wissen trägt dazu bei die Vitalraten von Kabeljau- und Dorschlarven unterschiedlicher Herkunft zu vergleichen und um Wachstumsraten aus einschlägigen Konditionsschätzern abzuleiten. Ich habe außerdem die Änderungsraten der biochemischen Kondition, die Schwellenwerte der selbigen und den Zeitraum der Lebenserwartung als Kenngrößen für die physiologischen Prozesse unter Bedingungen des Futterentzugs verglichen und habe dabei artspezifische Anpassungen zur Steigerung der Hungerresistenz in acht verschiedenen Fischarten gefunden. Außerdem habe ich eine Reihe von sub-lethalen, chronischen Effekten auf die Vitalraten von Dorscheiern und –larven beschrieben, die mit Alizarin Complexone markiert wurden und ich habe daraus Empfehlungen zur Abschätzung dieser Einflüsse formuliert, die für zukünftige Besatzmaßnahmen von Relevanz sein mögen. Außerdem beobachtete und beschrieb ich den stimulierenden Effekt von einzelligen autotrophen Mikroalgen und heterotrophen Protisten auf den gesteigerten und verfrühten Fresserfolg von Dottersacklarven des Dorsches.

Diese Arbeit richtet sich gleichermaßen an Fischereiwissenschaftler und Aquakulturexperten, die mit Kabeljau und Dorsch arbeiten, wie an den geneigten Leser, der sich für die Eigenschaften der frühen Lebensstadien von Meeresfischen und für die grundsätzlichen Zusammenhänge von Wachstum und Kondition interessiert.

Outline of publications

The following overview outlines the four publications included in this thesis and the contribution of the co-authors to the manuscripts.

Manuscript I:

Growth and condition of Eastern Baltic cod larvae – influence of key environmental factors

S. Meyer, K. Hüssy, J.G. Støttrup, M.A. Peck

Stefan Meyer conducted the experiments, analyzed samples and data and wrote the manuscript. Myron A. Peck planned the experiments and helped to analyze the data and to write the manuscript. Josianne G. Støttrup helped to conduct the experiments and to write the manuscript. Karin Hüssy helped to analyze the data and to write the manuscript. The manuscript is prepared for publication in Canadian Journal of Fisheries and Aquatic Sciences, a peer-reviewed journal.

Manuscript II:

On the edge of death: Rates of decline and lower thresholds of biochemical condition in food-deprived fish larvae and juveniles

S. Meyer, E.M. Caldarone, M.A. Chícharo, C. Clemmesen, A.M. Faria, C. Faulk, A. Folkvord, G.J. Holt, H. Høie, P. Kanstinger, A. Malzahn, D. Moran, C. Petereit, J.G. Støttrup, M.A. Peck

All co-authors contributed original research data to the manuscript. All co-authors contributed to write the manuscript. Myron A. Peck and Stefan Meyer developed the conceptual framework of the analysis. Stefan Meyer managed the creative group-process. This manuscript was published in the Journal of Marine Systems 93 (2012) 11–24, a peer-reviewed journal.

Manuscript III:

Sublethal effects of Alizarin Complexone marking on Baltic cod (*Gadus morhua*) eggs and larvae

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All authors planned and Stefan Meyer and Sune R. Sørensen conducted the experiments and analyzed the data. Stefan Meyer wrote the manuscript. Josianne G Støttrup, Myron A. Peck

and Sune R. Sørensen contributed to the writing. This manuscript was published in Aquaculture 324–325 (2012) 158–164, a peer-reviewed journal.

Manuscript IV:

Role of heterotrophic protists in first feeding by cod (*Gadus morhua*) larvae

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All authors planned and Julia L. Overton and Stefan Meyer conducted the experiments. All authors contributed to the writing and analysis of the data. This manuscript was published in Marine Ecology Progress Series 410 (2010) 197-204, a peer-reviewed journal.

Introduction

1. Cod (*Gadus morhua*)

Cod, usually only referred to as "the fish", has attracted a lot of human attention ever since the first sea-going nations sustained their living from this abundant and rich blessing of the seas. The common cultural history of cod and man has seen pioneers, like the medieval Basque fishermen that sailed from Europe to Newfoundland to fill their ships with salted cod for sell on the European market, and it has seen warriors, who used to be Icelandic fishermen that violently defended their inherited fishing rights against intruders (Kurlansky, 1997). All these people put their fate and existence into the hands of the fish; a fish that seemed to be so abundant and fertile, that under no circumstances it would ever be possible to diminish. But it happened. The major cod fisheries in the Northwest Atlantic broke down as a result of excessive fishing mortality (Hutchings, 1996). The manifold socioeconomic and ecological implications of this collapse have drawn public attention to a discussion that until hitherto has almost exclusively been led by fisheries experts and that is still ongoing (Jantzen, 2010). It has become clear that mankind can no longer put itself into the trusted hands of cod, but in turn has to take responsibility and action to mitigate and restore his misconduct.

In this respect, a special mandate has fallen to fisheries scientists on both sides of the Atlantic and worldwide, to come up with scientifically sound hypothesis and deduced recommendations that will help all involved stakeholders making the right decisions to overcome this crisis. Cod has for more than a century been studied by almost all disciplines of natural science and a wealth of knowledge has been accumulated. Some of this shall be reported here to give an impression on the complexity of the system, but it has to be realized that probably all the literature on this topic will easily fill a library.

2. Autecology of cod

Cod (*Gadus morhua* Linneaus, 1758) is a teleost fish belonging to the family Gadidae, order Gadiformes, class Actinopterygii. The Family Gadidae contains about 50 species of which many are economically highly important, like haddock (*Melanogrammus aeglefinus*), Alaska pollock (*Theragra chalcogramma*) and cod. Most of these are marine species and live on the continental shelves around the North Atlantic, except for one species, *Lota lota*, which is a freshwater fish (Cohen et al., 1990). Cod can reach a maximum body size of 200 cm in length and around 100 kg in weight. Its maximum life-span is reported to be 25 years. The species is considered a demersal groundfish, with juveniles preferring shallow (10-30 m) and adults deeper (150-200m, up to 600 m) water depths (Cohen et al., 1990). Its geographic distribution ranges over the whole North Atlantic from Cape Hatteras to Ungava Bay along the North American coast, east and west coasts of Greenland, around Iceland, coasts of Europe from the Bay of Biscay to the Barents Sea, including the region around Bear Island (Cohen et al., 1990).

3. Cod populations and habitats

Based on genetic studies, it is assumed that Atlantic cod and Pacific cod (*Gadus macrocephalus*) descended from a common ancestry line about 3.5 million years ago (Bigg et al., 2008). Genetic relationships within the Gadidae family are described in Coulsen et al.

(2006). The species *Gadus morhua* is made up from an unknown number of more or less distinct populations that are mainly separated by their spawning grounds and only to a lesser extent by their geographic distribution or environmental factors (Imsland and Jonsdottir, 2003; Ruzzante et al., 1999; Sundby, 2000). Different subpopulation structures of North Atlantic cod stocks have been investigated and were described with the metapopulation concept, which takes into account migration and genetic exchange between geographically distinct subpopulations (Kent Smedbol and Wroblewski, 2002). The genetic and phenotypic discrimination of stock structures is considered an essential prerequisite to cod stock management and subject of ongoing research (Ruzzante et al., 1999).

The investigation of life history differences between these populations has also been intensely studied (Imsland and Jonsdottir, 2003). The complexity of the marine habitats occupied by cod has induced adaptive differentiation to temperature (Bradbury et al., 2010). Extensive field and laboratory work on reaction norms and genotype x environment interactions, partly conducted in common-environment experiments (Purchase and Brown, 2000; 2001), did not indicate as clear environmental adaptations as were found for other fish species, e.g. counter gradient variation in Atlantic silverside (*Menidia menidia*) (Conover and Present, 1990). But several important traits of cod early life stages, like activity, metabolism (Hunt von Herbing and Boutilier, 1996) and growth (Folkvord, 2005; Harrald et al., 2010), exhibited thermal adaptation to their environment. This topic is further elaborated in Manuscript I, describing fundamental growth and condition traits of Eastern Baltic cod larvae under controlled laboratory conditions and relating these results to previous studies on Atlantic cod larvae.

4. Cod in the Baltic Sea

Cod is a marine species that migrated into the Baltic proper some 7100 yrs ago, when sea level rise and land settling opened the Danish sound (Nielsen et al., 2003). At this time, the young ecosystem was invaded by a number of marine species and, until present day, both the relatively short period of time and the continued fluctuation in salinity prevented the establishment of a more diverse and potentially more stable ecosystem (Kautsky and Kautsky, 2000).

The Baltic Sea used to be a cod-dominated environment in the 1980s, supporting substantial fisheries efforts and suppressing the dominance of other, e.g. clupeid, fish species. Recruitment failure caused the Eastern Baltic cod stock to decline in the 1990s (Bagge et al., 1994) and the causing mechanisms were identified to be related to a decrease in reproductive volume, i.e. the water masses containing sufficient oxygen and a high enough salinity for successful spawning and egg development (MacKenzie et al., 2000; Plikshs et al., 1993), egg predation by clupeids (Köster and Möllmann, 2000) and a spatial-temporal mismatch of larvae and their preferred zooplankton prey, *Pseudocalanus elongatus* (Hinrichsen et al., 2002). Along with the diminishing dominance of cod, clupeid species were increasing in abundance, further accelerating the so called regime shift (Alheit et al., 2005). Severe changes in all trophic layers of the Baltic ecosystem were the consequence. Current projections for near future development of climatic and hydrographic changes in the Baltic, especially the projected temperature increase and salinity decrease (Meier, 2006), suggest continued dynamic in the Baltic environment. Nevertheless, the Eastern Baltic cod stock in ICES

subdivision 25, east of the island of Bornholm, has recently recovered because of increased recruitment success and successful fisheries management practices (Eero et al., 2012).

5. Key environmental factors

In poikilothermic fish, temperature is the main important factor regulating the rate of cellular chemical reactions and processes (Brett, 1979). Most fish have distinct temperature optima for growth (Brett, 1979), which tend to decrease with increasing body size (Otterlei et al., 1999; Pedersen and Jobling, 1989). These and other norms of reaction determine the influence of key environmental factors on life history traits, not only in larval fish. These reaction norms and their interactions are one of the major keys to understanding the effect of climate change and human exploitation on ecosystem functioning. For fisheries scientists, especially those working with the early life stages of fish and other marine organisms, one principal of ecosystem functioning is expressed in recruitment hypothesis. These conceptual models try to describe the processes that determine year class recruitment to fish populations and fisheries stocks (Hjort, 1914b; Houde, 2008), i.e. they try to make an educated guess on how many more fish will be available for fisheries in the following years. Some of these models are actually based on guessing (stochastic processes), whereas others are more formally relating cause, e.g. hydrographic forcing, to effect, e.g. reproductive effort (Köster et al., 2005b). For the Eastern Baltic cod stock, the causal framework determining recruitment success has not only been investigated and understood, this knowledge has also been used to improve fisheries management strategies for sustainable fisheries (Köster et al., 2009). An ecosystem approach to fisheries management requires a great deal of understanding on the dynamics of the system (Möllmann et al., 2011).

The Baltic Sea is a highly dynamic ecosystem in almost any abiotic or biotic factor. The dynamics are driven by global processes, like climate change (Neumann, 2010), and they affect the largest (MacKenzie et al., 2002) as well as the smallest (Ennet et al., 2000) of the Baltic inhabitants. The Baltic is an almost marine habitat in the west, near the Danish Isles, and it is almost a freshwater environment in the east, in the Gulf of Bothnia. Salinity is changing as a function of saline water inflow events from the North Sea and the cumulated freshwater run-off from 1.7 million km² land area (Kautsky and Kautsky, 2000). The deep basins, five in total with a maximum depth of 459 m, are essentially anoxic, whereas the shallower regions have a high primary production ($5.9 * 10^8$ kg carbon per year) (Kautsky and Kautsky, 2000). The main water masses of the Baltic are permanently stratified by a combined thermo- and halocline at around 60 to 80 m depth, with salinities above ranging from 6 to 8 psu and 10 to 14 psu below. Surface water temperatures range from 0 °C and 180 days of ice coverage per year in the northern parts up to 20 °C in the summer (Kautsky and Kautsky, 2000).

Biotic factors are mainly determined by species interactions, across taxa and trophic levels. The Baltic Sea is comparably poor in biodiversity compared to full marine habitats and the number of (marine) species decreases rapidly from west to east. The Baltic food web structure is well described in its complex interactions between nutrients (N, P, DOM, POM), bacteria, cyanobacteria, phytoplankton, zooplankton, zoobenthos, fishes and mammals and carbon fluxes (Sandberg, 2007; Sandberg et al., 2000). Zooplankton, the main food source for the

early life stages of many fish species, has recently undergone severe changes, and these dynamics were mutually linked to fish stocks and climatic events (Möllmann et al., 2000).

6. Controlled laboratory-based experiments

The effects of these key environmental factors on Baltic cod larvae have been the main motivator for this thesis and the following chapters will relate the results of controlled laboratory experiments to Baltic cod early life history traits in the field. The experiments were conducted under controlled laboratory conditions in the facilities of Bornholm's Salmon hatchery (Bornholms Lakseklækkeri, Nexø, Denmark) in the years 2007 and 2008, at this time keeping a wild-caught broodstock of Eastern Baltic cod in the framework of a Danish restocking project (RESTOCK, Støttrup et al., 2008a). Larvae were hatched from spontaneously spawned eggs that were incubated in the hatchery facilities. Whereas one proportion of larvae was released as yolk sac larvae to restock the population, others could be used for land-based experiments. The results of the experiments conducted in the framework of the RESTOCK project are reported elsewhere (Overton and Støttrup, 2008; Støttrup et al., 2008a).

Manuscript I deals with the effect of temperature and feeding levels on growth and condition of Eastern Baltic cod larvae. Manuscript II is a meta-analysis of species- and body size specific adaptations of fish larvae and juveniles in response to food deprivation; this manuscript contains original data sets from Eastern Baltic cod larval experiments conducted in the year 2007 and 2008. Manuscript III discusses the acute and sublethal effects of marking Eastern Baltic cod larvae with a fluorescence dye, Alizarin Complexone. Manuscript IV takes a close look at the window-of-opportunity, i.e. the time period between onset of larval first feeding and the point of no return and how it is affected by the presence of different phytoplankton organisms. These studies are, to the best of my knowledge and understanding, the first of their kind to rear Eastern Baltic cod larvae for an extended time period (exogenously feeding stage, pre-metamorphosis) and to investigate their growth and condition.

7. Aquaculture of cod

The aquaculture of cod has mainly utilized other populations of the species, pioneered by Norway and Canada who sought alternative ways of sustaining their market claims for cod. Starting with the capture-based fattening of wild specimen in net cages, cod aquaculture managed to close the species life cycle pretty rapidly and the first commercial hatcheries were implemented in the mid-1980s (Brown et al., 2003). The species is among the most promising cold-water fish species for mass-rearing, mainly because of market considerations and fry availability (Tilseth, 1990). The average annual production quantity of Atlantic cod in the years 2008 and 2009 is estimated at 22.000 tonnes worldwide (FAO, 1997-2007). Understanding the principles of spawning and reproduction of cod (Kjesbu et al., 1991), is a key prerequisite for its successful industrial implementation, but further obstacles exist in the grow-out phase (early gonad maturation and escapee problematic) and marketing (recovering wild stocks, new stock exploitation, low market price) (Jørstad et al., 2008). An alternative, or at least complementary aquaculture to cod aquaculture, is the production of fry for stock enhancement.

8. Restocking and stock enhancement

The first reports on land-based reproduction and offspring release of Atlantic cod date back to 1878 in Gloucester Station in Massachusetts, USA and 1882 in Flødevingen, Norway. The latter case is attributed to G.M. Dannevig, name-giver of the marine research station at the same spot (Williams, 1991). A few years earlier, in 1865 G.O. Sars had discovered the pelagic egg stages of cod and already one year later artificially reproduced cod by strip-spawning of captured males and females.

Numerous works have investigated the aquacultural requirements for stock enhancement of cod and have greatly contributed to the biological understanding of the species (Blom et al., 1994; Svåsand, 1995). But later assessments on the viability of stock enhancement success of Atlantic cod, e.g. in Norwegian coastal waters, did not indicate any sustainable increase in cod abundance by stock enhancement (Chan et al., 2003; Svåsand et al., 2000).

In the Baltic, similar endeavors for stock enhancement and restocking were undertaken. These included the production and release of yolk sac larvae, either for practical reasons of easier logistics and based on the assumption of stocking success (Larsson and Pickova, 1993; Pickova and Larsson, 2003) or because they were based on the rationale of overcoming the specific recruitment bottle-neck of the population (Støttrup et al., 2008b). As an essential prerequisite for the responsible stock enhancement approach (Blankenship and Leber, 1995), released specimen needed to be marked and recaptured, in order to assess the stocking success. The work described in manuscript III is a contribution to the successful marking of Baltic cod eggs and larvae with a fluorescent dye for identification upon recapture.

9. Condition concepts

A common aspect of all the experiments conducted in the framework of this thesis is the application of condition concepts for larval fish. In general, condition describes the "state of being" of an organism on different levels of biological organization, from cell to population, and throughout ontogeny and habitats using morphological, histological, biochemical or ethological proxies. The fundamental principles of condition concepts in larval fish have been reviewed by Ferron and Leggett (1994). They can be used to draw conclusions on its nutritional condition (Blaxter and Ehrlich, 1974), survival probability (Dabrowski, 1975) or growth potential in the field (Suthers, 1998) and the laboratory (Fey and Hare, 2008). Some condition proxies are highly species and life stage specific (e.g., head and eye morphology) (Bisbal and Bengtson, 1995), whereas others are more generally applicable (e.g., gut epithelium integrity) (Ehrlich et al., 1976; Kjørsvik et al., 1991). Combinations of several proxies and measures are frequently used and expressed as indices to normalize for body size or mass. Most condition proxies describe the current, i.e. "ad hoc", condition of the organism which means that it is not possible to draw any conclusion on the direction of condition change and its reversibility. This can be problematic when the examined organism is close to its point of no return (PNR) (Blaxter and Hempel, 1963). The PNR is the point in time when a food depleted fish larva has suffered irreversible damages from starvation so that it cannot survive even when it reinitiates foraging. The PNR is of high relevance on the level of an individual fish larva, because it is an important landmark in the larva's individual life history trajectory (longitudinal perspective), but it is also an important demographic parameter on the

level of cohorts and populations (cross-sectional perspective), because it will determine starvation induced mortality rates. Manuscript IV deals with this aspect in more detail.

Among the most frequently used biochemical condition proxies for larval fish is the RNA-DNA ratio. This metric has been used as condition index for fish since the 1970s (Bulow, 1970), but even earlier notions on the utilization of RNA- and DNA-based condition metrics for other organisms are documented (Le Pecq and Paoletti, 1966; Sutcliffe, 1965; Van Dyke and Szustkiewicz, 1968). The first application in larval fish was described by Buckley (1979), when he related the RNA-DNA ratio of a fish larval homogenate, containing around 200 individuals, to food availability and growth rates. The same author, just 5 years later, proposed a numerical model to calculate growth rates from RNA-DNA ratios (Buckley, 1984). The model had been validated for eight marine fish species and there existed good justification to assume its general applicability to even more species. The only precondition that had to be met was that the ambient water temperature at time of capture had to be known. This innovative technique enabled, for the first time ever, large scale sampling campaigns and processing of great numbers of samples in an easy and cost-efficient was, compared to other concurrent methods like otolith analysis. Since this time, the RNA-DNA ratio has been used in many marine and freshwater habitats on numerous species of fish, crustaceans and other taxa. ASFA lists 189 entries on peer-reviewed publications using “RNA”, “DNA” and “fish” or “crustacean” or “copepod” as search string (date of query: 01. April 2012).

The functional principle of RNA-DNA ratio is simple. The method analysis the bulk content of all RNA types and DNA contained in a specific tissue or whole body crude homogenate and expresses them as a ratio. RNA in a fish cell is mainly made up of mRNA and tRNA which are both involved in protein biosynthesis. The quantity of both RNAs changes with biosynthesis rate, hence growth, because growth in fish larvae is mainly realized by protein build-up and it is therefore the enumerator of the RNA-DNA ratio. The DNA content is a function of cell number, because the DNA quantity per cell is determined by chromosome size. DNA is therefore the denominator in the RNA-DNA ratio, because it normalizes RNA quantity to body mass. Various methods for the RNA-DNA analysis have been proposed, the earliest using the UV-absorption characteristics of nucleic acids and later protocols using unspecific (e.g., Ethidiumbromide) and specific (e.g., Hoechst 33258, SYBR green II and others) nucleic acid fluorescence dyes. The main RNA-DNA protocol being used in the context of this thesis is described by Caldarone et al. (2001), adapting the method to a microplate fluorescence reader and thereby greatly increasing the work efficiency of the assay. The Appendix contains a detailed description of the modifications of this method used in the framework of this thesis.

Manuscript I:

I. Growth and condition of Eastern Baltic cod larvae – influence of key environmental factors

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Keywords

RNA-DNA ratio, otolith size, size-at-age, temperature, feeding levels, larval growth model

Abstract

Abiotic and biotic factors affect the growth and survival of marine fish larvae. Examining these factors under controlled laboratory conditions allows us to disentangle the processes affecting recruitment dynamics in field populations. We examined the effects of three different ambient water temperatures (7, 10, 13 °C, *T*) and feeding levels, ranging from food depletion to ad libitum feeding, on growth (*G*) in standard length and dry weight as well as biochemical condition (RNA-DNA ratio, *sRD*) and otolith size of the larvae of Eastern Baltic cod. Larvae were reared up to 32 days post hatch and grew up to standard lengths of 7.0 mm and dry weights (*DW*) of 297 µg. A series of models were developed, relating the aforementioned metrics to each other (length – weight, body size – otolith size, size-at-age, *G*-*sRD*-*T*) to provide condition-based growth and survival proxies for applications in field process studies. Although observed growth rates (*DW*-specific growth rates ranging from 4.7 to 22.1 % d^{-1}) of Eastern Baltic cod were somewhat lower than those previously reported for other, oceanic Atlantic cod populations at the same temperatures, morphological and biochemical condition indices were comparable. Various generalized linear models were parameterized to estimate recent growth from *sRD*, but all of these models failed to correctly estimate the negative growth rates observed for food-depleted larvae. On the other hand, comparably simple length – weight models were able to correctly identify food-depleted larvae and otolith size models could be used to estimate size and age. This study is the first to successfully rear exogenously feeding Eastern Baltic cod larvae in the laboratory and our growth and condition metrics will be useful when applied to field-caught larvae and for the correct parameterization of feeding and growth models for the larvae of this population.

1. Introduction

1.1. Cod in the Baltic Sea

The two Atlantic cod (*Gadus morhua*) stocks in the Baltic Sea are among the best studied stocks due to their relevance for commercial fisheries and their case study character representing a marine fish in a brackish environment. Numerous studies have been published on the biology of the species (Bagge et al., 1994), the trophodynamic role in the ecosystem (Möllmann et al., 2008) and the ups-and-downs of human exploitation (Cardinale and Svedang, 2011; Möllmann et al., 2011). It is remarkable that the Eastern Baltic cod stock, after two decades of overexploitation and being almost commercially extinct, has recently recovered to a significant spawning stock biomass and is considered to be again within safe biological limits (Köster et al., 2009). Favorable hydrographical conditions for recruitment success and reduced fisheries-induced mortality were the identified triggers for this peerless example of a cod stock recovery (Eero et al., 2012).

1.2. Experimental work with cod

Understanding how abiotic and biotic interactions lead to a recruitment bottle-neck in the early life stages, as it is thought to be the case for Eastern Baltic cod, requires a wealth of understanding on the effects of key environmental factors (Köster et al., 2005b). Besides extensive field studies and numerical modeling approaches, a large part of our current understanding of cod early life stage dynamics stems from laboratory-based rearing trials and experiments (Folkvord et al., 2010; Otterlei et al., 1999; Peck et al., 2004a; 2006). This work has been facilitated by improvements in commercial aquaculture of the species since the early 1980s (Dahl et al., 1984) and viable hatchery protocols being implemented on both sides of the North Atlantic since the mid-1990s (Brown et al., 2003). Altogether, this knowledge has enabled complex conclusions on the role of climate change, fisheries, pollution and other driving factors on Atlantic cod population dynamics.

1.3. Population specific reaction norms

Atlantic cod is a successful, keystone species in North Atlantic waters whose populations often display different traits and life histories as the result of more than 3.5 million years genetic adaptation (Bigg et al., 2008; Coulson et al., 2006). The genetic and phenotypic distinction of extant populations of Atlantic cod is ambiguous (Bentzen et al., 1996) and strong indications for population-specific reaction norms exist (Hutchings et al., 2007). For example, Norwegian and Northwest Atlantic cod larvae grow and survive best when exposed to light regimes of their original habitat (Puvanendran and Brown, 1998), but colder-water populations have a higher temperature-specific metabolic efficiency than their lower latitude siblings (Hunt von Herbing and Boutilier, 1996). The early life stages of Baltic cod exhibit unique physiological and morphological adaptations to their brackish environment (Nissling et al., 1994b). Most of the experiments conducted on early life stages have focused on endogenously feeding life stages (egg and yolk sac larvae). For example, population specific reaction norms of growth and survival have been described from fertilization until yolk exhaustion (Nissling, 2004; Pickova et al., 1997; Wieland et al., 1994). The lack of research on more developed life stages hampers our ability to understand prey requirements for larval survival and growth of cod in the Baltic. Consequently, larval growth models for Baltic cod

that integrate the interaction of abiotic and biotic factors are lacking and no comparison of the growth physiology has been made between populations of cod in the Baltic and elsewhere.

1.4. Larval growth and condition estimates

Different techniques for the in situ characterization of larval growth have been proposed and applied in laboratory and field trials (Ferron and Leggett, 1994). Changes in larval body length and weight, developmental stage and other morphometric landmarks (e.g. otolith size) are usually normalized to larval age or time of observation (Fuiman et al., 1998). The latter can be difficult or impossible to attain in cases when the exact age is not known or when it is not possible to obtain multiple samples from the same population over time. Opposed to this, indirect growth estimates are based on ad hoc assessment of morphological (e.g. Fulton's K or otolith increments width) or biochemical (e.g. digestive enzyme activity or RNA-DNA ratio) condition parameters. A common feature of these condition measures is that they can be used to derive instantaneous growth rate estimates from measurements of larval metabolism (catabolism/anabolism) and are therefore sensitive to the modulating effects of abiotic and biotic factors. A condition-based growth model requires empirical calibration and validation before it can be reliably applied to field-caught individuals. An interspecific RNA-DNA larval growth model has previously been proposed that estimates protein-specific growth rates of well-fed fish larvae reared at water temperatures between 4 and 28°C (Buckley et al., 2008) whereas a similar, temperature-corrected model for nine species of food-deprived larvae revealed species- and life stage-specific adaptive strategies during starvation (Meyer et al., 2012b). So far, no models estimating somatic growth from biochemical or morphological condition exist for Eastern Baltic cod; fundamental data on condition and growth rate of larvae reared under controlled laboratory conditions are lacking.

Size-at-age, rather than just body size, is the recommended metric to derive accurate estimates of larval mortality rates in the field, because growth rates of field cohorts are not necessarily homogenous (Pepin, 1993). It is also an oversimplification to assume, that all slow growing larvae are selectively removed from the cohort by mortality (predation and cannibalism) or advection, because in some years, the environment may not be as selective against slow growers who would contribute to field cohorts (Robert et al., 2007). Mesocosm experiments with Atlantic cod larvae and juveniles indicated specific genotype x environment interactions as potential causes for growth heterogeneity and habitat selectivity (Paulsen et al., 2009).

Unlike some previously published growth models for larval fish, our models were parameterized not only for larvae that were assumed to be growing at maximum age- and size-specific limits (e.g., Otterlei et al., 1999) but also at lower (food-limited) growth rates. Comparing our growth models to models that were parameterized with laboratory data from other Atlantic cod populations will demonstrate if these models are applicable to Baltic cod larvae. For this study, we reared Eastern Baltic cod larvae at different temperatures and feeding levels from onset of first feeding until the late pre-metamorphose stage and assessed somatic growth rates, biochemical condition (RNA-DNA ratio) and otolith size. From these data, we develop the first, empirically parameterized models for the growth of larval cod in this population. These models will provide helpful tools for the correct interpretation of datasets and conclusions on habitat selectivity and recruitment dynamics.

2. Material and Methods

2.1. Broodstock and egg incubation

Eastern Baltic cod broodstock fish were collected in March 2006 east of Bornholm (55° 03' 00''N, 15° 11' 50'' E) and held at Bornholm's Salmon Hatchery, Nexø, Denmark, in a fully recirculated system using artificial salt water (Tropic Marin sea salt). Broodstock husbandry is described in Støttrup et al. (2008a). Eggs were collected from spontaneous group spawning in spring and summer 2007 and were incubated in 100 L cylindrical, black, PVC tanks at 7°C and a salinity of 16.0 psu. Most eggs hatched on day 12 post fertilization and only larvae that hatched within a defined 24 h period (day 0 post hatch, dph) were used for experiments. Three distinct batches of eggs hatching on June 21st, July 27th and August 20th 2007 were used for three subsequent experiments (A, B-1 and C, respectively).

All dry weight and RNA-DNA samples from experiment B-1 (10 °C rearing temperature, year 2007) were destroyed due to a malfunction of a liquid nitrogen storage container. Dry weights and RNA-DNA ratios were excluded from the analysis, but standard length data were retained. To partly overcome this gap in the dataset of the present study, previously published data from another experiment at 10 °C, using an almost identical methodology, were used. In short, Meyer et al. (2012a) used the same tank setup and sampling scheme, but provided only one (high) feeding level. To avoid potentially confounding effects from Alizarin Complexone marking applied in this study, only data from unmarked larvae were used. This experiment will be referred to as B-2.

2.2. Experimental design

On day 2 post hatch, 500 larvae were randomly loaded into each of twelve 25 L rearing tanks (stocking density 20 larvae L^{-1}). Rearing tanks were randomly distributed among four water bathes in a temperature controlled room. Water temperature in the rearing tanks was set to 7 °C at time of loading and in case of experiment B-1, B-2 and C was slowly increased to 10 and 13 °C over a period of one and two days, respectively. Tanks were aerated with airlift pumps, partitioned with a 320 µm sieve preventing larvae from being sucked into the airlift. Rearing tanks were filled with 0.2 µm filtered artificial salt water and live *Nannochloropsis* sp. (1.5 * 10^6 cells mL^{-1} nominal density) to obtain "green water" conditions. Tanks were illuminated with strip-lights at a surface irradiation intensity of 350±20 lx (mean ± range) and continuous (24 h) light.

From 4 dph onwards, larvae were offered nauplii of the calanoid copepod *Acartia tonsa* enriched with *Rhodomonas* sp. at a nominal concentration of 1 nauplius mL^{-1}. Four hours after feeding, 20 larvae were randomly removed from the tanks and inspected for live prey items in their guts. The first samples for growth rate estimates were taken when at least 50 % first feeding larvae were observed, as was the case on 8 dph (experiment A, 7 °C), 6 dph (experiment B-1, 10 °C), 4 dph (experiment B-2, 10 °C) and 7 dph (experiment C, 13 °C), respectively.

Three feeding levels were adjusted in triplicate rearing tanks. In experiment A, nominal zooplankton densities of 0.5 (low), 1.0 (medium) and 5.0 (high) nauplii mL^{-1} were adjusted manually each day at 9.00 AM. Three additional replicate tanks were fed at high feeding

levels to provide well-fed larvae for food-deprivation trials. Because zooplankton density estimates from the rearing tanks were difficult to obtain in a reliable quality, the method for adjusting feeding levels was changed from density-based to duration-based-feeding in experiments B-1, B-2 and C. Each morning at 9.00 AM, zooplankton was added to all tanks to obtain a nominal density of 5 nauplii mL^{-1}. After 3 h (low), 9 h (medium) and 23 h (high), 38 µm sieves were placed under the airlift outlets of each tank to collect the majority of the initial zooplankton quantity (>90 % within 1 h, data not shown).

Samples were taken in 4, 3, 3 or 2 day intervals in experiments A, B-1, B-2 and C, respectively. For each sampling, 5 (experiment A) or 8 larvae (B-1, B-2 and C) were randomly removed from each replicate tank, anaesthetized with 50 mg L^{-1} MS222 and photographed with a dissecting microscope and a digital camera (Leica DC 300). Larvae were rinsed with distilled water and stored individually in liquid nitrogen prior to freeze-drying and RNA-DNA analysis.

Fasting trials were performed in experiment A (starting on 8, 12, 17 and 25 dph), B-1 (6, 9, 13, 16, 19 and 23 dph), B-2 (9 dph) and C (7, 10, 13, 16 and 18 dph) to assess the effect of food deprivation on growth and RNA-DNA ratio. Four slightly aerated 4 L tanks, filled with green-water of adequate temperature and salinity, were stocked with 30 to 100 larvae each, to obtain comparable larval size-based stocking densities. No food was added to the tanks. Samples (3 to 8 larvae) were taken every one to two days until 100 % larval mortality was reached.

2.3. Sample processing

Samples were transferred from liquid nitrogen to -80 °C at the end of the experiments. Samples were freeze-dried for 16 h (Christ Alpha 1-4) until constant weight. Larval standard length (*SL*) was measured with an image analysis software tool (ImageJ 1.41o, Wayne Rasband) to the nearest 0.1 mm and dry weight (*DW*) to the nearest 0.1 µg (SE2 Ultra Micro, Sartorius).

The RNA-DNA ratio of whole, individual larvae was analyzed using a fluorescent-dye, microplate assay, modified after Caldarone et al. (2001). In short, freeze-dried larvae were homogenized in 1 % Sarcosil-Tris-EDTA-buffer using an ultrasonic disruptor, diluted with Tris-EDTA-buffer and two aliquots were mixed with Ethidiumbromide in a 96-well plate. Fluorescence was measured at 520/605 nm (excitation/emission) in a microplate fluorometer (Xenius XC, SAFAS) and the average of the two aliquots was used. Subsequent addition of specific restriction enzymes (R 6513 and D 4263, Sigma Aldrich) eliminated RNA and DNA from the samples. Concentrations were determined based on calibration curves using highly-purified 18S + 28S rRNA from calf liver and calf thymus DNA (R 0889 and D 4764, Sigma Aldrich). A standardization factor (SF_{Pi} = 0.32±0.02, mean ± SE) for inter-laboratory comparison of RNA-DNA values was calculated based on the RNA and DNA standard curve slope ratios of all nucleic acid assays performed in this study (Caldarone et al., 2006). Only standardized RNA-DNA values (*sRD*) will be given in this manuscript.

Larval otoliths were dissected from re-hydrated, freeze-dried larvae under a dissecting microscope and fine insect needles. In most cases, Lapillus and Sagitta from each side were

removed, washed with distilled water and embedded in Crystal bond 509 resin (Structure Probe, Inc., USA) on a microscope glass slide. Otoliths were oriented in the mounting resin in a way that the diameter was maximized when viewed from above. Pictures were taken with a light microscope (Olympus AHBS/AH-2) and a digital camera (Leica DC 300) at 40x and 100x magnification. Otolith radius, diameter and perimeter were measured with an image analysis software tool (ImageJ 1.41o, Wayne Rasband) to the nearest 1 µm.

2.4. Data analysis and statistics

Growth rates were calculated based on body size data from subsequent replicate tank samplings. The following calculations utilized data obtained from each replicate tank, based on the assumption that body sizes between a) replicate tanks and b) subsequent sampling events were independent from each other and that c) larvae within a sampling event were representative for the whole tank population. Growth rate, i.e. change in body size with time, was assumed to be either linear (*SL*) or exponential (*DW*).

The following formulas were used to calculate relevant growth parameters (modified after Ricker, 1979):

(1) $AGR = \frac{BS_{i+x} - BS_{i+y}}{t_{i+x} - t_{i+y}}$

(2) $RGR = \frac{AGR}{BS_{i+y}}\ or\ RGR = \frac{AGR}{BS_{i+x}}\ or\ RGR = \frac{AGR}{\sqrt[2]{BS_{(i+x)} * BS_{(i+y)}}}$

(3) $SGR = 100 \times RGR$

(4) $G = \frac{ln(BS_{i+x}) - ln(BS_{i+y})}{t_{i+x} - t_{i+y}}$

(5) $SGR = 100 \times (e^{G} - 1)$

(6) $BS = a \times t + b$

(7) $BS = c \times e^{G \times t}$

with *AGR* = absolute growth rate in length (mm d^{-1}) or weight (µg d^{-1}), *RGR* = relative growth rate (d^{-1}), *G* = instantaneous rate of growth (d^{-1}), *SGR* = specific growth rate (% d^{-1}), *BS* = body size (either *SL* in mm or *DW* in µg), *t* time in days, *i* a running index (*i* = 1, …, *n*) for the sampling events, *x* and *y* = integers excluding zero, *a*, *b* and *c* constants and *e* = base of the natural logarithm.

For growth rate back-calculation from biochemical condition, different types of growth rates were calculated, either referring to a specific growth interval between two sampling events (later event: (*i*+*x*), earlier event: (*i*+*y*)) or integrating the change in body size for a longer

period of time ($\int_{j=(i+y)}^{(i+x)} t_j$) (Figure I-1). Note, that these growth rates may be prospective (forecast), retrospective (hindcast) or simultaneous in relation to the sampling event of interest (indicated by *i*). Prospective growth rates relate biochemical condition on sampling event *i* to the observed change in body size after sampling, retrospective rates relate to the time period before sampling and simultaneous relate to a time period starting before and ending after the sampling. The different growth rate types are:

1. "life-time" growth rate, tank-wise fitting of a linear (6) or an exponential (7) regression model to body size data over the full time span of observation (all sampling events, $i=1,\ldots,n$) , simultaneous growth rate for all sampling events, slope *a* or exponent *G* of regression represent relative and instantaneous rate of growth, respectively;

2. "stanza" growth rate, retrospective for one or more time increment(s), i.e. change in body size between the onset of the current growth stanza (Ricker, 1979) or developmental stage (e.g. post-yolk sac phase) and sampling, ($(i+y)<(i+x)$, $x=0$) assuming linear or exponential growth, using formula (1) or (4) respectively;

3. "step-wise" calculation, retrospective for one or more increments of time, i.e. change in body size from one sampling to the next, ($(i+y)=(i+x)-1$, $x=0$, $y=-1$) or to the 2nd next ($y=-2$), 3rd next ($y=-3$) or 4th next ($y=-4$), assuming linear or exponential growth, using formula (1) or (4) respectively;

4. "retrospective regression", tank-wise fitting of a linear (6) or an exponential (7) regression model to body size data over a defined period of time before sampling (t_1 ,…, t_i), integrating the previous two, three or four samplings, slope *a* or exponent *G* of regression represent relative or instantaneous rate of growth, respectively;

5. "prospective regression", tank-wise fitting of a linear (6) or an exponential (7) regression model to body size data over a defined period of time after sampling (t_i ,…, t_n), slope *a* or exponent *G* of regression represent relative or instantaneous rate of growth, respectively;

6. "simultaneous regression", tank-wise fitting of a linear (6) or an exponential (7) regression model to body size data over a defined period of time around sampling (t_1 ,…, t_i ,…, t_n), slope *a* or exponent *G* of regression represent relative or instantaneous rate of growth, respectively.

Models were fit by a Generalized Linear Model approach, goodness of model fit was expressed as Akaike's Information Criterion (*AIC*) and pseudo-R^2 values. Parameters are indicated with standard error (S.E.), unless stated otherwise. Parametric tests (t-test, ANOVA) and non-parametric tests (Mann-Whitney U test, Kruskal-Wallis tests, Jonckheere-Terpstra test for ordinal scaled variables) were used. Normality and heterogeneity of residuals was

respected. Significance was set to p=0.05. All analyses were performed with PASW 18 (SPSS Inc.).

3. Results

3.1. Somatic growth

Average (± S.D.) egg size (diameter) of developing eggs (early blastula stage) of the egg batches used in experiments A, B-1, B-2, and C were 1.7±0.05, 1.54±0.07, 1.59±0.04 and 1.52±0.03 mm, respectively. At the time of replicate tank loading, larvae were in a mixotrophic phase, i.e. still utilizing their endogenous yolk reserves. When 50 % first feeding was observed, between day 6 and 8 dph, yolk sac sizes were 0.15±0.1, 0.12±0.07, 0.35±0.18 and 0.22±0.1 mm² in experiment A, B-1, B-2 and C, respectively. Changes in yolk sac size (area) versus time suggested that yolk reserves were exhausted (i.e. reduced to ≤ 0.1 mm²) by 50 % of the larvae at 8.3, 6.2, 7.4 and 7.7 dph in experiments A, B-1, B-2 and C, respectively.

Initial larval body size at first sampling was 4.2±0.03 mm (experiment A, 8 dph), 4.5±0.04 mm (B-1, 6 dph), 4.7±0.03 mm (B-2, 4 dph), 4.6±0.02 mm (C, 7 dph) *SL* and 35.9±1.3 µg (A), 50.3±0.7 µg (B-2), 43.5±1.2 µg (C) *DW*. The experiments were conducted for 8 to 24 days from the onset of first feeding until final sampling (range across all 33 replicate tanks, Table I-1 and Table I-2, Figure I-2 and Figure I-3). Body size variability in replicate tanks increased throughout each experiment. The coefficient of variability (CV = mean SD^{-1}) of dry weight ranged in average from 5 to 60 %, and was positively correlated to larval age, duration of the experiment, and/or body size. Temperature had a significant effect on the increase in variability in relation to age, but not with respect to body size. Variability was not influenced by the three feeding levels (High, Medium and Low), but variability in food-depleted replicate tanks at 13 °C increased more rapidly than variability in any other treatment.

Tank-wise "life-time" *SL*-growth rates ranged from 0.043±0.016 mm d^{-1} in a low feeding level replicate tank at 13 °C (experiment C) up to 0.227±0.028 mm d^{-1} in a high feeding tank at 10 °C (experiment B-1). Growth rates were positively correlated to feeding level at 13 °C (Jonckheere-Test, H=27.0, p=0.003) but not at 7 °C and 10 °C (p>0.05). High feeding level *SL*-growth rates in both 10 °C experiments (B-1 and B-2) were significantly different from each other (M-U test, p=0.02), indicating a batch or experiment effect not related to water temperature. Tank-wise "life-time" *DW*-growth rates ranged from 0.046±0.013 d^{-1} (SGR_{DW}: 4.7 % d^{-1}) at low feeding level at 7 °C up to 0.2±0.018 d^{-1} (SGR_{DW}: 22.1 % d^{-1}) at 13 °C and high feeding level. They were not significantly different between feeding levels at 7 °C (experiment A, K-W test, H=6.0, p=0.051) and at 13 °C (experiment C, K-W-Test, H=2.9, p=0.236). High feeding level tank-wise "life-time" *DW*-growth rates were significantly higher in 13 °C (0.152 - 0.2 d^{-1}, experiment C) than at 10 °C (0.057 - 0.096 d^{-1}, experiment B-2) and 7 °C (0.073 - 0.087 d^{-1}, experiment A, K-W test, H=6.2, p=0.044), whereas the latter two were not significantly different from each other (p>0.05).

Dry weight specific type 2 growth rates ("stanza") ranged from -0.114 d^{-1} (food depleted, 13 °C) to 0.310 d^{-1} (high feeding level, 13°C) across all 141 valid data points. All negative

growth rates were found in food depleted tanks, whereas some food depleted tanks also had positive growth rates, indicating unrepresentative sampling and short-term effects of feeding on gut content. Positive growth rates were equally distributed among the remaining (positive) feeding levels and were 0.064(±0.025 S.D.) d^{-1} at 7 °C (A), 0.108(±0.024) d^{-1} at 10 °C (B-2) and 0.168(±0.047) d^{-1} at 13 °C (C). The growth rates at different temperatures were all significantly different from each other ($p<0.001$). A multiple linear model

(A) G (d^{-1}) = -0.073±0.015 + 0.017±0.001**T* + 0.134*10^{-3}±0.006*10^{-3}**DW* ($p < 0.001$; $R^2 = 0.65$; n = 93)

explained 65 % of the observed variability in dry weight specific type 2 growth rates of feeding larvae. A similar model

(B) *AGR* (mm d^{-1}) = -0.195±0.022 + 0.009±0.001**T* + 0.039±0.003**SL* ($p < 0.001$; $R^2 = 0.57$; n = 160)

explained 57 % of observed variability in *SL*-specific absolute growth rate of feeding larvae.

3.2. Length – weight relationship

A linear regression model was fitted to ln-transformed *SL* and *DW* value pairs of individual larvae of experiments A, B-2 and C. As mentioned above, data from B-1 were excluded due to a consistently lower allometric offset value (data not shown), indicating that dry weight was likely lost due to thawing of the samples. Ln-transformed *SL* could explain 88.7 % of the observed variability in ln-transformed *DW*:

(C) ln(*DW*) = 4.175±0.037*ln(*SL*) - 2.597±0.062 ($p < 0.001$; $R^2 = 0.887$; n = 1597)

The *SL-DW* plots of each treatment group and their respective regression models are provided in Figure I-4. Stepwise expansion of the regression model into a generalized linear model including water temperature (°C) and age (dph) improved predictability of the model by only 0.6 %, even though both variables were significant ($p<0.05$).

Standardized residuals from the simple linear ln(*SL*)-ln(*DW*) regression model (C) were not significantly different (K-W test, adj. significance level for multiple, pair-wise comparison, $p<0.05$) between experiments A (7 °C) and C (13 °C), but both were significantly different from B-2 (10°C, Figure I-5). Average (±S.E.), unstandardized residuals were 0.033±0.010, -0.106±0.008 and 0.043±0.009 in 7 °C, 10 °C and 13 °C, indicating a batch effect rather than an effect of water temperature. In experiment A (7 °C), residuals of all feeding levels were significantly different from each other (adj. $p<0.05$), except for the pair-wise comparison of feeding level Low and Medium (adj. $p=1.0$, $p=0.242$). Average (±S.E.), unstandardized residuals increased with feeding level (food-depleted: -0.062±0.015, Low: 0.048±0.020, Medium: 0.088±0.019 and High: 0.154±0.0156), indicating a positive functional relationship between feeding and *SL-DW*. In experiment B-2 (10 °C), the two available feeding levels were significantly different ($p<0.001$) and unstandardized residuals were higher in feeding larvae (-0.077±0.008) than in food-depleted larvae (-0.274±0.021). In experiment C (13 °C), residuals from food-depleted larvae were significantly lower (adj. $p<0.001$, -0.088±0.011) than all other feeding levels. Average, unstandardized residuals increased with feeding levels.

Low (0.076±0.018, unstandardized residual) and High (0.142±0.016) were significantly different (adj. $p<0.05$, tested on standardized residuals) from each other, but not from Medium (0.108±0.019).

The standardized residuals of food deprived larvae decreased with time of food-deprivation (Figure I-5). In experiment A (7 °C), standardized residuals of food-deprived larvae were already significantly different from average residuals of high feeding level larvae (adj. $p<0.01$), but not from medium and low feeding larvae, after one day of food-deprivation. In experiment C (13 °C), residuals were significantly different (adj. $p<0.05$) from all (positive) feeding levels within one day. The decrease continued for at least one more day in both experiments (A and C), before it evened out. In experiment B-2, no samples were taken after one day of food-deprivation. Standardized residuals on day 2 were significantly lower than high feeding level average. Prolonged food-deprivation, i.e. 3 or 4 days, did not significantly affect residuals.

3.3. Otolith – body size relationship

A total of 895 otoliths (630 Lapilli, 265 Sagittae) from 341 individuals were analyzed. Larval Asterisci were too small to dissect and mount and were therefore not included. From all larvae, 97 individuals (178 lapilli, 37 sagittae) stemmed from pilot rearing experiments containing relevant ages and/or body sizes reared at 7 or 10 °C. These larvae stemmed from three further spawning events and were reared for maximally 23 days post hatch under the same conditions as in experiment A and B-1. *SL* of these larvae ranged from 3.8 to 7.3 mm and *DW* from 25.8 to 246.2 µg.

Lapillus and sagitta diameter (i.e. Feret's diameter aka maximum caliper) were chosen as the main otolith metrics. Lapillus diameter ranged from 26 µm (5 dph) to 80 µm (27 dph) and sagitta diameter ranged from 17 µm (8 dph) to 155 µm (27 dph). The lapillus-sagitta-diameter ratio (*LS*-ratio) could be calculated for 136 individuals and ranged from 0.6 to 1.7. A linear regression model using age (dph) as the predictor-variable was able to explain only 13 % of the observed variability in *LS*-ratio, whereas the same model using body size (*SL*), explained 71 % of the variability

(D) $LS\text{-ratio} = -0.135\pm0.007 * SL\ (\text{mm}) + 1.809\pm0.049$ (all $p<0.001$).

The model calculated a *LS*-ratio of one, i.e. when lapillus size equals sagitta size, at a larval body size of 6.0 mm *SL*. A subset of *LS*-ratios from 66 individuals, stemming from 7, 10 and 13 °C water temperature and similar body sizes ranging from 5.2 to 6.7 mm *SL*, were not significantly different from each other, indicating that temperature had no effect on *LS*-ratio ($p=0.156$).

Lapillus diameter (*LD*) and sagitta diameter were found to be correlated with larval age and body size (all following *LD*-models: n=336, *SD*-models: n=139, Figure I-7). Multiple linear models were used to predict *LD* and *SD* from larval age (dph) and *T*.

(E) $\ln(LD) = 3.362\pm0.082 - 0.016\pm0.004 * \text{age} - 0.025\pm0.009 * T + 0.005\pm0.001 * \text{age} * T$ (all $p<0.001$, *AIC*: -591.1, pseudo-R^2: 0.794)

(F) $\ln(SD) = 3.977\pm0.404 - 0.071\pm0.019*age - 0.160\pm0.044*T + 0.014\pm0.002*age*T$ (all $p<0.001$, *AIC*: 26.3, pseudo-R^2: 0.637)

The relationship between body size and *LD*, *T* and age was parameterized as follows:

(G) $SL = -11.762\pm0.566 + 4.998\pm0.170*\ln(LD) - 0.101\pm0.017*T$ (all $p<0.001$; AIC: 603.2, pseudo-R^2: 0.791)

(H) $SL = -14.716\pm0.728 + 6.293\pm0.270*\ln(LD) - 0.188\pm0.022*T - 0.051\pm0.008*age$ (all $p<0.001$; AIC: 571.0, pseudo-R^2: 0.768)

(I) $\ln(DW) = -7.662\pm0.354 + 3.471\pm0.107*\ln(LD) - 0.061\pm0.011*T$ (all $p<0.001$; AIC: 290.8, pseudo-R^2: 0.584)

(J) $\ln(DW) = -9.104\pm0.467 + 4.103\pm0.173*\ln(LD) - 0.103\pm0.014*T - 0.025\pm0.005*age$ (all $p<0.001$; AIC: 272.7, pseudo-R^2: 0.665)

The same types of models were parameterized using *SD*, *T* and age as predictors:

(K) $SL = -4.801\pm0.417 + 3.188\pm0.119*\ln(SD) - 0.124\pm0.022*T$ (all $p<0.001$; AIC: 226.3, pseudo-R^2: 0.848)

(L) $SL = -4.620\pm0.407 + 3.488\pm0.148*\ln(SD) - 0.177\pm0.027*T - 0.036\pm0.011*age$ (all $p<0.001$; AIC: 218.4, pseudo-R^2: 0.859)

(M) $\ln(DW) = -1.997\pm0.241 + 1.959\pm0.069*\ln(SD) - 0.057\pm0.013*T$ (all $p<0.001$; AIC: 73.5, pseudo-R^2: 0.868)

The next highest complex model structure building on model (M), including age as predictor, was not significant and is therefore not displayed.

Ln-transformed Lapillus diameter was plotted against dry weight-specific growth rates to check for growth rate effects (Figure I-8). Simple linear regressions indicated that for feeding larvae, otolith size and growth rates were correlated, indicating size-specific growth. Food depleted larvae had, in average, slightly smaller otoliths than feeding larvae.

3.4. Biochemical condition

Absolute *RNA* (µg) and *DNA* (µg) contents were correlated to larval body size. Linear models explained 92.5 % of the variability in *DW* when based upon *DNA* and 84.3 % of the variability when based upon *RNA*:

(N) $DW\ (\mu g) = 23.783\pm2.57 + 22.288\pm0.212*DNA - 3.136\pm0.255*T$ (all $p<0.001$, pseudo-R^2: 0.925)

(O) $DW\ (\mu g) = -19.376\pm3.799 + 23.392\pm0.337*RNA - 2.174\pm0.352*T$ (all $p<0.001$, pseudo-R^2: 0.843)

The standardized RNA-DNA ratio (*sRD*) of individual larvae ranged from 0.7 to 6.0 across all 942 individuals included in the data set. Replicate tank-wise average *sRD* was variable throughout the course of the experiment in all treatments and no clear linear correlations to

body size or water temperature were apparent (Figure I-3). The *sRD* values were related to either shorter periods of positive or negative growth rate (i.e. growth rate types) or to residuals of length – weight or otolith-body size relationships (see below).

3.5. Growth models

Growth rates of feeding and fasting larvae (all feeding levels included from food-depleted to ad libitum) were related to *sRD* and water temperature by means of generalized linear models including the variables water temperature (*T*), tank wise average *sRD* and an interaction between both variables (*T***sRD*). Model parameters are summarized in Table I-3.

Type 1 ("life time") growth rates were related to the geometric mean of all tank-wise average *sRD* data, including 55 valid data points. The highest model fit was obtained with a simple model including only the variable *sRD* and a constant intercept. This model explained 11.5 % of the observed variability (pseudo-R^2), whereas a fully parameterized model was not significant (omnibus test $p>0.05$) and still explained 12.7 % of variability. It can be concluded that a general positive trend existed between "life-time" growth rates and *sRD* under constant experimental conditions (*T* and nominal feeding levels). The low predictive capability of this model emphasized the need to utilize another method for estimating the growth rate of larvae.

Type 2 ("stanza") growth rates could only be calculated for one ontogenetic stanza starting with the day of yolk exhaustion (less than 10 % yolk sac area) or starting with the onset of food deprivation and ending at the final day of the experiments (or final day of food deprivation in case of food depletion trials). The best model fit with all significant model parameters was able to explain 24.9 % of the observed variability in type 2 growth rate with *sRD* and *T* as explanatory variables. No significant interaction between *sRD* and *T* was found. When the same model was fitted to feeding larvae only (no food depletion trials), it was able to explain 66.6 % of the observed variability in growth rate

(P) $G\ (d^{-1}) = -0.142 \pm 0.030 + 0.021 \pm 0.007 * sRD + 0.019 \pm 0.001 * T$ (all $p<0.001$, pseudo-R^2: 0.666, n=93)

The time lag in response of *G* – *sRD* relationship was assessed with the retrospective model type 3 "single step" for time periods of one to four sampling events, equaling a time period of two (13 °C, one event interval) to 16 (7 °C, four event intervals) days. The first "single step" growth model type 3 (one sampling event retrospective, y= -1) was able to explain only 7.6 % of the observed variability using *T* and *sRD* as predictor variables. All other model structures in this sub-data set contained at least one insignificant variable and failed to explain more variability. The unbalanced design of the data set, i.e. the number of G – *sRD* duplets decreased with increasing length of time intervals, limits the absolute comparability of the model runs. Comparisons could only be made within the same sub-data set (same *G* – *sRD* duplets). For each of these sub-data set, the predictive capacity of the simplest possible model, i.e. including only one variable *T***sRD* and an intercept, was set to one and the other model pseudo-R^2 were expressed in relative terms. In general, increased retrospectivity improved the predictive capacity for all model structures. The highest predictive capacity was achieved when growth was calculated over 3 or 4 sampling events, explaining around 70 % of observed variability. The different model structures varied in their predictive capacity by factors of ~1.5

to ~2.8, indicating that model complexity was gaining importance in these more retrospective models. In summary, the highest predictive capacity (69 %) and best goodness of fit (lowest *AIC*) was achieved with a three sampling event retrospective model including *sRD* and *T*, but no *sRD***T* interaction (Figure I-6).

Type 4 "regression" growth rates had a generally higher predictive capacity than their respective "single step" counterparts. The best predictive capacity was achieved with a four-event regression and *sRD* and *sRD***T* predictor variables (77.7 % explained variability). Absolute parameter values of this models were comparable to "single step" model parameters.

Type 5 (single event prospective) and type 6 (double event simultaneous) growth rates did not have a higher predictive capacity than their retrospective counterparts. No type 5 growth rate model could be found that had all significant parameters. A type 6 growth rate model using *sRD* and *T*, but not *sRD***T*, explained 20.1 % of the observed variability.

Standardized residuals of the best fit model for the present data set, type 3 "single step" (y= -3) using *sRD* and *T* as predictors, were related to body size, age and feeding level to identify trends. A significant positive trend was found in relation to body size ($p<0.001$), indicating that growth rates of larvae smaller than 150 µg *DW* are systematically overestimated by the model and larvae >150 µg are underestimated in their recent growth rate (max. *DW* of model parameterization: 300 µg) (Figure I-9). Age had no significant influence on residuals ($p>0.05$), indicating that the body size effect might not be solely attributable to size-at-age. The residuals of feeding levels (high, medium, low, food depleted) were not significantly different from 0 ($p>0.05$). High feeding level residuals from experiment A (7 °C) were significantly higher than 0 (Bonferoni-corrected $p<0.05$) in a multiple comparison of feeding level and temperature. Expanding the above mentioned model by larval body size, improved predictive capacity to 80.3 %

(Q) $G\ (d^{-1}) = -0.237(\pm0.028) + 0.039(\pm0.007)*sRD + 0.017(\pm0.002)*T + 3.6*10^{-4}(\pm0.67*10^{-4})*DW$

(all $p<0.001$, *AIC*: -215.1).

3.6. Model residuals

Residuals of the length – weight relationship model were aggregated to replicate tank level, i.e. individual larval residuals were averaged to a replicate tank sampling event mean value. Residuals were positively correlated to *sRD*, but not *T*. A linear model, using *sRD* as a single predictor, was able to explain 7 % of the observed variability in residuals ($p<0.001$). Adding *T* to the model significantly improved predictive capacity to 31.7 % (model fit $p<0.001$), but the *T*-coefficient was not significantly different from 0 ($p>0.05$), indicating that the variability in the data set was caused by a similarly structured influential factor, but not *T*. Experiments at different *T* utilized different batches of cod larvae, stemming from different broodstock individuals and spawning events. The model was therefore now parameterized separately for each egg batch that was used in experiments A, B-2 and C. All three models were now highly significant ($p<0.001$) and contained only significant model parameters. Predictive capacity increased to 20.6 %, 52.6 % and 43.0 % and regression slopes were 0.389±0.095, 0.817±0.121 and 0.909±0.121 in A (n=67), B-2 (n=43) and C (n=77), respectively. This

confirmed the above mentioned notion of a maternal effect on the length – weight relationship.

Aggregated residuals of the length – weight relationship model (C) were also related to observed type 2 ("stanza") growth rates and were able to explain 32.9 % of the observed variability ($p<0.001$, slope: 0.077±0.010, n=134), even though the model contained a large proportion of negative growth rates from food depleted tanks. Adding *T* to the model did neither improve predictive capacity nor was the *T*-coefficient significant. When being related to the smaller data set of type 3 growth rate ("single step", y= -3, using *sRD* and *T* as predictors, used for *sRD* – *G* model), 43.0 % of observed variability in growth rate could be explained ($p<0.001$, slope: 0.052±0.008, n=52). No trend with *T*, feeding level or egg batch could be found when relating length – weight model and *sRD* – *G* model residuals to each other.

Standardized residuals of the length – weight model were used to predict residuals of the otolith – body size model. For the case of the *SL* – *LD* model (G), a significant negative trend (slope: -0.401±0.058, R^2 0.218, $p<0.001$) indicated that positive residuals from the former model were correlated to negative residuals of the latter and vice versa. No trend could be found for the ln(*DW*) body size – otolith model.

The highest representation of food depleted larvae was included in growth rate type 2 ("stanza"). This model, even though it had a lower predictive capacity for the full data set, was used to validate the sensitivity for larvae under conditions of prolonged food depletion. The model performed poorly in correctly identifying the negative growth rates of food depleted larvae. The type 2 model was therefore re-parameterized with a limited data set, containing all valid *sRD* – *G* duplets of the positive feeding levels and only negative growth rates of food depleted larvae, based on the assumption that starvation needs to cause a loss in body mass and that if that this is not the case this is a sampling artifact. The new model was parameterized as follows:

(R) $G\ (d^{-1}) = -0.216\pm0.032 + 0.031\pm0.012*sRD + 0.007\pm0.001*sRD*T$ (all parameters $p<0.01$, n=126, pseudo-R^2: 0.409, *AIC*: -262.6).

The *sRD***T* interaction shows, that the T effect is minimized at low *sRD* and that the curves converge in the case of food depletion and hence negative growth rates.

Body size effects in a selection of the previously mentioned metrics were visualized by a regression of dry weight against standardized model residuals from the *G* – *sRD* model (type 3 retrospective, y= -3, using *sRD* and *T* as predictors), the type 2 "stanza" model (R), length – weight relationship model (C) and otolith – length model (E) (Figure I-9).

A previously published multi-species *sRD***T* – *G* model (Buckley et al., 2008) was used to estimate growth rates from *sRD* values of the same data set as used for the parameterization of the type 2 "stanza" model. The calculated *G* of this model were regressed against observed type 2 "stanza" growth rates (slope: 1.439±0.200, intercept: -0.049±0.018, $p<0.001$, R^2 = 0.271, n = 141), indicating that the previously published model overestimated type 2 growth

rates by a factor of ~1.4. Figure I-10 shows that especially food depleted larval growth rates were not correctly estimated by the model.

4. Discussion

This study is the first published attempt to successfully rear exogenously feeding larvae of Eastern Baltic cod and to examine the effects of temperature and prey to develop larval growth models for this population. Our work provides some of the fundamental laboratory-based calibration data that are needed to apply species-specific, or to validate generic, condition proxies and growth-models to the Eastern Baltic cod population.

Despite the complexity of recruitment dynamics, field sampling campaigns and laboratory studies have been conducted to either directly explain variability in year class success or to contribute metrics for bio-physical modeling approaches. For example, Houde (1989) and Pepin (1991) related ambient water temperature in the field to larval growth and survival, incorporating laboratory data on the temperature dependence of egg development and yolk absorption rates in their calculations. Buckley et al. (2004) used laboratory-based growth reference data from Caldarone et al. (2003) to conclude food-limited growth and vulnerability to climate change for cod and haddock (*Melanogrammus aeglefinus*) larvae on Georges Bank. Daewel et al. (2011) used a spatially explicit, individual-based model to estimate larval cod and sprat(*Sprattus sprattus*) survival in the North Sea, incorporating laboratory data on the effect of light, temperature and prey characteristics on foraging success (food consumption rate) and other parameters of the balanced bioenergetics budget to estimate somatic growth. These and many other studies rely, at least in part, on laboratory-based calibration data that provide direct or indirect estimates of growth and survival. Various condition concepts have been developed to explain the link between growth and survival, and have helped to bridge the gap between laboratory measurements and field application (see Ferron and Leggett, 1994 for an extensive review of this subject). Both species-specific and generic condition proxies have been described from larval morphology, histology, biochemistry or larval behavior.

Since larval growth and survival in the field has been hypothesized to be a function of various temporal and spatial match-mismatch dynamics during critical periods in larval ontogeny (Cury and Roy, 1989; Cushing, 1990; Hjort, 1914b; Hjort, 1926; Iles and Sinclair, 1982; Lasker, 1975) and, more recently, throughout the whole early life phase (Houde, 2008) it has become clear that no single environmental factor can be used to predict recruitment success. Pelagic early life stages of marine fishes and other marine organisms are passive recipients of their environment and are subjected to a variety of physical factors from hydrodynamics (e.g., large-scale advection to small-scale turbulence) to levels of abiotic factors (e.g., temperature, salinity, oxygen, light). The effects of this physical forcing on the growth and survival of individuals can often be modulated by either intrinsic or extrinsic biotic factors such as predation, prey quality and quantity, diseases, changes in individual and cohort behavior. The earliest documented notion of the complexity of these influential parameters and the myriad possible significant interactions suggested to Hjort (1914a) that it would be impossible to model recruitment success.

4.1. Somatic growth of Eastern Baltic cod larvae

Early life stage characteristics of Eastern Baltic cod larvae in the current experiments were comparable to previously reported laboratory-based data in terms of yolk sac larval size (Nissling, 2004), first feeding success (Nissling et al., 1994b), yolk absorption (Trippel et al., 2005) and early (up to 6 dph) somatic growth (Nissling et al., 1994b). No further published data on laboratory-reared exogenously feeding and growing Baltic cod larvae were available.

Larval size at the start of the experiments was presumably influenced by maternal effects. The broodstock fish that were used for the 2007 experiments (A, B, C) were the same that were used in the following year for experiment B-2, i.e. the fish were one year older, were fed on a commercial cod diet and had increased in body size. The greater yolk sac size and longer yolk sac phase of the B-2 experiment fish is therefore not surprising, because a high correlation between female body size and egg diameter has previously been described (Vallin and Nissling, 2000). The greater size-at-age of this batch implicates a greater metabolic mass already at the embryonic stage. This greater mass would have been mainly yolk reserves in the egg, but during embryogenesis has been catabolized to a greater size-at-hatch. In our experiments, this initial size advantage was not profound enough to outperform the temperature effect on somatic growth rate, but it consistently influenced the length – weight relationship. A consequence of this difference was that the final dry-weight-at-age of high feeding level B-2 (10 °C) larvae was almost the same as that of high feeding level larvae from experiment C (13 °C), with almost identical ages in both treatments (20 and 19 dph, respectively). The consequence of a seemingly subtle difference in larval size-at-hatch is, that larvae stemming from a larger female experience a fitness advantage in comparison to their cohort siblings that were spawned by a smaller female and that those larvae have a higher probability to survive throughout early ontogeny, because they will sooner have grown to a body size that is less vulnerable to predation and advection (Houde, 2008). This mechanism of maternal effects is well described both for Atlantic and Baltic cod populations (Saborido-Rey et al., 2003; Vallin and Nissling, 2000).

4.2. Morphological condition and growth

The residuals of weight at length were highly correlated to biochemical condition (*sRD*) and *sRD*, in most cases, was able to identify larvae within the different feeding levels, but not at different water temperatures. The same holds true for the relationship between length – weight residuals and growth rates calculated with one of the growth models (type 2 “stanza”). Higher growth rates were associated with positive length – weight residuals and vice versa. Length – weight relationship residuals were also highly (negatively) correlated to residuals of the body size (*SL*) – otolith size (*LD*) model. These morphology-based condition proxies were therefore univocally correlated to larval growth and condition, making them useful tools for condition assessment of field caught larvae.

Larvae experiencing food depletion and hence slower growing larvae, had a lower allometric offset value than feeding larvae, indicating that food depleted larvae and larvae feeding at lower than ad libitum rations were not able to build up somatic tissue as their ad libitum feeding siblings. Similar body size and density relationships were previously described for cod larvae (Folkvord, 2005) and, more generally for adult fish, as Fulton’s K (Ricker, 1975).

In the field, those poor condition larvae have a higher specific density and exhibit less swimming activity and are therefore more susceptible to sinking. In the stratified water column of the Bornholm basin, larvae are negatively buoyant in comparison to the low salinity surface waters (e.g. ~9 PSU in the upper 50 m, Huwer et al., 2011). Sinking larvae have a high risk of suffocating in hypoxic areas below the halocline containing less than 2.5 mg/l oxygen (Nissling, 1994) or to starve, because of limited zooplankton abundance and light intensity at depth. The upward migration of newly hatched Baltic cod larvae and the horizontal location at optimal temperature, salinity and prey concentrations is therefore considered a crucial mechanism in the recruitment of this population (Grønkjær et al., 1997). For this reason, a correct identification of nutritional status of field sampled larvae just based on residuals of length – weight relationship, we found for the larvae in our experiments, is appealing, especially because this metric is relatively easy to obtain and was unaffected by water temperature. The previously mentioned maternal effects though might limit the applicability of this method, unless it is possible to quantify the maternal contribution to size-at-age, e.g. by analysis of otolith hatch-check size (Grønkjær and Schytte, 1999).

4.3. Otolith-body size relationship

We provided metrics for the growth of Baltic cod larval lapillus and sagitta, the former being the otolith of choice for larval cod otolith studies and the latter being used throughout in the later life stages of cod. Age and body size relationships to otolith size were readily explained by linear models, over the observed range in body size (~4 to ~12 mm *SL*) and age (5 – 33 dph). Temperature had the strongest impact on the age determination from otolith size, but maternal effects were also apparent here. The greater size-at-hatch of the B-2 batch (10 °C) caused a systematic offset of those data points in the body size – otolith size plot and, less clearly, in the otolith size – age plot, too. These larvae had a larger body size than their siblings from other batches with similar otolith size, limiting the universal applicability of this metric as a somatic-size-based condition proxy. Sagitta diameters increased at a higher rate than lapillus diameters, eventually increasing the lapillus – sagitta ratio (*L/S* ratio) above 1 at a body size of ~6 mm *SL*. *L/S* ratio was a bad predictor for larval age, but good for size, irrespective of temperature. Both otolith diameter metrics were closely related to larval age, when water temperature was included in the model. This makes them a very useful tool for larval age determination. Lapillus increments are routinely used in cod larval age assessment and fulfill the main criteria for a robust age back-calculation, e.g. no sudden changes in increment width (Stevenson and Campana, 1992). No otolith increments of the present data set were analyzed.

The otoliths of Eastern Baltic cod larvae from the field have previously been analyzed in shape, symmetry and hatch-check-size (Grønkjær and Schytte, 1999; Grønkjær and Sand, 2003; Grønkjær et al., 1997), but no conclusive information on the relationship to body size and age was parameterized. Laboratory reared Baltic cod larvae have been used to validate the daily deposition of otolith increments and inferences on biochemical condition (unstandardized RNA-DNA ratios) were drawn, but no further information on somatic growth were provided (Clemmesen and Doan, 1996). Otterlei (2002) investigated lapillus radius-at-age of two oceanic Atlantic cod populations and found their growth rate to be affected by temperature and Hüssy (2008) described otolith growth Atlantic cod juveniles reared at

different temperatures and feeding ratios. In the latter case, indications for an otolith size effect on increment deposition were found and potentially attributed to the metabolic activity of the endolymphatic epithelium. We observed an effect of feeding levels on dry-weight-specific growth rate and otolith size in our feeding Baltic cod larvae and hypothesize that early life stage differences in feeding success may be the foundation for later stage otolith growth effects.

4.4. Growth rate estimates from RNA-DNA ratio

Previous work indicated that food depleted larvae and juveniles exhibited species- and life stage- specific differences in their ability to withstand the effects of starvation which uncouples any generic relationship between condition and growth (Meyer et al., 2012b). We hypothesized that previously published generic models were not sufficiently parameterized for larvae feeding at lower than ad libitum rates (Buckley et al., 2008; Caldarone et al., 2003).

In a field sampling campaign, it is highly likely to find larvae in all various states of nutritional condition and growing at rates ranging from high (maybe as high as the specific reference growth rate) to low (as low as possible without dying). The range in growth rates exhibited by such a larval cohort is a function of key environmental factors and intrinsic and extrinsic biotic factors. Compared to the larvae of cod in most other populations, Baltic cod larvae experience a more variable environment with respect to abiotic factors, especially water temperature, because this is highly variable in their habitat. Cod larvae in the Bornholm basin hatch at depths around 60-70 m (Bagge et al., 1994) close to the halocline and at water temperatures around 6 °C (MacKenzie et al., 1996). Survival throughout the egg phase is mainly determined by the reproductive volume (MacKenzie et al., 2000), the water body with oxygen concentrations above ~2.5 mg/l (Wieland et al., 1994) and a salinity of > 11 PSU (Nissling and Westin, 1991) that is needed to maintain the embryos in neutral buoyancy. Yolk sac larvae need to migrate to the upper water layers to forage (Grønkjær and Wieland, 1997) and to actively swim between different depths strata in search for zooplankton (Hinrichsen et al., 2003; Hinrichsen et al., 2002; Huwer et al., 2011). During these early life stages, cod is prone to predation, cannibalism and other types of selective removal (Köster and Möllmann, 2000; Schaber et al., 2011; Voss et al., 2011), which in turn influences the range of observed conditions in a field sampling. Any kind of useful condition proxy and growth and survival estimator therefore needs to be calibrated on the greatest possible range of expected states of condition.

Condition can be used to derive recent somatic growth rate estimates from an “ad hoc” physiological/metabolic state of condition. The metric of choice, RNA-DNA ratio, is thought to be sensitive to changes in nutritional condition and somatic growth rate with a time lag of “several days” (Buckley and Lough, 1987). The exact temporal dynamics of the underlying physiological mechanism of protein biosynthesis and tissue deposition are not yet fully understood. In experimental practice, sampling frequencies have been chosen arbitrarily and/or based on the working hypothesis. In the latter case, longer (40 degree-days) sampling intervals have been used when larvae were reared under constant temperature and feeding levels and no change in condition was expected (Caldarone, 2005). Shorter time intervals were used when feeding levels were changed (Clemmesen, 1994) or when other growth rate

modulating effects were expected (Meyer et al., 2012a). In our study, we were looking for both. For the growth/feeding trials we arbitrarily set the sampling interval to 2, 3 or 4 days, resulting in 26, 30 and 28 degree-day intervals, at 13, 10 and 7 °C respectively, and in the food deprivation trials, we sampled daily whenever possible. In the former case, we were not expecting any changes in growth and condition, because environmental conditions were kept constant. In the latter case, we were expecting a change in condition from the moment larvae were food depleted. We then related the RNA-DNA ratio of each sampling event to one of our 6 different types of growth rate calculations. These were either conventional hindcasts of recent growth (Caldarone, 2005), which we did for up to 4 sampling intervals, or they were forecasts or simultaneous estimates of present or future growth.

The result of this exercise was that the highest predictive capacity across the whole data set was obtained using the model that related condition and temperature to larval growth calculated over the last 3 and 4 intervals (i.e. 6 to 16 days in total), the latter model being parameterized with only 28 out of initially 141 data points. One conservative conclusion from this tendency is: the longer, the better, meaning integrating growth over longer periods of time improves the "signal-to-noise" ratio (growth to growth variability ratio). This variability, that we wanted to minimize by means of constant rearing conditions, is a case in point for the laboratory artifacts associated with sub-optimal larval rearing methods. The larvae in our experiments were indeed growing well, but there was high inter-individual variability in growth within replicate tanks. For example, in the low feeding level tanks, some larvae were able to grow at ad libitum feeding rates and "poor condition" larvae could be found in all tanks at any time. To ultimately overcome this variability of groups, it would have been necessary to relate individual growth rates to individual RNA-DNA ratios. The experimental design and the need for independent sampling prevented this since in longer-term growth experiments with a high degree of sibling interaction, *sRD* of individuals within the same tank cannot be considered to be independent from one another. Within the replicate tank design, it would either had been necessary to increase sample size and/or to use percentiles of sampling populations (Meyer et al., 2012b) or to find other means of reducing cohort variability, e.g. by technically improving rearing conditions.

Ontogenetic differences in *G – sRD* were difficult to grasp with the short duration (covering only the early larval stages) of the current experiments. The type 2 model ("stanza") and the type 1 model (single step, hindcast for one sampling event) used virtually the same sub-set of data and still had very different predictive capacities. The higher predictive capacity of the stanza model is mainly caused by the lower variability in the tank population at first sampling. The young exogenously feeding larvae at the end of their yolk sac phase were still relatively homogenous in body size and it was therefore easier to obtain a representative sample. Opposed to this, the later sampling events that were used to calculate type 3 "single step" growth rates contained more variability in body size and growth rate accuracy was masked by this effect.

The highest scatter in the data set was introduced by growth rate estimates for food deprived larvae. These larvae were supposed to stem from initially well-nourished groups and to lose dry weight over time of deprivation at a more or less constant rate. These negative growth rates would then be related to RNA-DNA ratios in a similar fashion as for feeding larvae. The

group average growth rates of these larvae were so variable that they ranged over the full range of observed data set, from ~-0.2 d^{-1} to ~0.4 d^{-1} (dry weight specific instantaneous rate of growth). When all positive growth rates of food depleted larvae were removed from the analysis, a model was able to explain 40 % of the observed variability in growth rate. Residuals of this model indicated that negative growth rates of food depleted larvae were still not adequately estimated. The same holds true for the application of a previously published *sRD*T – G* model (Buckley et al., 2008).

4.5. Comparison to cod reference growth data

Larval growth rates of Eastern Baltic and other populations of Atlantic cod were comparable. Comparison with the length – weight relationship of Otterlei (1999) indicated the following: 1) The length – weight relationship slope reported for Northeast Arctic (NA) cod larvae was 4.14 and for Norwegian coastal (NC) cod larvae was 4.01 whereas the slope of our Baltic cod (BC) length – weight relationship was 4.18; 2) the slope of the relationship was not affected by water temperature in any of the three populations. Comparison with the growth model in that study (Otterlei et al., 1999, his equation 4) indicated that: 1) at 7 °C, BC larval size-at-age (28 dph) was 171 µg at high feeding conditions, whereas NA size-at-age was calculated to be 258 µg and NC 333 µg; 2) at 10 °C (17 dph), BC: 119 µg, NA: 148 µg, NC: 183 µg and 3) at 13 °C (19 dph), BC: 318 µg, NA: 451 µg, NC: 585 µg.

The observed growth rates of our Baltic cod larvae were consistently lower than those of the two other Atlantic cod population, but this does not necessarily implicate that the larvae of this population have a lower size- or age-specific maximum growth rate. This difference can also be attributable to the experimental rearing environment. The experimental rearing facilities used in our study were far from best hatchery protocol recommendations in commercial cod aquaculture (Brown et al., 2003). Overall low survival rates until the end of the experiments support this assertion. Instead of claiming the growth rates of larvae in our experiments as reference growth rates for the population, we recommend to utilize the additional information on growth capacity that is inherent from the lower feeding levels. The nominal feeding levels that were adjusted in the rearing tanks were not necessarily representative for the feeding conditions experienced by a cod larva in the field, but they provided to be useful in generating a broad range of observed growth rates. Not surprisingly, growth rates in low feeding tanks were lower than those at higher feeding levels, but negative growth rates were not observed. This is remarkable, because it supports previous observations by Folkvord (2009) who reported the lowest dry-weight-specific growth rate of Atlantic cod larvae (~15 to ~50 dph) to be 4 % d^{-1}. These larvae, stemming from the same experiment as mentioned above (Otterlei et al., 1999), were also reared at lower feeding levels and under controlled laboratory conditions. Apparently, cod larvae are not capable of compensating situations of food limitation by extended fasting and down-regulation of activity level and maintenance metabolism. Instead, larvae must be able to forage on prey at rates that support 4 % d^{-1} growth or they will not survive. Mortality was not monitored in our experiments, but low feeding tanks consistently exhibited the lowest survival at the end of the trials (data not shown). The food deprivation trials in our experiments and physiological effects of starvation are discussed in more detail elsewhere (Meyer et al., 2012b). In summary, we hypothesize

that the parameterization of our *G* – *sRD* – *T* models, including larvae in all possible states of condition, will avoid overestimation of growth rates from field sampling campaigns.

4.6. RNA-DNA in field larvae

Previous work on field sampled Eastern Baltic cod larvae (Grønkjær et al., 1997) used a different methodology for RNA-DNA ratio analysis and cannot be directly compared to the present data set. Grønkjær et al. (1997) used RNA-DNA ratio over time of feeding and/or starvation of parallel reared Atlantic cod larvae to identify feeding and growing Eastern Baltic cod larvae in their field trawls. The conversion of RNA-DNA ratios to protein growth rates was not consistent with observed growth rates, which can be explained with later findings on the importance of correct intercalibration of RNA-DNA methodologies (Caldarone et al., 2006). But the size- and age-class specific minimum and maximum RNA-DNA ratios of Atlantic cod larvae were successfully used to identify high condition (and presumed fast growing) Baltic cod larvae above the halocline (Grønkjær et al., 1997).

More recently, Huwer et al. (2011) sampled Eastern Baltic cod larvae from two field stations near/in the Bornholm basin and used standardized RNA-DNA ratios to calculate protein-specific growth rates, using the same methods as we used in the present study (Buckley et al., 2008; Caldarone et al., 2006). The *sRD* values of Huwer et al.'s larvae ranged from ~1.4 to ~2.4 at water temperatures between 5 and 15 °C. These *sRD* values were used to calculate protein-specific growth rates (% d^{-1}) mostly close to 0 and hardly higher than 5 % d^{-1} (using the model provided by Buckley et al., 2008). Similar *sRD* values in our study were mainly found in the early phase (~20 dph, ~5-6 mm *SL*) of the low feeding level replicate tanks at 7 °C. At this time, these tanks were growing at a dry weight-specific growth rate of 1.9 to 3.9 % d^{-1} (20 dph, type 3 growth rate, y= -3), which is only marginally higher than the calculated growth rate from Huwer et al.'s station 569 (ref. their Figure 4). This finding confirms not only the applicability of the previously published generic *sRD-T-G* model (Buckley et al., 2008) for application in feeding Eastern Baltic cod larvae, but also Huwer et al.'s conclusion about food-limited growth at this station.

5. Conclusion

The current results show that the interaction of *T* and *sRD* is less relevant than the actual temporal perspective of growth rate back-calculation. Not surprisingly, the best results will be achieved when a longer time period of growth and constant abiotic and biotic conditions are integrated. This situation is, apparently, limited to the controlled conditions of the laboratory. In turn, the practical consequence for field application of *sRD* is that in apparent situations of high experienced environmental fluctuation, it might be favorable to use a less retrospective model with lower predictive capacity to gain a more recent estimate of *G* or condition. Otolith size suggests itself as a useful and simple to assess longitudinal estimator of integrated larval growth history. The inherent growth variability of individuals within the same environment requires that growth histories be examined on field-caught individuals, e.g. by investigating growth trajectories from otoliths, and not merely instantaneous estimates of growth, like RNA-DNA ratio. The metrics provided in this study will help to resolve the role of growth –

survival – condition relationships in recruitment dynamics of Eastern Baltic cod larvae and to get a bit closer to the Holy Grail of fisheries science (Houde, 2008).

6. Figures

Figure I-1 Conceptional illustration of growth rate type calculations. Open circles are simulated size-at-age data of sampling groups, bold black circles indicate sampling event of interest (i), black arrows indicate direction of growth rate estimate (forecast, simultaneous or hindcast), dotted and solid lines indicate stepwise calculations, curves indicate regressions.

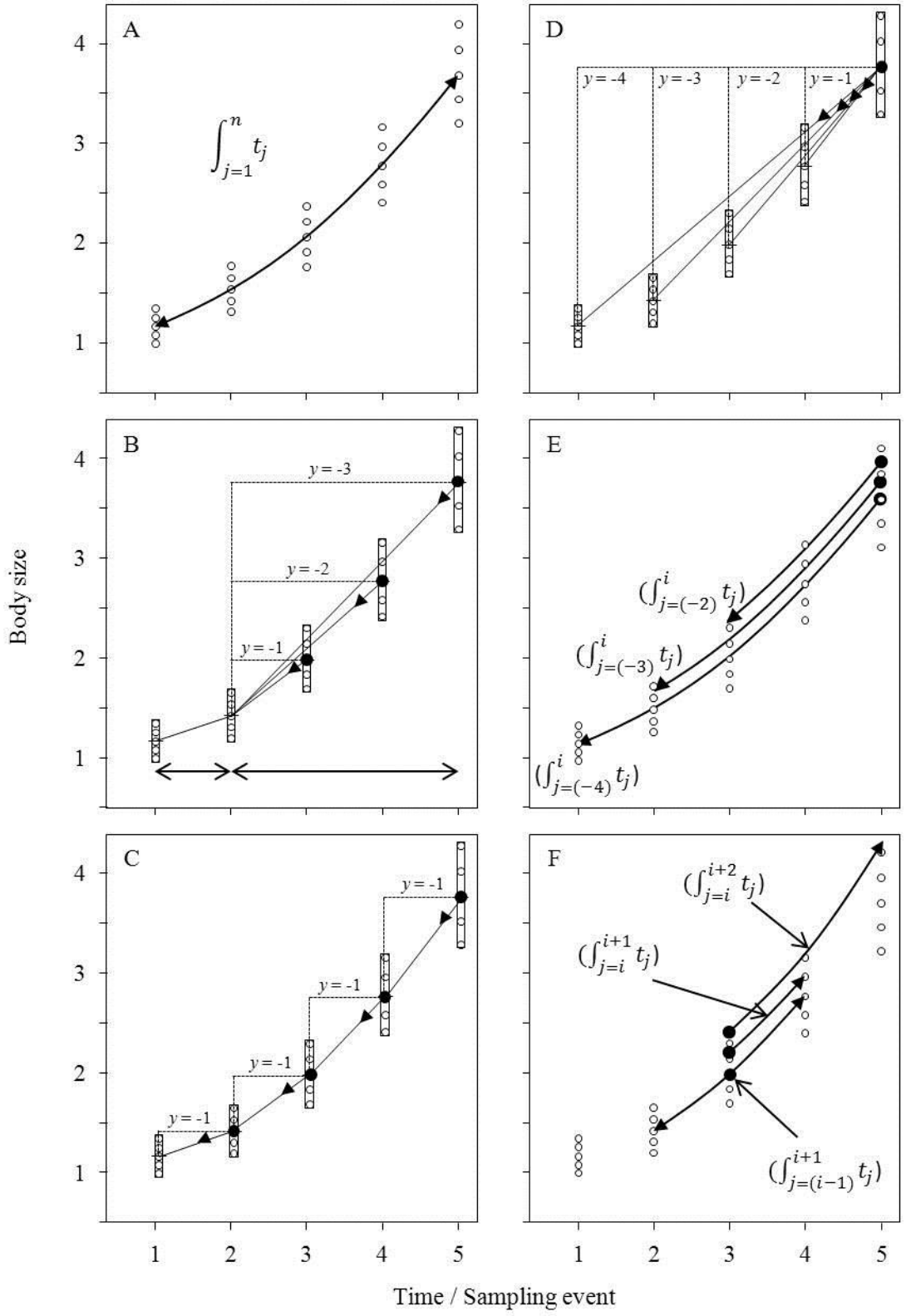


Figure I-2 Eastern Baltic cod larval body size at age. Given are replicate tank average (±S.E.) standard length (*SL*, mm, cubes, left y-axis) and dry weight (*DW*, µg, circles, right y-axis) against age (days post hatch, dph) at three water temperatures (7, 10 and 13 °C) and three nominal feeding levels (Low, Medium and High).

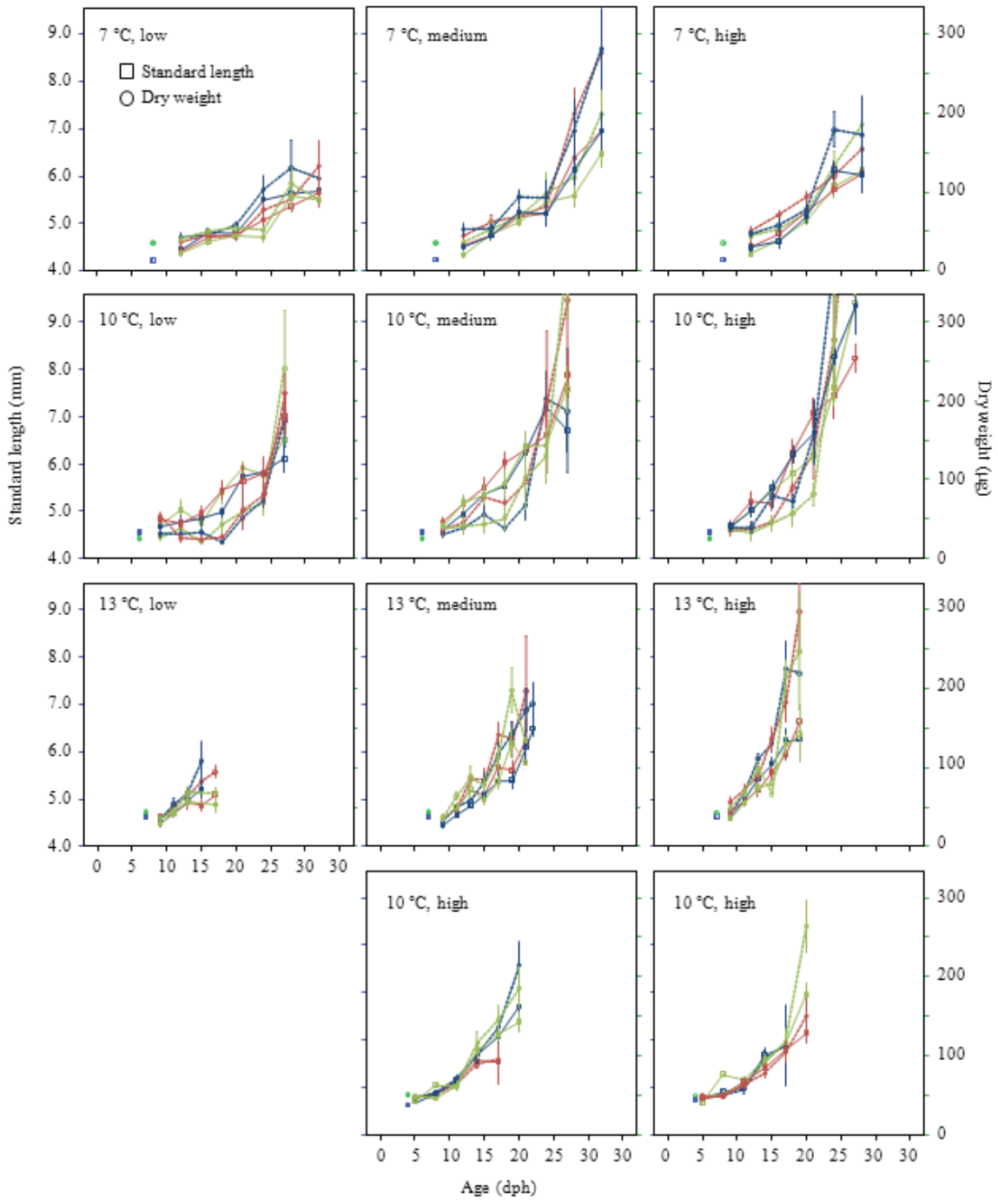


Figure I-3 Eastern Baltic cod larval body size and biochemical condition at age. Given are replicate tank average (±S.E) dry weight (*DW*, µg, circles, left y-axis) and standardized RNA-DNA ratio (*sRD*, dimensionless, triangles, right y-axis) against age (days post hatch, dph) at three water temperatures (7, 10 and 13 °C) and three nominal feeding levels (Low, Medium and High).

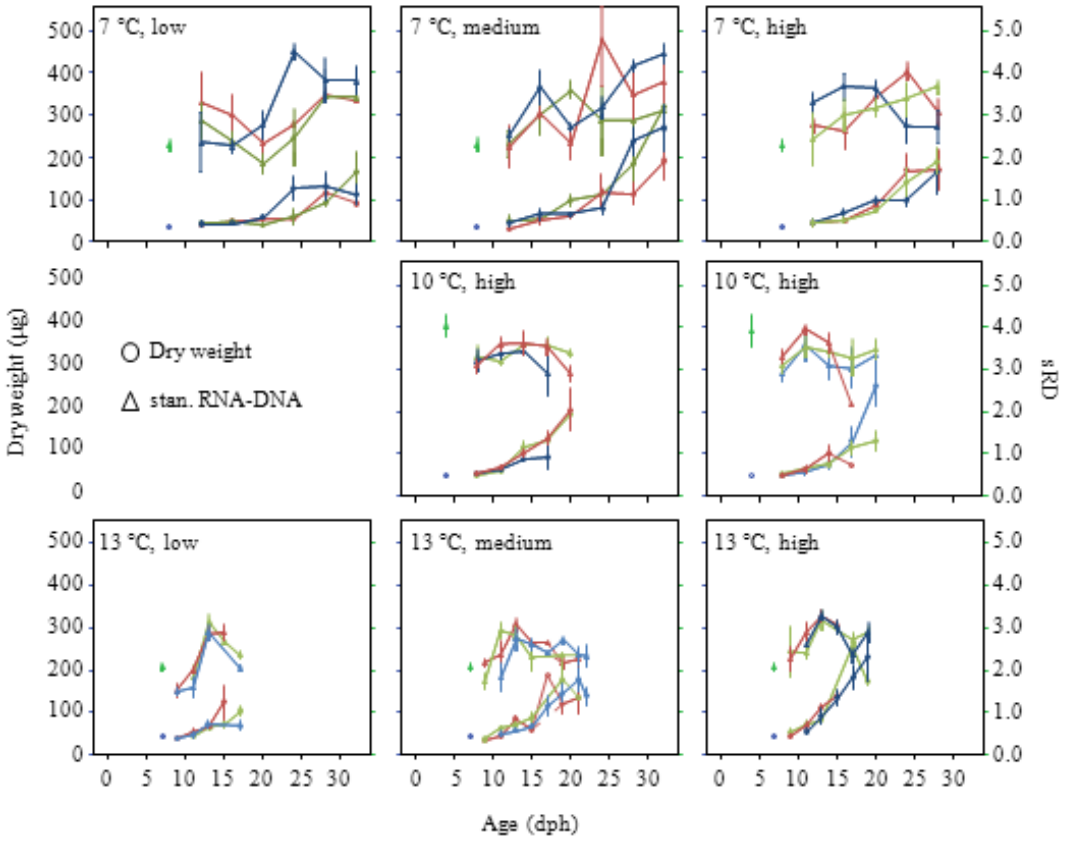


Figure I-4 Eastern Baltic cod length –weight relationship. Given are standard length (*SL*, mm) and dry weight (*DW*, µg) on a log-log-scale for all treatments (temperatures: 7, 10 and 13 °C, nominal feeding levels: High, Medium, Low and food-depleted). Ln(*SL*)-ln(*DW*) – regression lines are given for the best model fit for the whole data set (model (C), red line) and for each respective treatment (black lines).

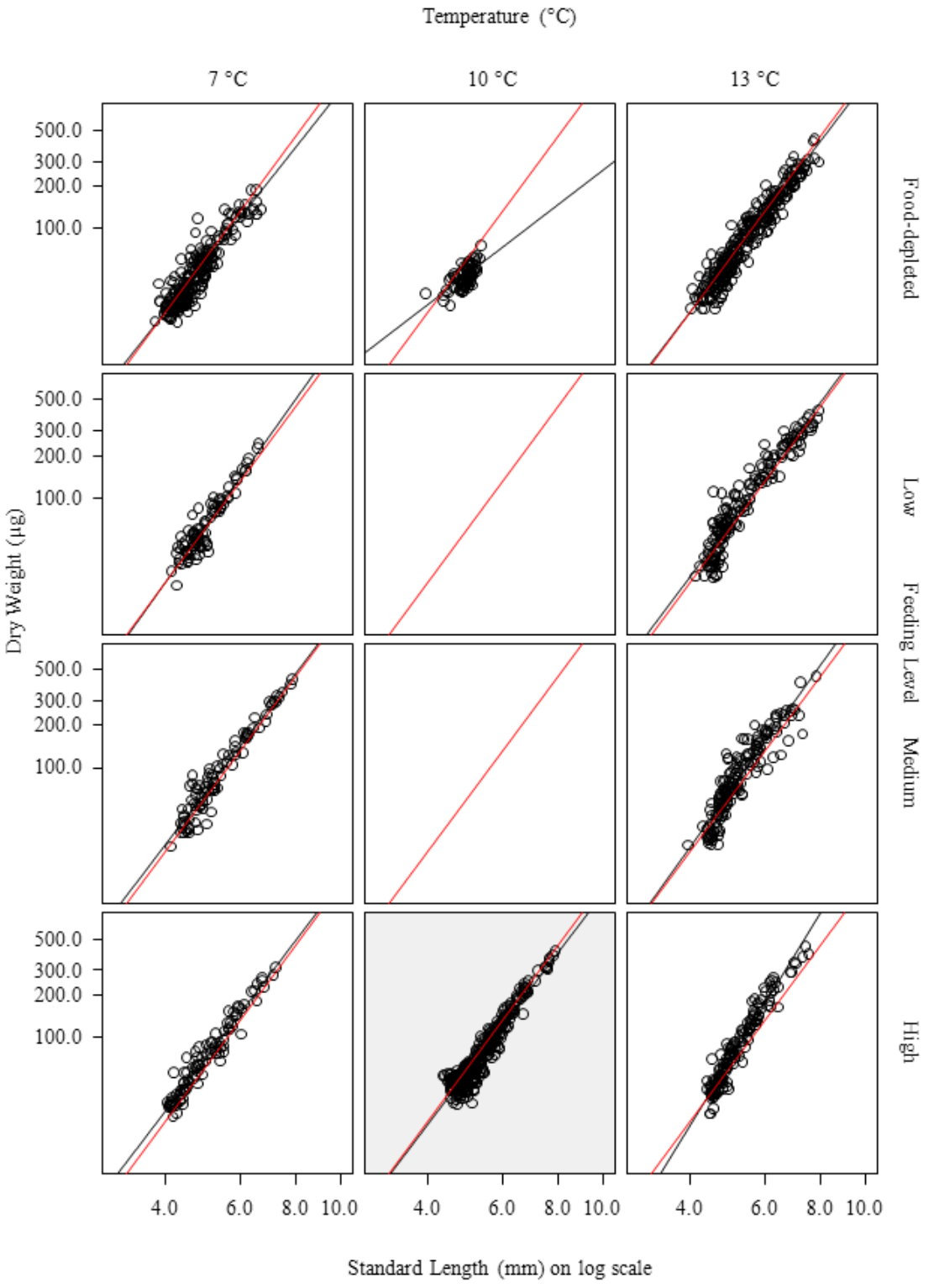
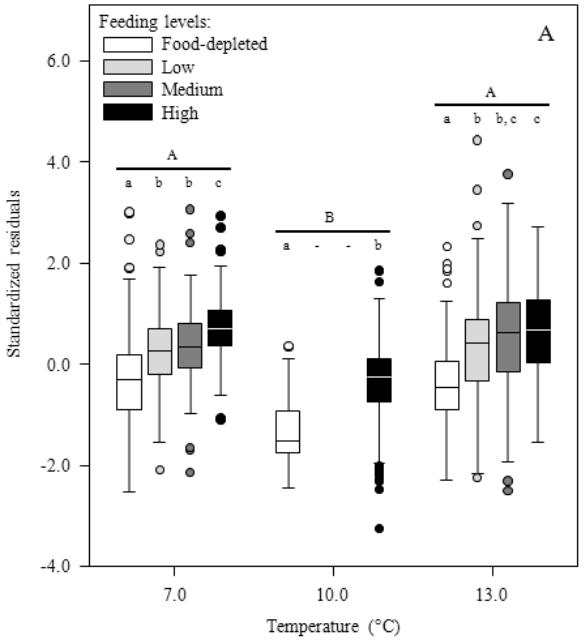


Figure I-5 Eastern Baltic cod length –weight relationship regression model residuals. A: Boxplots of standardized residuals from a length – weight regression model (C) for each treatment (temperatures 7, 10 and 13 °C, nominal feeding levels High, Medium, Low and food-depleted). Significant differences between temperature groups are indicated by capital letters and significant differences between feeding levels within a temperature group are indicated by lower case letters. B: Boxplots of standardized residuals from a length – weight regression model (C) for low feeding level treatments at three temperatures against days of food-depletion. Lower case letters indicate significant difference between residuals within one temperature group.



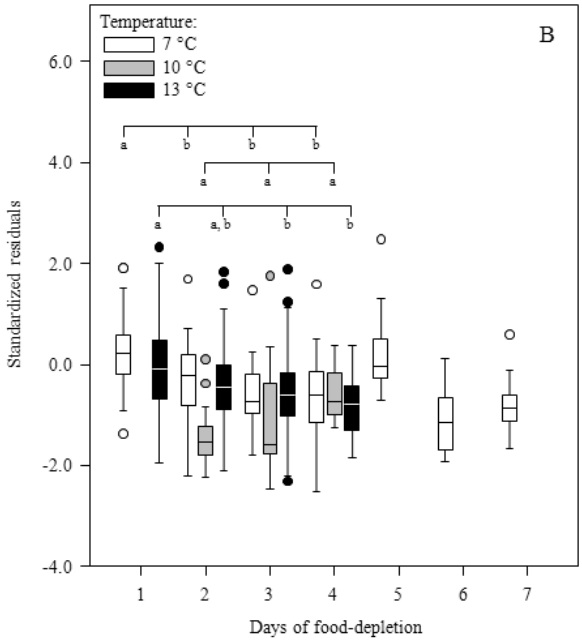


Figure I-6 Dry weight-specific instantaneous rates of growth of Eastern Baltic cod larvae, type 3, retrospective (y= -3) of 52 replicate tanks from experiments A (7 °C), B-2 (10 °C) and C (13 °C) including all feeding levels plotted against replicate tank average *sRD* values. These *G – sRD* duplets were used to parameterize a *G – sRD* back calculation model (see Table I-3) with a predictive capacity of 69 %. The *G – sRD* model regression lines are given. Error bars indicate standard error of *sRD* mean (x-axis) and standard error of *G – sRD* model estimate (y-axis).

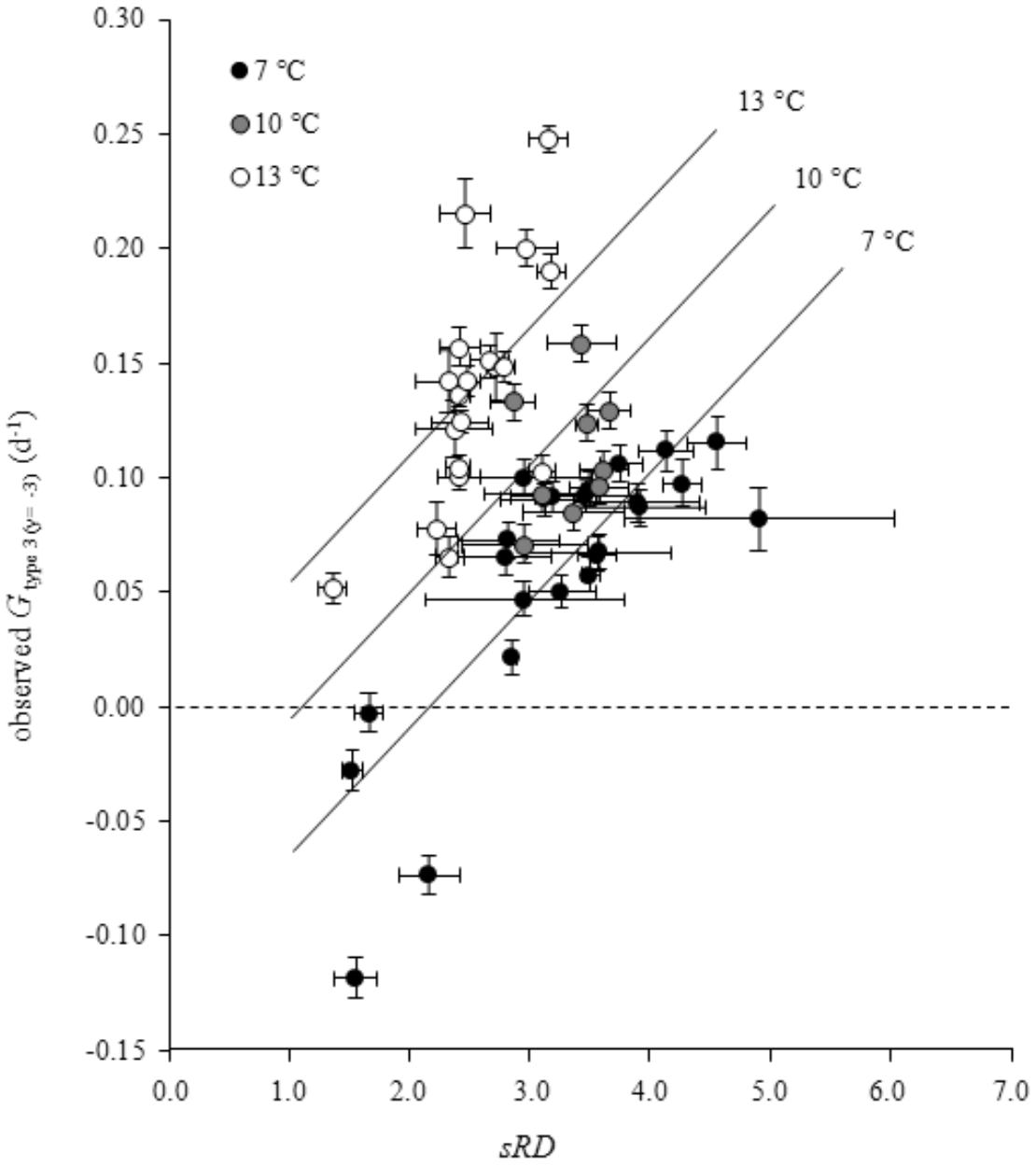


Figure I-7 Eastern Baltic cod larval otolith metrics. Ln-transformed sagitta diameter plotted against age (dph, A), standard length (mm, B), ln-transformed dry weight (dimensionless, C) and ln-transformed lapillus diameter against age (dph, A), standard length (mm, B), ln-transformed dry weight (dimensionless, C). Symbols indicate water temperature. These graphs include up 341 larvae (895 otoliths) from all feeding levels.

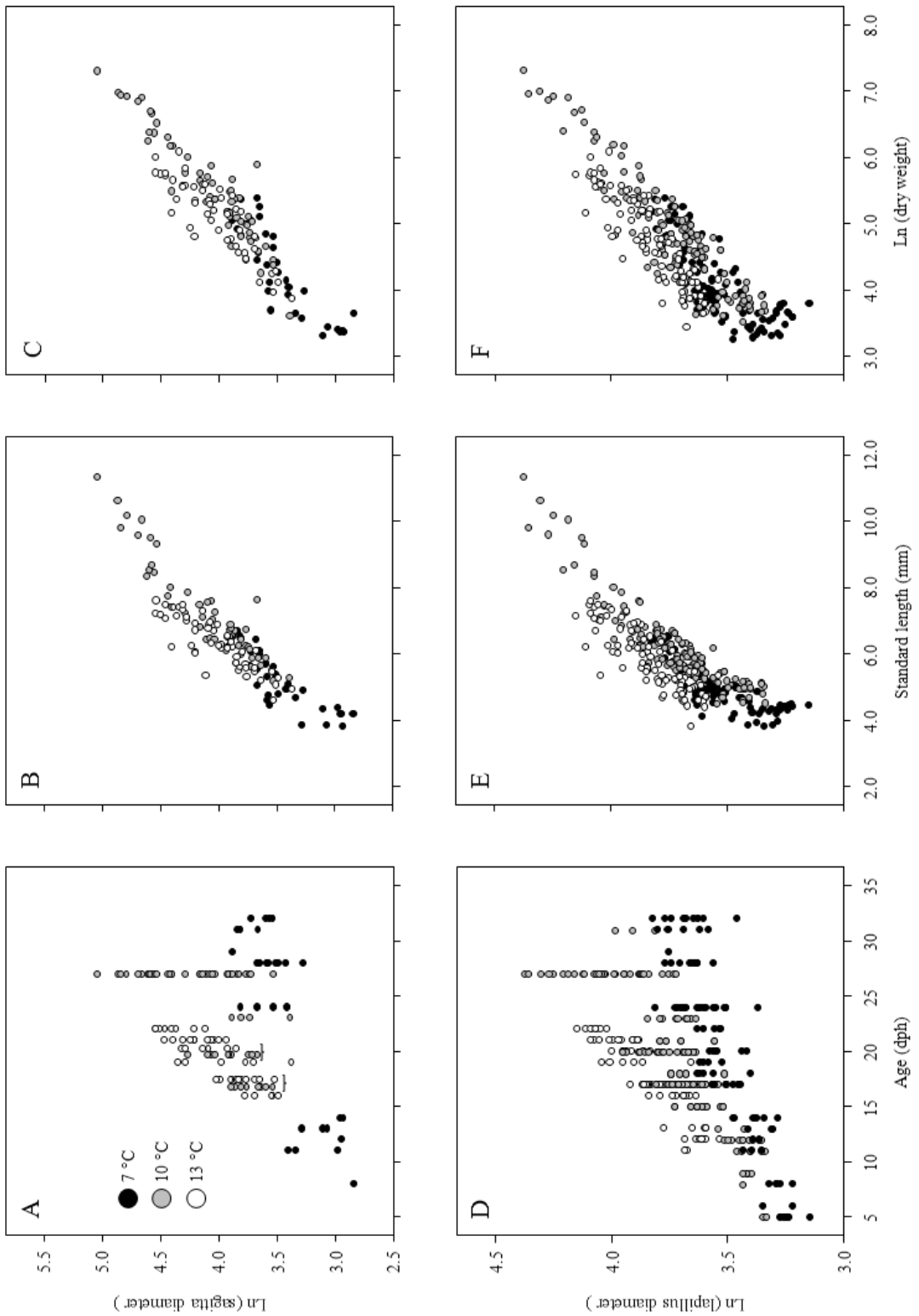


Figure I-8 Eastern Baltic cod larval otolith size against growth rate. Ln-transformed lapillus diameter plotted against dry weight specific growth rate (% d^{-1}) calculated with growth rate type 2 ("stanza") for individual larvae. Symbols and regression lines indicate feeding levels.

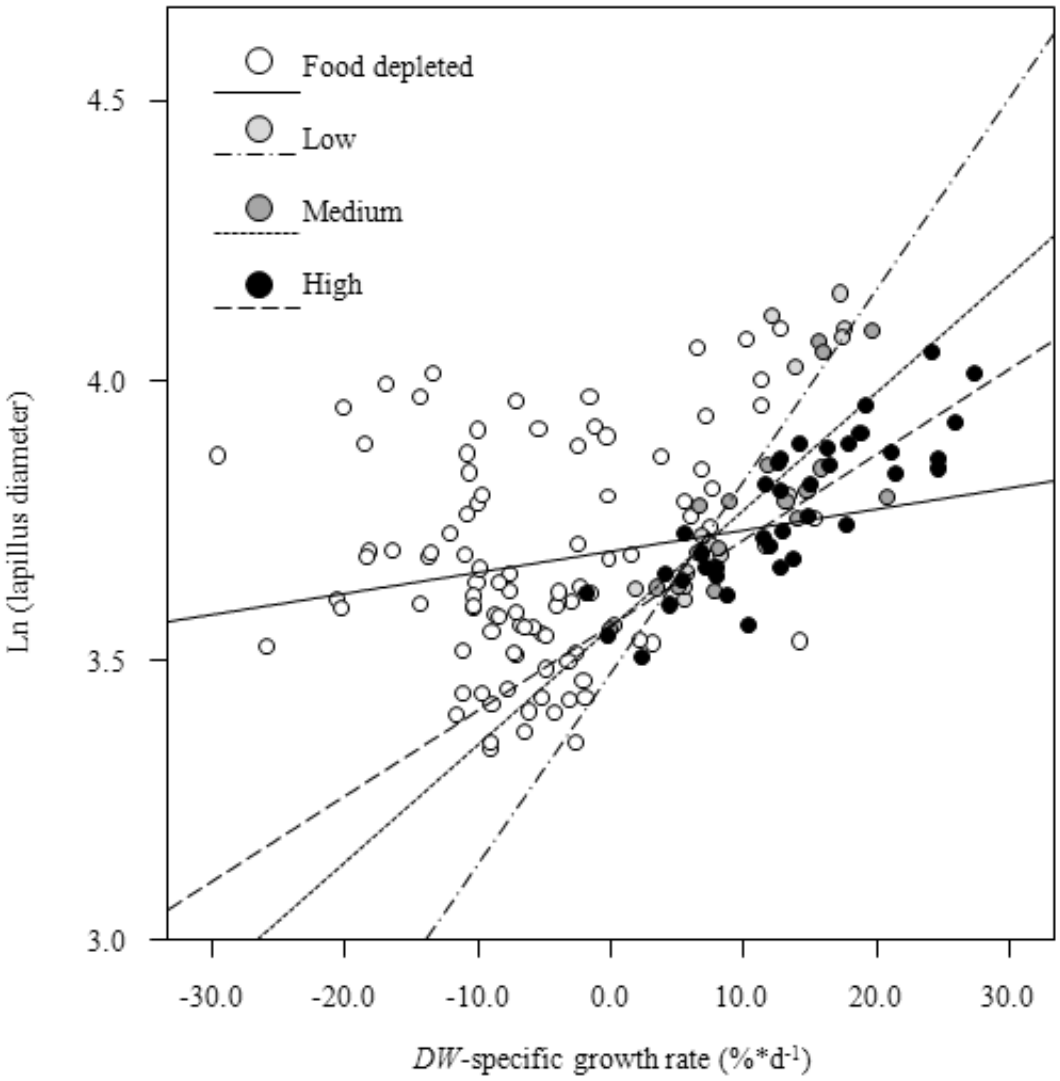


Figure I-9 Body size effects in various Eastern Baltic cod larval growth and condition models. Standardized residuals of A: length – weight relationship model (C), B: length – otolith size model (G), C: instantaneous rate of growth, type 3 (y= -3) – sRD – T model (Table I-3), and D: instantaneous rate of growth, type 2 ("stanza") – sRD – T model (R, without positive growth rates of food depleted larvae) plotted against ln-transformed dry weight. Symbols indicate water temperature. Inlets: same plots, symbols indicate feeding levels.

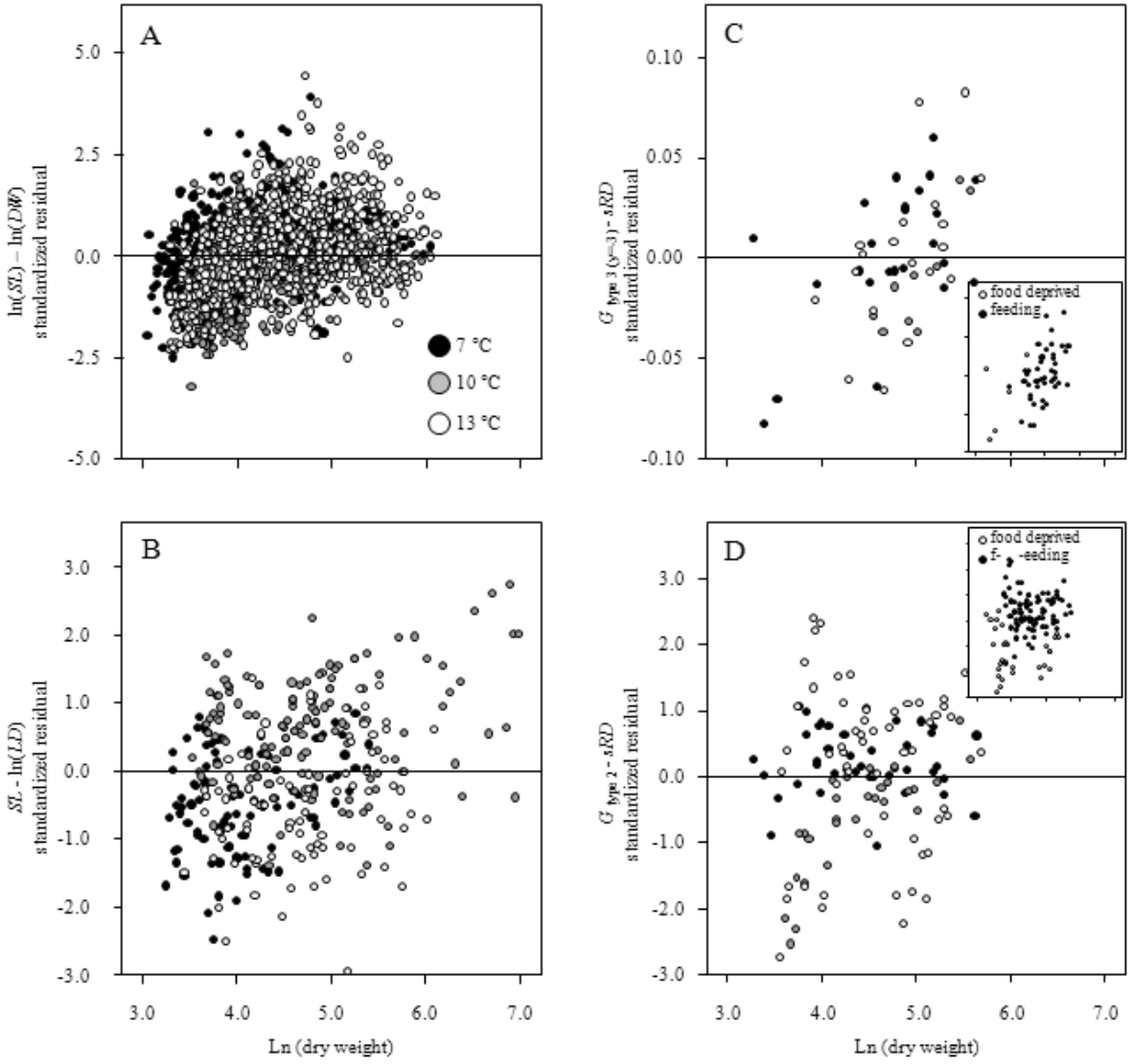
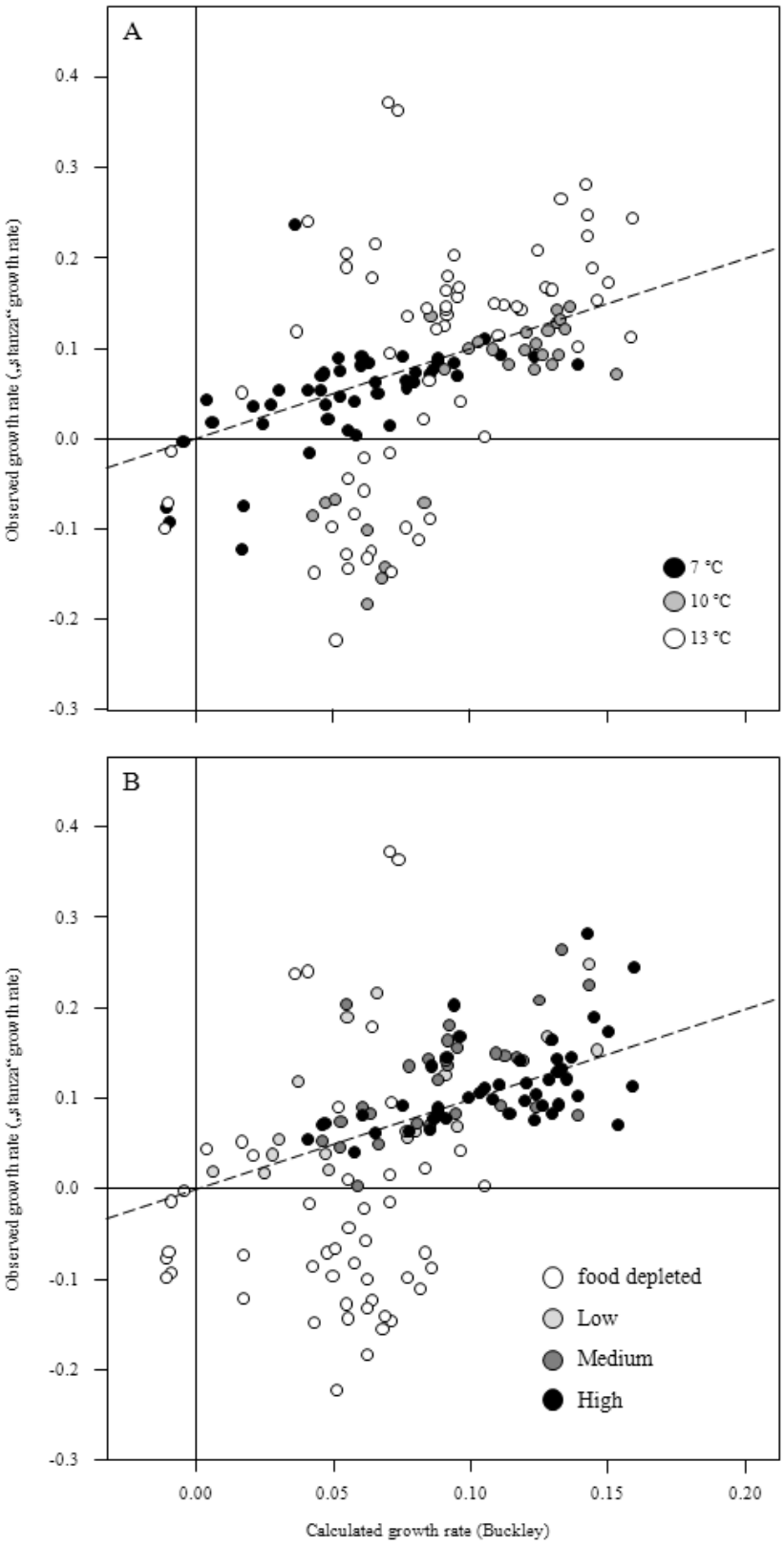


Figure I-10 Dry weight-specific instantaneous rates of growth (% d^{-1}) of Eastern Baltic cod larvae, type 2 ("stanza") plotted against (protein-specific) growth rates calculated with a previously published sRD-T-G model (Buckley et al., 2008), A: symbols indicate water temperature, B: symbols indicate feeding levels. Dashed line: y=1*x.



7. Tables

Table I-1 Summarized information on Eastern Baltic cod larval growth experiments A (7 °C), B-1 (10 °C), B-2 (10°C) and C (13 °C) at three different, nominal feeding levels. Given are replicate tank-wise age (dph) at initial sampling (coinciding with 50 % first feeding) and final sampling, the number of sampling events, final body size (*SL* and *DW*) and the type 1 ("life-time") growth rate parameters (*SL*: absolute growth rate, *AGR*; *DW*: instantaneous growth rate, *G*) of each replicate tank. * indicate significant fit of type 1 growth rate regression model.

Exp ID	T (°C)	Nom. feeding level	Tank No.	Initial age (dph)	Final age (dph)	No. of sampl. events	Final *SL* (mm) ±SD	Final *DW* (µg) ±SD	Type 1 AGR_{SL} ($mm*d^{-1}$)	Type 1 G_{DW} (d^{-1})
A	7	High	1	8	28	6	6.0 ±0.9	173.1 ±106.5	0.103 ±0.017*	0.087 ±0.012*
			2	8	28	6	6.1 ±0.8	154.8 ±92.0	0.094 ±0.005*	0.073 ±0.003*
			3	8	28	6	6.1 ±0.4	185.6 ±45.9	0.101 ±0.012*	0.085 ±0.009*
		Medium	4	8	32	7	6.5 ±0.6	198.9 ±81.5	0.088 ±0.009*	0.072 ±0.006*
			5	8	32	7	7.0 ±0.9	281.2 ±114.9	0.106 ±0.015*	0.082 ±0.009*
			6	8	32	7	6.9 ±0.8	275.8 ±100.1	0.110 ±0.016*	0.084 ±0.011*
		Low	7	8	32	7	5.7 ±0.4	117.7 ±38.6	0.067 ±0.007*	0.059 ±0.008*
			8	8	28	6	5.6 ±0.6	111.0 ±47.2	0.056 ±0.014*	0.046 ±0.013*
			9	8	32	7	5.7 ±0.7	133.3 ±71.9	0.059 ±0.003*	0.056 ±0.008*
B-1	10	High	1	6	27	8	9.3 ±1.7	-	0.227 ±0.028*	-
			2	6	27	8	9.0 ±1.6	-	0.192 ±0.030*	-
			3	6	27	8	8.2 ±0.8	-	0.183 ±0.017*	-
		Medium	4	6	27	8	6.7 ±1.3	-	0.120 ±0.011*	-
			5	6	27	8	7.9 ±1.3	-	0.149 ±0.013*	-
			6	6	27	8	7.6 ±1.4	-	0.134 ±0.016*	-
		Low	7	6	27	8	6.9 ±0.9	-	0.097 ±0.015*	-
			8	6	27	8	6.5 ±1.4	-	0.086 ±0.014*	-
			9	6	27	8	6.1 ±0.9	-	0.082 ±0.009*	-
B-2	10	High	1	4	17	6	5.5 ±0.7	94.6 ±70.0	0.076 ±0.009*	0.057 ±0.009*
			2	4	20	7	6.4 ±0.7	185.9 ±82.8	0.115 ±0.011*	0.093 ±0.013*
			3	4	20	7	6.8 ±0.7	234.6 ±104.8	0.133 ±0.013*	0.097 ±0.010*
			4	4	20	7	7.0 ±0.7	264.0 ±106.1	0.123 ±0.020*	0.096 ±0.016*
			5	4	20	7	6.1 ±0.7	150.4 ±70.7	0.089 ±0.008*	0.069 ±0.009*
			6	4	17	6	5.9 ±0.8	113.0 ±89.8	0.091 ±0.015*	0.070 ±0.013*
C	13	High	1	7	19	7	7.4 ±1.0	407.8 ±182.1	0.215 ±0.031*	0.200 ±0.018*
			2	7	19	7	6.7 ±0.8	297.5 ±117.7	0.170 ±0.018*	0.152 ±0.014*
			3	7	19	7	6.4 ±1.0	247.4 ±125.8	0.177 ±0.022*	0.152 ±0.029*

Medium	4	7	21	8	6.2 ±1.1	197.8 ±154.1	0.111 ±0.015*	0.123 ±0.014*
	5	7	19	7	6.2 ±0.7	198.0 ±65.1	0.113 ±0.026*	0.123 ±0.019*
	6	7	23	9	6.3 ±0.8	214.1 ±102.5	0.115 ±0.015*	0.121 ±0.010*
Low	7	7	17	7	4.9 ±0.3	67.6 ±11.9	(0.043 ±0.016)^{ns}	(0.110 ±0.059)^{ns}
	8	7	17	7	5.1 ±0.4	95.0 ±32.9	0.046 ±0.009*	0.097 ±0.018*
	9	7	15	6	5.1 ±0.4	153.3 ±77.0	0.070 ±0.020*	0.160 ±0.049*

Table I-2 Summarized information on Eastern Baltic cod larval food deprivation trial in experiments A (7 °C), B-1 (10 °C), B-2 (10°C) and C (13 °C). Given are replicate tank-wise age (dph) at initial sampling (onset of food deprivation) and final sampling, the presence of yolk reserves (*YS*), initial stocking density, number of sampling events, initial body size (*SL* and *DW*) and the type 1 ("life-time") growth rate parameters (*SL*: absolute growth rate, *AGR*; *DW*: instantaneous growth rate, *G*) of each replicate tank. * indicate significant fit of type 1 growth rate regression model.

Exp ID	T (°C)	Trial #	Initial Age (dph)	Final Age (dph)	*YS*	Initial stocking ($tank^{-1}$)	No. of Sampl. Events	Initial *SL* (mm)	Initial *DW* (µg)	Type 1 AGR_{SL} ($mm*d^{-1}$)	Type 1 G_{DW} (d^{-1})
A	7	1	8	13	+	100	6	4.2	35.9	n.s.	-0.060*
		2	12	16	-	50	5	4.5	42.6	n.s.	-0.108*
		3	17	24	-	50	6	4.8	61.4	n.s.	n.s.
		4	25	31	-	30	4	5.2	88.0	n.s.	n.s.
B-1	10	5	6	13	+	100	8	4.5	-	-0.060	-
		6	9	15	-	100	7	4.8	-	-0.072	-
		7	13	18	-	100	6	4.9	-	n.s.	-
		8	16	21	-	100	6	5.4	-	n.s.	-
		9	19	25	-	50	4	5.7	-	n.s.	-
		10	23	32	-	50	6	6.7	-	n.s.	-
B-2	10	16	9	13	-	30	4	5.0	54.4	n.s.	-0.082*
C	13	11	7	10	+	75	4	4.6	43.5	n.s.	n.s.
		12	10	13	-	75	4	4.9	61.5	n.s.	-0.114*
		13	13	17	-	75	5	5.4	95.6	n.s.	n.s.
		14	16	19	-	75	3	5.9	172.8	n.s.	n.s.
		15	18	21	-	75	3	6.3	214.0	n.s.	n.s.

Table I-3 Eastern Baltic cod larval growth rate types against *sRD* model parameters. Dry weight-specific instantaneous rates of growth (G, d^{-1}) were calculated, using the six different types (methods) described above, for the time interval or integral between two samplings (later point in time: ($i+x$) and earlier point in time: ($i+y$)) and in perspective to the sampling event of *sRD* (i = 1, …, n). Included are all valid duplets of growth rate and tank-wise average *sRD* containing at least 3 individual larvae. All feeding levels are equally included (food-depleted to ad libitum). The number of duplets (N) is different between the growth rate types, because of the different time intervals / integrals involved in growth rate calculation. Significant model parameters (±S.E.) are indicated with p<0.05: * and p<0.001: ** or are marked as not significant (n.s.). The parameterized models were tested with an omnibus-test (p<0.05: *and p<0.001: **; n.s. not significant). Pseudo-R^2 values are derived from multiple linear regression model fits (results not shown). Relative predictive capacity is the fraction of model pseudo-R^2 in relation to *sRD*T* model R^2 (grey: highest fraction with all sign. parameters). Akaike's Information Criteria (AIC) are given for models using the same data set (fitting with different parameters within a growth rate type, grey: lowest AIC).

Growth rate type	perspective	*(i+x)* & *(i+y)*	*N* of *G* – *sRD* duplets	*sRD*	*T*	*sRD*T*	intercept	p	R^2	Rel. pred.	AIC
Type 1, "life time"	simultaneous	i=1, …, n	55	0.129±0.134 n.s.	0.016±0.031 n.s.	-0.005±0.003 n.s.	-0.365±0.335 n.s.	n.s.	0.127	1.98	-65.1
				0.084±0.030*	0.006±0.007 n.s.	-	-0.258±0.125*	*	0.126	1.97	-67.0
				-	-0.013±0.007 n.s.	0.008±0.003*	-0.051±0.076 n.s.	*	0.113	1.77	-66.2
				0.062±0.032 n.s.	-	0.002±0.003 n.s.	-0.196±0.075*	*	0.123	1.92	-66.8
				-	-	0.005±0.003 n.s.	-0.121±0.066 n.s.	n.s.	0.064	1.00	-65.2
				0.074±0.027*	-	-	-0.174±0.068*	*	0.115	1.80	-68.3
Type 2, "stanza"	retrospective	x=0, y<x	141	-0.017±0.046 n.s.	-0.010±0.012 n.s.	0.010±0.005*	-0.058±0.133 n.s.	**	0.274	1.43	-256.7
				0.081±0.013**	0.016±0.003**	-	-0.321±0.059**	**	0.249	1.30	-253.9
				-	-0.005±0.003 n.s.	0.008±0.001**	-0.105±0.035*	**	0.192	1.01	-258.6
				0.018±0.013 n.s.	-	0.007±0.001**	-0.159±0.034**	**	0.271	1.42	-258.1
				-	-	0.007±0.001**	-0.135±0.030**	**	0.191	1.00	-258.2
				0.054±0.012**	-	-	-0.080±0.034*	**	0.126	0.66	-234.4
Type 3 "single step"	retrospective	x=0, y=-1	141	0.085±0.089 n.s.	0.023±0.023 n.s.	-0.002±0.009 n.s.	-0.337±0.254 n.s.	*	0.076	2.00	-74.3
				0.068±0.023*	0.018±0.006*	-	-0.291±0.111*	*	0.076	2.00	-76.2
				-	0.001±0.007 n.s.	0.006±0.002*	-0.100±0.067 n.s.	*	0.044	1.16	-75.4
				0.002±0.025 n.s.	-	0.006±0.002*	-0.097±0.065 n.s.	*	0.070	1.84	-75.3
				-	-	0.006±0.002*	-0.094±0.056 n.s.	*	0.038	1.00	-77.3
				0.038±0.022 n.s.	-	-	-0.020±0.060 n.s.	n.s.	0.014	0.37	-70.2

Table I-3 continued.

Type 3 “single step”	retrospective	x=0, y=-2	98	-0.006±0.045 n.s.	-0.005±0.012 n.s.	0.008±0.004 n.s.	-0.062±0.130 n.s.	**	0.301	1.77	-196.5
				0.071±0.013**	0.016±0.004**	-	-0.270±0.060**	**	0.278	1.64	-195.4
				-	-0.003±0.004 n.s.	0.007±0.001**	-0.078±0.036*	**	0.173	1.02	-198.5
				0.011±0.014 n.s.	-	0.006±0.001**	-0.112±0.035*	**	0.300	1.76	-198.4
				-	-	0.007±0.001**	-0.097±0.030*	**	0.170	1.00	-199.7
				0.048±0.013**	-	-	-0.045±0.036 n.s.	**	0.129	0.76	-178.9
Type 3 “single step”	retrospective	x=0, y=-3	52	0.042±0.027 n.s.	0.015±0.009 n.s.	0.002±0.003 n.s.	-0.223±0.078*	**	0.692	2.78	-191.7
				0.056±0.007**	0.020±0.002**	-	-0.261±0.035**	**	0.690	2.77	-193.4
				-	0.003±0.002 n.s.	0.006±0.001**	-0.107±0.021**	**	0.421	1.69	-191.4
				-0.005±0.008 n.s.	-	0.007±0.001**	0.089±0.024**	**	0.672	2.70	-190.6
				-	-	0.007±0.001**	-0.099±0.020**	**	0.249	1.00	-192.0
				0.024±0.012*	-	-	0.026±0.035 n.s.	*	0.077	0.31	-138.7
Type 3 “single step”	retrospective	x=0, y=-4	28	-0.018±0.030 n.s.	0.004±0.009 n.s.	0.004±0.003 n.s.	0.028±0.094 n.s.	**	0.734	1.50	-133.6
				0.012±0.010 n.s.	0.013±0.002**	-	-0.057±0.048 n.s.	**	0.723	1.48	-134.5
				-	0.009±0.002**	0.002±0.001 n.s.	-0.025±0.022 n.s.	**	0.731	1.50	-135.3
				-0.029±0.006**	-	0.005±0.001**	0.062±0.030*	**	0.732	1.50	-135.4
				-	-	0.005±0.001**	-0.043±0.030 n.s.	**	0.488	1.00	-119.3
				-0.035±0.009**	-	-	0.215±0.028**	*	0.350	0.72	-112.6
Type 4 “regression”	retrospective	x=0, y=-2	75	0.065±0.030*	0.024±0.009*	-0.002±0.003 n.s.	-0.265±0.087*	**	0.557	2.73	-245.9
				0.046±0.008**	0.018±0.002**	-	-0.211±0.036**	**	0.554	2.72	-247.4
				-	0.004±0.003 n.s.	0.005±0.001**	-0.076±0.021**	**	0.371	1.82	-243.1
				-0.008±0.009 n.s.	-	0.006±0.001**	-0.048±0.027 n.s.	**	0.517	2.53	-241.4
				-	-	0.006±0.001**	-0.063±0.020*	**	0.204	1.00	-242.7
				0.024±0.011*	-	-	0.035±0.035 n.s.	*	0.056	0.27	-193.2

Table I-3 continued.

Type 4 “regression”	retrospective	x=0, y=-3	49	0.043±0.029 n.s.	0.016±0.010 n.s.	0.001±0.004 n.s.	-0.216±0.084*	**	0.685	3.66	-191.2
				0.052±0.008**	0.019±0.002**	-	-0.241±0.036**	**	0.684	3.66	-193.1
				-	0.002±0.002 n.s.	0.006±0.001**	-0.094±0.020**	**	0.412	2.20	-191.0
				-0.004±0.007 n.s.	-	0.007±0.001**	-0.081±0.026*	**	0.667	3.57	-190.4
				-	-	0.007±0.001**	-0.090±0.020**	**	0.187	1.00	-192.1
				0.014±0.012 n.s.	-	-	0.060±0.036 n.s.	n.s.	0.029	0.16	-140.0
Type 4 “regression”	retrospective	x=0, y=-4	28	-0.026±0.025 n.s.	0.000±0.008 n.s.	0.005±0.003 n.s.	0.056±0.076 n.s.	**	0.777	1.41	-145.3
				0.014±0.008 n.s.	0.012±0.002**	-	-0.057±0.040 n.s.	**	0.754	1.37	-144.5
				-	0.008±0.002**	0.002±0.001*	-0.022±0.018 n.s.	**	0.769	1.39	-146.3
				-0.025±0.005**	-	0.005±0.001**	-0.052±0.024*	**	0.777	1.41	-147.3
				-	-	0.005±0.001**	-0.037±0.025 n.s.	**	0.552	1.00	-129.8
				-0.030±0.008**	-	-	0.198±0.025**	*	0.335	0.61	-118.7
Type 5 “single step”	prospective	x=1, y=0	126	-0.111±0.127 n.s.	-0.039±0.033 n.s.	0.019±0.012 n.s.	0.239±0.363 n.s.	*	0.068	0.89	-18.1
				0.082±0.032*	0.012±0.008 n.s.	-	-0.284±0.147 n.s.	*	0.049	0.64	-17.7
				-	-0.011±0.009 n.s.	0.009±0.003*	-0.070±0.085 n.s.	*	0.097	1.28	-19.4
				0.032±0.033 n.s.	-	0.005±0.003*	-0.174±0.089 n.s.	*	0.057	0.75	-18.8
				-	-	0.007±0.003*	-0.124±0.073 n.s.	*	0.076	1.00	-19.8
				0.062±0.029*	-	-	-0.110±0.082 n.s.	*	0.034	0.45	-17.6
Type 6 “regression”	simultaneous	x=1, y=-1	83	-0.010±0.052 n.s.	0.002±0.014 n.s.	0.005±0.005 n.s.	-0.029±0.157 n.s.	**	0.210	1.00	-177.1
				0.040±0.014*	0.016±0.004**	-	-0.170±0.064*	**	0.201	0.96	-178.1
				-	0.005±0.004 n.s.	0.004±0.001*	-0.057±0.037 n.s.	**	0.224	1.07	-179.0
				-0.017±0.014 n.s.	-	0.006±0.001**	-0.007±0.040 n.s.	**	0.210	1.00	-179.1
				-	-	0.005±0.001**	-0.034±0.032 n.s.	**	0.209	1.00	-179.6
				0.015±0.014 n.s.	-	-	0.062±0.041 n.s.	n.s.	0.014	0.07	-162.7

Manuscript II:

II. On the edge of death: Rates of decline and lower thresholds of biochemical condition in food-deprived fish larvae and juveniles

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Abstract

Gaining reliable estimates of how long fish early life stages can survive without feeding and how starvation rate and time until death are influenced by body size, temperature and species is critical to understanding processes controlling mortality in the sea. The present study is an across-species analysis of starvation-induced changes in biochemical condition in early life stages of nine marine and freshwater fishes. Data were compiled on changes in body size (dry weight, *DW*) and biochemical condition (standardized RNA-DNA ratio, *sRD*) throughout the course of starvation of yolk-sac and feeding larvae and juveniles in the laboratory. In all cases, the mean biochemical condition of groups decreased exponentially with starvation time, regardless of initial condition and endogenous yolk reserves. A starvation rate for individuals was estimated from discrete 75^{th} percentiles of sampled populations versus time (degree-days, *Dd*). The 10^{th} percentile of *sRD* successfully approximated the lowest, life-stage-specific biochemical condition (the edge of death). Temperature could explain 59 % of the variability in time to death whereas *DW* had no effect. Species and life-stage-specific differences in starvation parameters suggest selective adaptation to food deprivation. Previously published, interspecific functions predicting the relationship between growth rate and *sRD* in feeding fish larvae do not apply to individuals experiencing prolonged food deprivation. Starvation rate, edge of death, and time to death are viable proxies for the physiological processes under food deprivation of individual fish pre-recruits in the laboratory and provide useful metrics for research on the role of starvation in the sea.

Keywords

RNA-DNA ratio, starvation rate, mortality threshold, time to death, percentile approach

1. Introduction

The recruitment (year class) strength of marine fish species can vary by orders of magnitude between years and is normally governed by processes that affect mortality rates during the first year of life (Houde, 2008). For example, changes in prey availability, resulting from temporal and spatial matches and mismatches of larvae and their prey, can alter larval growth rates and consequently the duration of the pre-recruit period when larvae are particularly susceptible to predation mortality (Bailey and Houde, 1989). Depending upon the degree of mismatch with prey production and availability, food-deprived larvae may die of starvation or weakened larvae may be more vulnerable to predation (Skajaa et al., 2004). It is therefore critical to not only assess the degree of food limitation in the sea, but to also understand how the physiological process of starvation changes with species and/or life stage to gain a mechanistic understanding of the role that prey deprivation plays in the recruitment process.

The nutritional condition of marine fish early life stages has been evaluated using the ratio of nucleic acids (RNA-DNA ratio, *RD*) for more than two decades (Buckley, 1984; Buckley et al., 2008). RNAs are essential for the biosynthesis of proteins and can vary depending on nutritional condition, while DNA levels in a cell remain fairly constant (Buckley et al., 1999; Bulow, 1987). Recently, an inter-calibration of *RD* measurements derived from different fluorometric protocols (Caldarone et al., 2006) has allowed multi-species comparisons of protein-specific growth rates and *RD* in marine fish larvae resulting in a general model relating growth rate and *RD* to temperature (Buckley et al., 2008). During food deprivation, declines in *RD* have been observed in larvae of Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*), reflecting the cessation of protein synthesis and somatic growth (e.g., Clemmesen, 1994; Grønkjær et al., 1997; Suneetha et al., 1999). Still, *RD* is not usually considered an indicator of starvation because some life stages and species can compensate for extended periods of food deprivation, either by catabolizing energy reserves within muscle and liver tissue or by utilizing embryonic yolk reserves, sometime leading to ambiguous patterns in changes in *RD*. Direct methods to describe and quantify the phenomenon of starvation have included observations on feeding success (gut content) (Bochdansky et al., 2008), measurements of otolith microstructure (Johnson et al., 2002), and histological examination of digestive tissues (Kjørsvik et al., 1991; Theilacker and Watanabe, 1989). However, the potential influences of life stage and/or species relationships on nutritional deficiency, *RD*, and starvation in marine fish early life stages are not yet clarified.

Laboratory trials have contributed a wealth of knowledge on factors and processes affecting larval growth and feeding, and have often identified clear inter-individual differences in vital rates. Such phenotypic variability may arise from physiology (Peck et al., 2004a) or genetic differences among individuals (Case et al., 2006; Clemmesen et al., 2003; Saborido-Rey et al., 2003), and can be exacerbated by behavioral interactions among individuals (Moran, 2007). Despite the best efforts to reduce this variability, laboratory-reared groups often contain individuals having different nutritional or growth status. Inter-individual differences in growth potential are likely to cause differences in the responses of larvae to food deprivation (e.g., time to mortality, ability to re-establish feeding) but, to our knowledge, this aspect of starvation response was unstudied. For example, laboratory trials normally use group mean values to describe the time course of changes in *RD* (Rooker and Holt, 1996;

Suneetha et al., 1999; Tanaka et al., 2008). However, the resulting functional model describing rates of starvation (e.g., rate of decrease in group mean *RD* versus time of food deprivation) will likely underestimate the rate occurring at the level of the individual.

Here, we propose an indirect method for addressing this problem based on the following assumptions: a) the condition of individuals at any point in time is stochastically distributed around a group mean value; b) the underlying function describing decline in *RD* with time of food deprivation is reasonably known (i.e. an exponential function); and c) larvae with a low nutritional condition suffer higher mortality rates than those in good condition (if starvation is the only source of mortality). Computing daily changes in the 75^{th} percentile of *RD* values appears to be a good approach to estimate starvation rate since individuals within upper percentiles represent a discrete sub-group of the population that is unlikely to shift in its relative ranking within a group in the short-term (Folkvord et al., 2009; Paulsen et al., 2009). Larvae in the upper percentiles (i.e., 75^{th} and above) will tend to survive for the longest times and will thus form an ever-increasing portion of the population on subsequent sampling days (Figure 1), yielding a better approximation of the "true" starvation rate. The 90^{th} and 10^{th} percentiles of the sampling population can be categorized as idealized start and end points, representing initial condition at onset of food deprivation and a final condition near starvation-induced mortality, respectively. In our review and synthesis of *RD* research, we attempt to validate this "percentile approach" as a method to represent daily changes in *RD* at the individual level during food deprivation by comparing it to a traditional approach that utilizes group mean values.

Although there is individual variability in starvation rates, ambient temperature and body size will have profound effects on aspects of metabolism, including energy losses due to routine respiration and excretion (Peck and Buckley, 2008; Peck et al., 2008). Protein synthesis and somatic growth rates are no exception to this rule and various methods for generalization and comparison have been proposed. For example, Buckley et al. (2008) reported that the best model describing instantaneous growth rates as a function of *RD* included a temperature interaction term (*T*x*RD*). Folkvord (2005) assessed intra-specific differences of larval cod from two distinct populations with the aid of a coupled size-temperature-growth model while Malzahn et al. (2003) used degree-days to normalize the temperature dependence of somatic growth rates in North Sea houting larvae. The present study attempts to parameterize a common function that quantifies and normalizes the contribution of temperature and body size on the change in biochemical condition (*RD*) of food-deprived individuals within controlled laboratory conditions. Our emphasis was to understand how intrinsic (body size, species) and extrinsic (temperature) factors contribute to differences in the response of individuals to starvation, including the rate of decrease in *RD* and lowest (threshold) values of biochemical condition, and the time to death. Our results are discussed with respect to utilizing *RD* to help understand starvation-induced mortality of larval fishes in the sea.

2. Material and Methods

2.1. Data set overview

We compiled previously published and unpublished data from laboratory-based, food deprivation experiments conducted on nine species of marine and freshwater fish larvae and juveniles, namely Atlantic cod, Atlantic herring, sprat (*Sprattus sprattus*), common goby (*Pomatoschistus microps*), southern flounder (*Paralichthys lethostigma*), vendace (*Coregonus albula*), North Sea houting (*Coregonus oxyrinchus*), haddock (*Melanogrammus aeglefinus*), and sea bream (*Sparus aurata*). Detailed protocols and methods utilized in the experiments are described in the original publications (Table 1). The common feature of all experiments was that groups of well nourished (either newly-hatched yolk-sac or previously *ad libitum* fed) fish larvae were deprived of food for at least three days and sampled (minimum of 5 fish per sample) at the start and on at least two more occasions during the experiment.

The combined data included measurements on 3542 individuals. Paired values of body size and RNA-DNA ratio (*RD*) were acquired from 15 experiments (Table 1). Experiments included distinct trials using different ambient temperatures and/or initial body sizes and life stages (Table 2). Across all trials, water temperature ranged from 2.6 to 24.1 °C and body size (mean initial dry weight, *DW*) ranged from 35.9 µg in young larvae to 43.2 mg in juveniles. Endogenous feeding yolk-sac larvae were included in 21 trials. Trial duration ranged from 2 to 36 days (Table 2). The termination of each trial did not necessarily coincide with fish mortality. To be included in our analysis, sampled larvae from an experiment must have been processed using only one technique for measurement of body size and one single-dye fluorescence-based protocol to determine bulk nucleic acid contents. If not stated otherwise, *DW* was measured after freeze-drying to the nearest 0.1 µg (for < 100 µg individuals) or 1.0 µg (for > 100 µg individuals). In some cases, *DW* was calculated from known relationships to standard length [Experiment H, herring, Harrer (2006); Experiment N, southern flounder, Bolasina et al. (2006) and Qin et al. (2008)], to protein content (Experiment D, cod and Experiment I, haddock, Caldarone, unpublished data), or to wet weight (Experiment J, sprat, Peck et al., 2004b).

2.2. RNA-DNA analysis

RD was measured in crude, whole body or muscle tissue homogenates using the non-specific, nucleic acid intercalating fluorescence dye ethidium bromide (Caldarone et al., 2001; Clemmesen, 1993; Suneetha et al., 1999; Wagner et al., 1998; Westermann and Holt, 1988). Subsequent addition of RNA- (and in some cases DNA-) specific restriction enzymes allowed the quantification of RNA and DNA in the same homogenate. In all protocols, standard reference materials (purified nucleic acids standards for RNA and DNA) were used to convert fluorescence yields into nucleic acid concentrations. In cases where only DNA standards were used, the slope of the RNA standard curve was assumed to be 2.2 times lower than for DNA (Le Pecq and Paoletti, 1966). All *RD* values were standardized based on the assay-specific ratio of the slopes of the standard curves (DNA slope/RNA slope), standardized to a reference slope ratio of 2.4, as described in Caldarone et al. (2006). Standardized RNA-DNA ratios (*sRD*) are referred to throughout the remainder of this manuscript. Assay-specific slope ratios and standardization factors are provided (Table 1).

Measurements of *sRD* were conducted on either whole body or muscle tissue homogenates (Table 1). We intentionally abandoned any effort to convert muscle tissue *sRD* to whole body *sRD*, realizing the unpredictable effects of differences in dissection protocols and potential differences in cell size (Olivar et al., 2009). However, muscle tissue *sRD* of early life stages was previously demonstrated to be affected by food deprivation and it does respond to the physiological process of starvation. Sample tissue types are therefore indicated throughout the manuscript and tissue types were treated separately in all analysis. We followed a similar approach for endogenous feeding, yolk-sac larvae (Table 2). The contribution of maternal RNA and yolk-sac dry weight substantially affect *sRD* and estimates of body size, and can introduce a bias to modeled growth estimates (Buckley et al., 2006). Nevertheless, yolk-sac larval starvation parameters provide a useful base for comparison of temperature- and species-specific physiological mechanisms of yolk mobilization.

2.3. Linearization of starvation rate

Following a percentile approach, starvation rates were calculated from regression models fit to the 75^{th} percentile of each sampling date *sRD* regressed on the duration (days) of food deprivation. Visual inspection of linear and exponential model residuals confirmed the assumption of an exponential decrease in most trials (data not shown). In order to express starvation rates as a linear relationship, values of *sRD* were natural logarithm- ($\log_e$) transformed. If not stated otherwise, all analyses utilized $\log_e$-transformed 75^{th} percentile *sRD* data. Only starvation rate parameters (rate of decrease and time to death) from significant linear regressions ($p \leq 0.05$) were included in further analysis.

2.4. Temperature-normalization of starvation rate

A subset of data was used to validate the degree-day normalization of starvation rates (Experiment D, #12-14; F, #17-23; I, #40-42; K, #44-48; L, #49+50; M, #52+53; Table 2). Only trials with different rearing temperatures in the same experiment were tested against each other to minimize uncontrolled effects (e.g., maternal/batch effects, body size and species differences). Time of food deprivation (days) was used as covariate in analysis of covariance (ANCOVA) to test for significant differences between starvation rates at different temperatures (independent variable). The ANCOVA was also conducted with temperature-normalized data, expressing time of food deprivation in degree-days (*Dd*). Temperature-normalization was considered effective when a significant effect of temperature (the independent variable) in the original data became insignificant after normalization.

2.5. Body size and life stage effect on starvation rate

A subset of data was analyzed to test the influence of body size and life stage on starvation rates, after temperature-normalization (A, #1-5; B, #6-10; E, #15+16; G, #24-29; G, #30-35; H, #36-39; Table 2). Only trials with different initial dry weight in the same experiment were compared to minimize uncontrolled effects (e.g., maternal/batch effects, species differences). Yolk-sac and exogenous feeding larvae were compared in three larval cod experiments (Experiments A, B and E). Only one experiment (Experiment G, herring) spanned a sufficient body-size range to compare early-stage, exogenous feeding larvae with older-stage larvae. Time of food deprivation, expressed in degree-days (*Dd*), was the covariate and mean initial dry weight the independent variable in an ANCOVA.

2.6. Population percentiles and time to death

Time to death was calculated as:

$$(1) \qquad time\ to\ death = \frac{log_e(sRD_{10th\ perc}) - log_e(sRD_{90th\ perc})}{starvation\ rate_{75th\ perc}}$$

where $\log_e(sRD_{10th\ perc})$ and $\log_e(sRD_{90th\ perc})$ represent the 10th and 90th percentile values of $\log_e$-transformed *sRD* for the sampled population and $starvation\ rate_{75th\ perc}$ represents the temperature-normalized starvation rate based on the 75th percentile of daily $\log_e$-transformed *sRD* (Figure 1). The 10th percentile of *sRD* values in any population of food-deprived individuals that expresses substantial variability in condition is presumed to approximate the lowest level of biochemical condition sustaining life. A direct estimate for this threshold level was difficult to derive from the present dataset because most of the food deprivation trials ended before larval mortality (33 out of 55 trials), and the 75th percentile values of *sRD* calculated on the last sampling day of these trials may not represent larvae ultimately close to death. Therefore, we assumed that the 10th percentile of the full sampled population (pooled over time) was a better approximation for the edge of death. The 90th percentile is thought to represent larvae having the highest species- and life stage-specific *sRD* and thus provides a normalized start-value for the onset of food deprivation.

2.7. Validation of the percentile approach

To evaluate the percentile approach, starvation rates based on the 75th percentile were compared to starvation rates calculated from the arithmetic mean of the population on each sampling day. Additionally, the starvation parameters '10th (edge of death)' and '90th (normalized start-value) percentile of *sRD*' were regressed against observed start- (day 0) and cut-off (final sampling day) values of condition. In the subset of trials that ended with larval mortality, two *sRD*-based estimates of the 'time to death', one calculated using percentile data (Equation 1) and the other using average starting and final *sRD* values ("mean-based"), were regressed against the observed time to death (i.e., trial duration). Only experimental trials yielding a significant slope parameter estimate (for each of the respective regressions) were included in this regression analysis.

In trials using whole body tissue preparations, the 10th percentile *sRD* was converted into somatic growth rates, using a multi-species, temperature-corrected model describing instantaneous growth rates as a function of *sRD* (Buckley et al., 2008) to evaluate this metric for fish larvae and juveniles under food depletion. The model parameters of their equation no. 1 (Buckley et al., 2008), including an interaction term between *sRD* and T, were used for the calculation.

All statistical calculations were performed with PASW Statistics 18 (SPSS Inc.). Criteria of normality, homoscedasticity, homogeneity of regression slopes, and independence of covariate and treatment effects were respected for Analysis of Covariance (ANCOVA) and multiple regressions (Field, 2009). Standardized regression coefficient ß was reported for multiple regressions. Significance level was set to $p \leq 0.05$. If not stated otherwise, means are given ± standard deviation, SD.

3. Results

As expected, *sRD* decreased with time of food deprivation in all 55 food deprivation trials. This was observed in whole body (*wb*) and muscle tissue (*mt*) preparations, and in yolk-sac and exogenous-feeding life stages. Examples of these trends for cod, herring and other species are provided (Figure 2-4). Starvation rates, i.e. significant linear regressions of the 75th percentile of $\log_e$-transformed *sRD* versus time (*Dd*) of food deprivation, were calculated for 34 trials and ranged from -0.0206 $*Dd^{-1}$ in 17 days post hatch (dph) exogenous-feeding cod larvae to -0.0030 $*Dd^{-1}$ in newly hatched, herring yolk-sac larvae (Table 2). Muscle tissue starvation rates ranged from -0.0043 $*Dd^{-1}$ to -0.0050 $*Dd^{-1}$ and were therefore significantly slower than the mean (-0.0091 $*Dd^{-1}$) whole body starvation rate (Mann-Whitney test, U = 5.0, z = -2.52, $p < 0.05$). When considering only whole-body preparations, starvation rates of yolk-sac larvae (-0.0089 ± 0.0047 $*Dd^{-1}$) did not differ significantly from exogenous-feeding larvae rates (-0.0093 ± 0.0038 $*Dd^{-1}$, Mann-Whitney test, U = 97.0, z = -0.689, $p > 0.05$). Starvation rates (mean ± S.D.; no S.D. indicated for n=1 observations) for taxa in the analysis, ranked in descending order, are *G. morhua* -0.0140(0.0043) > *M. aeglefinus* -0.0131 > grand mean -0.0087(0.0041) > *C. harengus* -0.0077(0.0021) > *P. microps* -0.0066(0.0007) > *P. lethostigma* -0.0050 > *C. albula* -0.0048(0.0001) > *C. oxyrinchus* -0.0045 > *S. sprattus* -0.0043 $*Dd^{-1}$. Trials in which the regression slope was not significant (21 trials) included occurrences for all species, temperatures and body sizes (indicating no bias).

3.1. Temperature-normalization of starvation rate

Temperature had a significant influence on $\log_e$-transformed 75th percentile s*RD* before temperature-normalization, i.e. when time of food deprivation was expressed in days (Table 2). Significant differences (ANCOVA) relative to the reference trial (highest temperature in the same experiment) were detected in every experiment in the subset of data, except for Experiment L (*C. oxyrinchus*, yolk-sac larvae). After degree-day normalization, no significant differences relative to the reference trial were detected, indicating that the time of food-depletion (x-axis) was successfully normalized so that differences in the decrease of *sRD* (y-axis) over time became insignificant. The only exception was one trial at the lowest temperature of Experiment K (*P. microps*, exogenous feeding larvae). This experiment spanned the broadest temperature range (14.3 °C) for a single species in the entire dataset. A failing of the *T*-normalization was observed between the two extreme ends of the *T*-range in this experiment, indicating a possible limitation in general applicability of this normalization procedure when applied over such a broad temperature range.

Temperature explained a significant proportion of variability in the starvation rates of the full dataset before *Dd*-normalization (linear regression, $B = -0.0056$ (0.0011), $p < 0.001$, $r^2 = 0.48$; Figure 5A). The slope of the regression indicated that faster starvation rates were found at higher water temperatures. After *Dd*-normalization, the slope of the regression was still negative but it was reduced by more than one order of magnitude (and not significantly different from zero), indicating that the *T*-effect was successfully channeled into *Dd* ($B = -0.0004$ (0.0001), $p = 0.027$, $r^2 = 0.16$; Figure 5C).

3.2. Body size and life stage effect on starvation rate

Body size was found to have a significant effect on starvation rate in four out of five experiments, but only when yolk-sac and exogenous-feeding larvae were equally included in the analysis (Experiments A, B and E). In these three experiments, starvation rates of yolk-sac larvae were significantly different (ANCOVA, Table 2) from the reference category. The reference category had the highest initial dry weight, whereas the yolk-sac larvae had the lowest initial *DW* in the respective experiment. When yolk-sac larvae were excluded, no significant difference was found. From the two experiments that did not contain any yolk-sac stages (Experiment G and H, both on *C. harengus*, dry weight range 207.4 to 6051.1 µg and 215.6 to 537.5 µg, respectively) it is apparent that at approximately 500 µg dry weight there is a break point for detecting body size differences in starvation rate. At sizes smaller than 500 µg dry weight (Experiment H) and heavier than 500 µg dry weight (Experiment G) body size had no effect on starvation rate. Exploratory analysis of starvation rates with respect to body size (here: $\log_{10}$ dry weight) indicated a significant correlation between starvation rate and body size in *Dd*-normalized starvation rates (B = -0.002 (0.001), p = 0.014, r^2 = 0.18; Figure 5D), but not in the original data (B = -0.005 (0.009), p = 0.591, r^2 = 0.01; Figure 5B). Examining starvation rates only in those experimental trials that used exogenous feeding larvae and whole body tissue preparations, increased the proportion of explained variability in starvation rate from 18 to 45 % (B = -0.005 (0.001), p = 0.002, r^2 = 0.45; Figure 5D).

3.3. Population percentiles and time to death

The value of *sRD* at the 10th percentile, the approximation of the lowest biochemical condition sustaining life, spanned almost one order of magnitude (range: 0.3 to 2.3) across all species and life stages (Table 2) and also was variable across temperature (Figure 6A) and body size (Figure 6B). It was significantly correlated with temperature when yolk-sac (B = -0.074 (0.028), p = 0.023, r^2 = 0.42) and exogenous-feeding stages (B = -0.084 (0.021), p = 0.001, r^2 = 0.48) were analyzed separately. The slopes of these regressions were nearly identical, but the intercept for exogenous-feeding larvae was higher (2.4 ± 0.3, estimator ± standard error, S.E.) than for yolk-sac larvae (1.7 ± 0.3). There was no significant correlation with body size (B = 0.260 (0.307), p = 0.409, r^2 = 0.04), but life-stage specific trends for cod and herring larvae were apparent. On average, yolk-sac larvae of these two species had lower 10th percentile values (1.1 ± 0.1, mean ± S.E.) than exogenous-feeding stages (1.7 ± 0.1), whereas life-stage-independent mean values for the 10th percentile *sRD* were very similar (1.5 ± 0.04 and 1.5 ± 0.2). The 90th percentile, the approximation for *sRD* at onset of food deprivation, ranged from 1.0 to 4.9 across species and life stages and was not correlated with water temperature, body size, species, or life stage (Table 2).

Time to death ranged from 33.4 to 409.7 *Dd* (Table 2) and was significantly correlated with temperature in both yolk-sac and exogenous-feeding stages (Figure 7A), but not with body size (Figure 7B). A multiple regression using temperature and body size (*DW*) as predictors explained 59 % of the observed variability, but *DW* alone was not significant (Temperature: B = 10.284 (1.632), ß = 0.76, p < 0.001, change in r^2 = 0.57; Dry weight: p = 0.59). The slopes of the regressions between time to death and temperature in yolk-sac larvae (B = 11.719 (1.797), ß = 0.85, p < 0.001, r^2 = 0.71) and exogenous-feeding larvae (B = 12.396 (2.420), ß =

0.85, $p < 0.001$, $r^2 = 0.72$) were both positive. Time to death increased by about 12 *Dd* with each degree rise in temperature for the two life stages.

3.4. Validation of the percentile approach

Starvation rates based on the 75th percentile *sRD* were regressed against starvation rates calculated from the arithmetic mean *sRD* of daily sampled populations (*B* = 0.997 (0.044), $p < 0.001$, $r^2 = 0.95$). The slope of the regression did not differ significantly from 1 (Confidence Interval (CI) range for slope estimator *B* = 0.907 to 1.088). The 10th percentile *sRD* (edge of death) for trials ending with larval mortality (22 trials, including yolk-sac and exogenous life stages, all tissue types) slightly overestimated the observed mean *sRD* on the last sampling day, but was nevertheless significantly correlated with it (*B* = 1.123 (0.100), $p < 0.001$, $r^2 = 0.86$). The slope of this regression did not differ significantly from 1 (CI range for slope estimator *B* = 0.914 to 1.332). The 90th percentile *sRD* (normalized start-value) of all trials (except for two outliers, both Experiment E) slightly underestimated the observed mean *sRD* at onset of food deprivation (*B* = 0.982 (0.026), $p < 0.001$, $r^2 = 0.96$). The slope of this regression did not differ significantly from 1 (CI range for slope estimator *B* = 0.929 to 1.035).

Calculated time to death was regressed against observed time to death (i.e., trial duration), including only experimental trials ending with larval mortality. The regression slope was significantly lower than 1 (*B* = 0.738 (0.041), $p < 0.001$, $r^2 = 0.97$; CI range for slope estimator *B* = 0.645 to 0.831), indicating an underestimation of trial duration by the percentile-based parameters. Visual inspection of residuals showed that the regression was influenced by a single trial (#49, *C. oxirynchus*, *mt*, yolk-sac stage). When this trial was excluded from the analysis, the regression slope was no longer different from one (*B* = 1.092 (0.108), $p < 0.001$, $r^2 = 0.93$; CI range for slope estimator *B* = 0.844 to 1.340). Time to death, calculated using both the percentile approach and the arithmetic mean, was regressed against trial duration (Figure 8). The percentile approach significantly underestimated trial duration (*B* = 0.793 (0.035), $p < 0.001$, $r^2 = 0.94$; CI range for slope estimator *B* = 0.721 to 0.865), whereas the slope of the population mean based estimator was not significantly different from 1 (*B* = 1.002 (0.044), $p < 0.001$, r^2=0.94; CI range for slope estimator *B* = 0.912 to 1.091). Residuals from a one-to-one line representing 100 % congruence of the calculated and the observed metric were positive in only 5 out of 34 cases, supporting the assertion that the larvae in most of the trials were sampled slightly before they were ultimately close to death. In contrast, in 17 out of 36 cases the population mean-based metric had negative residuals, indicating that predicted time to death was longer than that observed in the trial.

Instantaneous growth rates, calculated from 10th percentiles of whole body *sRD* using a multi-species, temperature corrected model describing instantaneous growth rates as a function of *sRD* (Buckley et al., 2008), ranged from -0.065 to 0.071 $*d^{-1}$ over all food deprivation trials. In yolk-sac stages, most of the calculated growth rates were negative (-0.026 on average, range from -0.063 to 0.008 $*d^{-1}$). In exogenous-feeding larvae, the mean growth rate was positive (0.007, range from -0.043 to 0.061 $*d^{-1}$).

4. Discussion

Natural variations in the abundance of fish stocks can be the result of numerous factors acting on all life stages. The fast growth and high mortality rates observed for larval fish have led researchers to conclude that processes acting during the larval stage have the potential to introduce major variability in recruitment levels of marine fishes. The ability to gain robust in situ growth estimates and distinguish individuals that are growing well from those growing poorly is critical (Bochdansky et al., 2008; Houde, 2008). Starvation and predation are considered to be the most important causes of mortality in the early life stages of fish (Bailey and Houde, 1989). The combination of low ability to detect and escape from predators, and high metabolic rate and limited energy reserves, make larvae vulnerable to mortality via both predation and starvation (Bochdansky et al., 2008; Fuiman and Cowan, 2003).

Biochemical condition (*RD*) is one of the most widely used growth indicators for marine fish early life stages (e.g., Buckley, 1984; Buckley et al., 2008; Chícharo and Chícharo, 2008; Clemmesen et al., 2003). Recent research has standardized this ratio (s*RD*) allowing comparison of measurements made in different laboratories (Caldarone et al., 2006). In our study, we used a novel approach to examine changes in *sRD* in food-deprived individuals to 1) identify species- and body size-specific s*RD* lower threshold values, 2) quantify the rate of change in s*RD* in food-deprived individuals, and 3) estimate the time required to reach threshold *sRD* levels in the early life stages of nine marine and freshwater fish species. These parameters are corner-stones of theories describing mortality via starvation in finfish pre-recruit life stages (Ferron and Leggett, 1994). Additionally, we demonstrated how inter-individual variability in s*RD* can be harnessed to provide better estimates of starvation trajectories using a percentile approach. We argue that the physiological rate of starvation is better estimated using this approach compared to traditional calculations that employ group mean values.

The low *sRD* values of food-deprived individuals in the present study yielded both negative as well as positive growth rates when applying the relationship described by Buckley et al. (2008). Our results suggest that this published general model (Buckley et al., 2008) and species-specific models (e.g., Caldarone, 2005; Caldarone et al., 2003) explaining the relationship between growth rate and *sRD* in feeding fish larvae do not apply to individuals experiencing prolonged food deprivation. In well-nourished individuals, growth potential can be well explained by a generic (inter-specific) relationship including *sRD* values and water temperature (Buckley et al., 2008). However, this generic relationship breaks down during food deprivation, when species- and life stage-specific responses are evident that likely reflect different adaptive strategies during starvation.

4.1. Temperature-normalization of starvation rate

In the present study, temperature effects on starvation rates within species were successfully normalized by the degree-day metric. There was, though, an unexpected positive correlation between temperature and calculated time to death, which was intended to be a temperature-corrected metric. This implies that at higher temperatures fasting larvae of similar size and life stage exhaust their energy reserves later. It can only be speculated if this is a systematic trend that is caused by failure of the degree-day approach to normalize responses at different

temperatures or whether this trend is rooted in life-stage and species-specific thermal sensitivity. Although still not common, the normalization of temperature-effects has helped researchers to reveal causal trends in poikilotherm metabolic rates (Fuiman et al., 1998; Neuheimer and Taggart, 2007). It was also described that for certain behaviorally controlled traits (e.g., swimming activity, prey ingestion and hence the ability to avoid death from starvation) considerable energy is invested to maintain high trait performance across a broader range of temperatures (Dell et al., 2011). A constantly high trait performance over a broad range in temperatures would be impossible to normalize with a simple numerical approach like the *Dd*-normalization. The current dataset is too limited to draw conclusions on the general mechanisms acting here. Further research on the thermal sensitivity of *sRD* in fish early life stages, especially comparing taxa with steno- and eurythermal tolerance ranges, is needed.

4.2. Body size and life stage effect on starvation rate

Starvation rates were not universally correlated to body size, but body size could explain a significant fraction of variability when limited to exogenous feeding stages, and when ambiguous species-tissue type combinations were excluded. This rather weak relationship suggests that a) the ~2.5 order of magnitude difference in body size covered by the regression was necessary to yield a biological signal and b) that the species that were excluded from the regression either indicate a species effect or are an artifact of life stage (endogenous yolk reserves) and tissue type. Our results further suggest that early stage exogenous-feeding larvae are particularly vulnerable to starvation under laboratory conditions. After absorbing their yolk reserves, larvae must start first feeding within a very limited window of opportunity. The window of opportunity is the time period between first feeding (closely related to mouth-gape opening and onset of foraging behavior) and the point of no return (Blaxter and Hempel, 1963). Overton et al. (2010) estimated the window of opportunity for Baltic cod yolk-sac larvae in the laboratory to be 5.6 days at 10 °C (56 *Dd*). At warmer temperatures (19°C), Yúfera et al. (1993) estimated the window of opportunity to be only 2 days (38 *Dd*) for sea bream. It appears that mixotrophic larvae, those undergoing the transition from yolk to exogenous feeding, rapidly deplete energy reserves when they are food-deprived. These young larvae have not yet had time to deposit energy-rich storage tissues (e.g., white trunk muscle or lipids within hepatic tissues) that could be used to increase starvation resistance. Very short times to death for cod larvae at exactly this transitional life stage were determined in our analysis (on average, well below 50 *Dd*). The rapid starvation rates as well as the highly variable condition levels of transitional life stage cod larvae in all trials were clear indicators of their pronounced vulnerability to starvation.

In sharp contrast to the larval life stage, juvenile sprat (25 to 35 mm length) exhibited high starvation resistance and were an exception to the usual exponential decrease in condition of individuals with time of food deprivation. Sprat deprived for 12 days were not yet beyond their point of no return and all individuals were able to successfully reinitiate feeding and growth (Peck et al., 2004b). The individual starvation rate trajectory of these juvenile sprat indicated that they have a different strategy to respond to food deprivation, compared to the linear decrease in *sRD* condition until mortality that we found for larval fish. Meta-analysis research investigating respiration rates in larval and later stage fish (Bochdansky and Leggett,

2001) demonstrated that metabolic control changes throughout ontogeny in a fashion not solely attributable to body size and storage tissue mass. After prolonged periods of starvation, clupeids (such as sprat and herring) display a continuous loss of body weight and decreasing somatic condition factors (*DW* per unit length) yet only show very modest declines in length (Hauss, 2008).

The 10th percentile *sRD* exhibited a significant negative trend with temperature in yolk-sac and exogenous feeding larvae, with lower 10th percentile *sRD* values at higher temperatures. Exogenously-feeding larvae had a consistently higher 10th percentile *sRD* than yolk-sac larvae at the same temperature. This 10th percentile *sRD* metric represents an important empirical approximation for the lowest species- and life stage-specific *sRD* level under starvation. Besides *sRD* (biochemical condition) larval growth rates are also affected by food depletion. It is assumed that growth rates decrease in a similar fashion as *sRD*, irrespective of species and life stage. This assumption is based on the relation between growth and condition, previously described by a multi-species model relating growth rate and *sRD* with respect to temperature in well-nourished larvae (Buckley et al., 2008). Clearly, growth not only depends upon temperature, but also on the input of energy and nutrients (e.g., Peck et al., 2003) and most research seeks to deal with well-feeding and growing organisms (i.e., when rates of anabolism exceed catabolism). Negative and positive growth rates are always examined in concert, implying a basic assumption that there are no major physiological differences in growing or mildly fasting animals, even though this is usually not explicitly stated. For our data, it was not possible to use the Buckley et al. (2008) growth model to predict (negative) growth rates for food-depleted larvae. We calculated growth rates from the 10th percentile *sRD* values of exogenous life stages from our data set that ranged almost equally from positive to negative, with a mean just slightly above zero. In the absence of external sources of nutrition and without endogenous reserves, the larvae in our dataset were clearly not capable of somatic growth, but this assertion was not supported by the *sRD* growth model.

When the same calculation was done for the 10th percentile *sRD* of yolk-sac larvae (i.e., when most of the endogenous reserves were already exhausted), growth rates were mainly negative, i.e. individuals in this life stage at the 10th percentile *sRD* were correctly identified to be in poor condition. Young yolk-sac larvae (prior to any "mixed feeding period"), totally rely on their endogenous yolk reserves for growth and development. *RD* values are initially high in the yolk-sac stage and have led to overestimates of somatic growth rates using *RD*-temperature models that had been successfully fitted to exogenous-stage larvae (Buckley et al., 2006). Besides potentially reduced net protein retention rates and RNA activity levels, the body weight or protein-specific growth rate of a yolk-sac larva is substantially underestimated if yolk mass is not excluded from the calculation (Buckley et al., 2006). *RD* can be an indicator of recent growth in yolk-sac larvae when protein accretion in the larval body is considered independently from weight loss due to yolk absorption. For the whole larval body, including the yolk reserves, maternal effects are important in imparting variability to nutritional condition of larvae. Egg size and quality determine the quantity and composition of yolk reserves that provide the initial protein synthesis machinery (e.g., maternal ribosomal RNA) (Clemmesen et al., 2003; Saborido-Rey et al., 2003) and can have a profound impact on starvation resistance.

A special case among yolk-sac larval strategies is apparent for North Sea houting (Malzahn et al., 2003). The authors determined that hyperplasia, an increase of cell numbers rather than cell size, characterized the development in this species up to 250 degree-days post hatch. Using the same muscle tissue *sRD* dataset, we demonstrated in our analysis that the endogenous-feeding houting larvae exhibited a) the lowest condition threshold (10^{th} percentile), which was presumably caused by low RNA concentration in the hyperplasia muscle tissue and b) the longest time to death, which was possible because of high levels of maternally-derived yolk reserves. Houting and vendace, the only freshwater taxa in the dataset, are both coregonids in the family Salmonidae, which generally are large at hatch and have large yolk reserves. They can accomplish a greater proportion of early ontogeny by feeding on endogenous yolk reserves. The hyperplasia mode of growth is very efficient for taxa having this life history strategy because it does not require any external food intake for ontogenetic progress. The hyperplasia mode is an adaptation to spawning and growing in a low temperature and/or food limited environment where it is advantageous for larvae, in addition to being large at hatch, to be well developed when either suitable temperatures for growth or adequate food sources are not available until later in the season. Vendace, for example, spawn in the winter and embryonic development can take place over a five-month period at low water temperatures (Karjalainen et al., 1991).

Tissue types may affect *RD* values. Tail and trunk muscle sections were used for *sRD* analysis in some of the experiments in our study. Olivar et al. (2009) systematically investigated the different contributions of tissue type *RD* (e.g., head, eyes, muscle, gut) relative to whole body *RD* and found that muscle tissue had consistently higher *RD* values than other tissues. This result was noted for pre- and post-flexion larval stages of two clupeids (5.7 – 30.8 mm) collected at sea and laboratory food deprivation trials with a paralichthyid (5.6 – 7.5 mm) species (Olivar et al., 2009). Muscle tissue is the most important energy-storage tissue in late pre-metamorphosis larvae, prior to stages in which lipid storage becomes important. Muscle growth is highly correlated with *sRD* because of its high protein synthesis rate. At the onset of food deprivation, protein turnover rates in muscle tissue decrease and protein reserves are mobilized to satisfy catabolic needs, based on histological or cell-cycle analysis in fish larvae (Catalán and Olivar, 2002; Catalán et al., 2007). This process proceeds with a reduction of ribosomal RNA and an increase of DNA content per unit dry weight (Bergeron, 1997). Therefore, the starvation signal can be strongly expressed in muscle tissue even though it only accounts for a part of the physiological response in whole body samples. For four species investigated by Olivar et al. (2009), the authors suggested a correction factor to account for tissue-type effects. It remains to be clarified how the decline in relative DNA content per unit dry weight throughout ontogeny, caused by formation of low-DNA organic matter such as bones and lipid storage tissue (Suthers, 1998), is differentially expressed in species with different morphometric growth strategies (Froese, 1990).

Larvae analyzed in our research exhibited some body size- and stage-dependent differences in starvation rate and thresholds, but the ability to resist starvation remained generally low over several orders of magnitude in dry weight. Miller et al. (1988) found that taxa with small larvae are more susceptible to starvation than taxa with larger larvae. Folkvord et al. (2009) suggested a trade-off between levels of energy storage and growth rate where species with

faster (slower) growing early life stages had little (more) starvation resistance but developed more rapidly (slowly) through life stages that were more vulnerable to predators. Jordaan and Brown (2003) identified clear tradeoffs between body size, growth performance and starvation resistance. In their laboratory study, cod larvae ~ 12 mm *SL* had the highest potential for growth but also starvation-induced mortality. In contrast, first-feeding cod yolk-sac larvae have been demonstrated to have a higher potential to withstand periods of prey deprivation (Overton et al., 2010). Other studies conducted on marine fishes (e.g., Bochdansky et al., 2008) reveal that larvae may pass through multiple "critical periods" where starvation resistance and growth capacity are linked. For the present dataset, the percentile approach improved the interpretation of *sRD* values of heterogeneous groups of larvae of different life stages and body sizes under food depletion. The a priori assumptions about the sampled population (stochastic distribution), the function describing the decline of *RD* over time of food depletion (exponential) and the selective mortality of individuals in low condition (edge of death), enabled the percentile approach to better represent the physiological process of starvation.

4.3. Population percentiles and percentile approach

A key premise of this study is that variability among individuals in growth potential and starvation resistance strongly determine responses by fish larvae cohorts to environmental conditions. It is necessary to move beyond simply using group averages in nutritional condition to characterize a cohort, either in the laboratory or in the field because the group-average metric does not accurately capture the heterogeneity in condition typically exhibited by fish early life stages. If we are to develop predictive models to describe how larval condition relates to survival and growth at later life stages, we must better define the distribution of the characteristics in individuals, for example values of condition indices, starvation resistance, and growth potential.

Monitoring individual larvae over time, even under controlled laboratory conditions, is extremely difficult. However, inferential methods can be developed such as those proposed by Folkvord et al. (2009), who used cumulative size distribution to derive subpopulation specific growth rates. In this case, a change in the cohort weight distribution over time suggested a higher mortality in smaller, slower-growing individuals. The rationale behind this approach is that relative size of a larva compared to other individuals of the same cohort is not likely to change in the short term; for example, ranks of cod larvae remained the same from yolk-sac to metamorphosis stage (Paulsen et al., 2009). Thus comparing larval growth is best achieved by comparing larval size from the same percentile of a population at consecutive samplings rather than comparing an individual's size with the mean size in the previous or following sampling. The same situation may be the case for larval condition, where condition of an individual on a given day will depend to some extent on its condition on the previous day. In the case of *RD*, the change in the RNA content of a larva is likely to be more important with respect to the short term change in biochemical condition because total DNA content, reflecting the number of cells in an organism, will not change dramatically from one day to the next (Clemmesen, 1994).

Repeated measurements of condition to determine an individual's condition trajectory under food depletion is virtually impossible in larval fish. Ferron and Leggett (1994) proposed a conceptual model for various condition proxies (morphological, histological and biochemical) under recurring feeding and starvation conditions. In their sense, condition responds to changes in feeding regime within boundaries defined by *ad libitum* feeding and food depletion. Depending upon the characteristics of a condition index or proxy (e.g., responsiveness, sensitivity), the direction of change in an individual's condition may not be apparent. This is especially true for *RD* that provides the *ad hoc* status of protein synthesis rate and recent growth rate, but does not indicate the direction of change in condition over time.

The percentile approach is a first step to overcome the inability to identify individual starvation trajectories and it provides a metric not otherwise available from population means. It must be realized though, that in our research the same dataset was used for development and application of this approach. Because these steps are not independent, careful validation was mandatory. Independent datasets were lacking and cross-validation, for example by step-wise exclusion of individual experiments and validation against the remaining experiments, would have required substantially more data. We therefore compared the percentile-based starvation parameters with the traditional and conservative approach of population mean-based estimates of *RD*. For starvation rates, we found that these two approaches produced almost identical results. This indicates that the percentile- and mean-based regression slopes of condition (*sRD*) against time of food deprivation were parallel in most trials and were only slightly off-set (higher intercept in 75^{th} percentile approaches). Most of the experiments applied sampling schedules designed for pooled estimates of *sRD* from replicate tanks, and presumably sampled only as many individuals from each tank as needed to obtain a "good" arithmetic mean. The predictive power of the percentile approach can be expected to increase with increases in the number of samples drawn from the experimental population. Stabilization of the 75^{th} percentile, i.e. when it changes not too abruptly over time of food depletion, suggests itself as a good indicator for sampling power. Both the 90^{th} and the 10^{th} percentiles of the sampled population proved to be valid approximations of larval condition at onset of food deprivation and the edge of death, respectively. Applying this finding, based on experimental populations in the laboratory, to populations in the sea it is possible to circumvent labor-intensive approaches to identify duration of starvation time in individuals, for example by measuring otolith increment widths (Baumann et al., 2005) or from histology (Ehrlich et al., 1976; Gisbert and Doroshov, 2003).

The calculated time to death was successfully validated with a rigorous selection of trials that terminated with larval mortality. Time to death, based on the percentile approach, slightly underestimated observed time to death (or more correctly: trial duration), which was expected because most individuals in the 10^{th} percentile fractions were not ultimately close to death. Experimental trials were terminated because of pre-determined experimental schedules and presumably the actual edge of death would have been reached later and with a lower 10^{th} percentile value of *sRD*. In contrast, the population mean-based calculation overestimated trial duration in most cases. We believe that, for these laboratory experiments, the percentile

approach was successfully validated and that it provides an accurate approximation of *sRD* starvation parameters at the level of individuals.

The percentile approach is relatively straightforward in laboratory research but more difficult to apply in the sea. In field research, it is necessary to derive the *sRD* metrics (10^{th}, 75^{th}, and 90^{th} percentiles), and to relate them not only to life stages, but also to population and habitat characteristics. These 10^{th} and 90^{th} percentile metrics have been previously applied in evaluations of marine fish early life stages. Clemmesen et al. (2003) reported that the distribution of *RD* values and patterns of percentiles of *RD* were strongly influenced by environments experienced by larvae in mesocosm research. Individuals in a mesocosm with warmer temperatures and higher prey concentrations had higher larval growth rates and *RD* values, and relatively large prey in their gut compared to individuals in a second mesocosm that was colder and had lower prey concentrations (Busch et al., 2009; Clemmesen et al., 2003). Moreover, the 10^{th} percentile value of *RD* was stable with time in the warm-high prey mesocosm, indicating a non-selective environment (predators were absent) whereas it increased rapidly during the first three weeks in the cold-low prey mesocosm, suggesting that only the fittest larvae were able to survive and successfully compete for food in the cool, prey-limited environment.

Evaluating changes in specific percentiles of *RD* has also yielded insight into how poor feeding and starvation act to control early life stage survival in the sea. In research on sprat larvae in the Bornholm Basin (Baltic Sea), Voss et al. (2006) reported that stable but relatively high 10^{th} percentile values of *RD* with increasing body size reflected a feeding environment with high copepod nauplii concentrations that was favorable for small, first-feeding sprat larvae in April and May. Lower 10^{th} percentile values of *RD* occurred in July, suggesting a less selective environment in which both fast-and slow-growing larvae survived. In the April-May period, larvae in lower nutritional condition suffered high mortality and were removed from the population, resulting in a rise in the 10^{th}percentile. A similar increase in the 10^{th} percentile values of *RD* was observed by Huwer et al. (2011) who examined nutritional condition of Baltic cod larvae during a period of prey limitation. These results support the proposal that during periods of abundant food supply, selective pressure for fast growth is relaxed and slow-growing larvae may experience improved survival (Meekan and Fortier, 1996).

4.4. Perspective

An empirical parameterization of the functional model describing changes in nutritional condition under food depletion will advance understanding starvation prevalence in the sea and will answer the questions: When and where are early life stages of fish pre-recruits exposed to starvation in the sea? How important is starvation as a cause of mortality? There is a substantial body of literature that indicates limiting prey levels are a source of larval mortality and a factor affecting recruitment success. In the Baltic Sea, empirical and model results indicate prey limitation controls survival of cod larvae (Köster et al., 2003). In a recent study, Huwer et al. (2011) utilized *sRD* measurements to identify Baltic cod larvae in poor nutritional condition within areas having low concentration of the preferred prey, *Pseudocalanus acuspes*. In the Northwest Atlantic, Buckley et al. (2010) identified ‘windows

for survival' of Atlantic cod and haddock larvae based upon seasonal and inter-annual differences in larval *RD* condition and prey abundance. Voss et al. (2006) utilized *sRD* measurements to reveal size-specific 'windows of survival' for Baltic sprat larvae that were linked to the availability of suitable prey. These results support Cushing's 'match–mismatch' hypothesis (Cushing, 1974; 1990) which emphasizes the key role of timing of prey production and its coincidence with early life stages of fish as a mechanism controlling recruitment in marine fishes. Our efforts to quantify starvation rates and times to death based upon thresholds in biochemical indices (*RD*) will contribute to progress in evaluating and understanding how prey limitation acts to affect early life stages. The advantage of our proposed *sRD*-derived measure of mortality risk is that it may be applicable as a first estimate of prey limitation effects in many environments and across a wide range of species.

4.5. Conclusion

Our analysis has shown that starvation-induced changes in condition in pre-recruit life stages of fishes can be described by a common function when temperature effects, life stage and species-specific differences are taken into account. Starvation rates were normalized with respect to temperature by expressing the duration of food deprivation on a degree-day basis. We fitted functional models to discrete percentiles of biochemical condition (standardized RNA-DNA ratio) in sampled populations to derive an estimate for starvation rates and mortality thresholds of biochemical condition on the level of an individual. Within narrow ranges of body sizes and life stages, we were able to quantify key aspects of starvation (initial condition, rate of decrease in condition, mortality threshold, and time to death) based on data from controlled laboratory trials. Although the selective loss of individuals in poor condition will undoubtedly differ between the laboratory and sea, our analysis represents a step towards a tailored condition index that takes into account species-, stage- and time frame-specific attributes of starvation (Suthers, 1998). Additional research is needed to address gaps in our knowledge of how different life stages and/or species are able to cope with periods of prey deprivation and how frequently cohorts of fish experience life "on the edge of death".

Table II-1 literature.

(Caldarone, 2005; Caldarone et al., 2003; Caldarone et al., 2001; Caldarone et al., 2006; Clemmesen, 1993; 1994; Faria et al., 2011; Faulk and Holt, 2009; Folkvord et al., 2009; Harrer, 2006; Malzahn et al., 2003; Suneetha et al., 1999; Wagner et al., 1998; Westermann and Holt, 1988)

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5. Figures

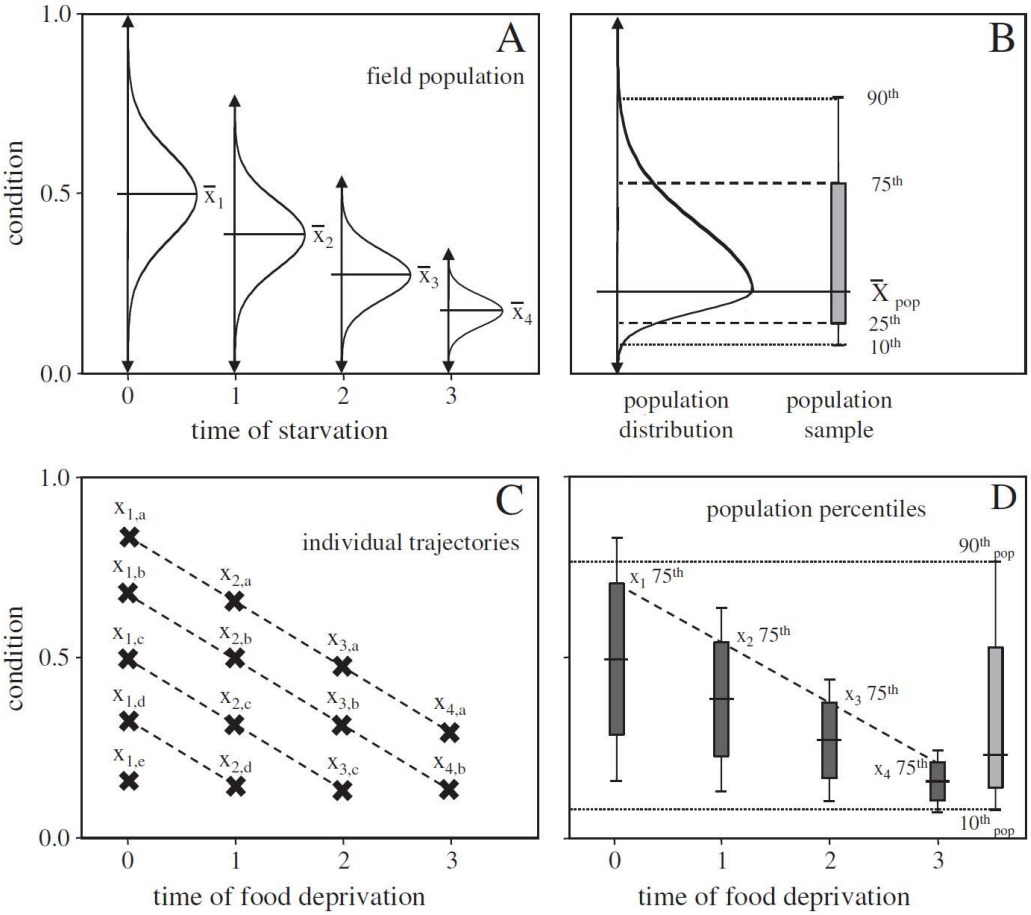

Figure II-1 Conceptual illustration of the percentile approach. Panel A: Field populations contain at any time individuals under food deprivation. Condition is a function of time of starvation and is stochastically distributed around unknown mean values for each time-step of starvation. B: Condition in field populations is stochastically distributed. Population sample percentiles are used to describe the shape of the underlying distribution. In this example, the population contains a high number of individuals in low condition; hence, the lower percentiles are closer to the population mean than the higher percentiles. C: Individual starvation trajectories of 5 individuals from the population in panel A on the course of starvation over 4 time steps. Slopes of starvation trajectories are parallel for each individual a-e. D: Population percentiles of an experimental sample population under food deprivation. The 75^{th} percentiles of each sampling time-step x_i decrease with a similar slope as in individuals a-e (panel C). Population percentiles represent highest (90^{th}) and lowest (10^{th}) possible condition under these environmental conditions.

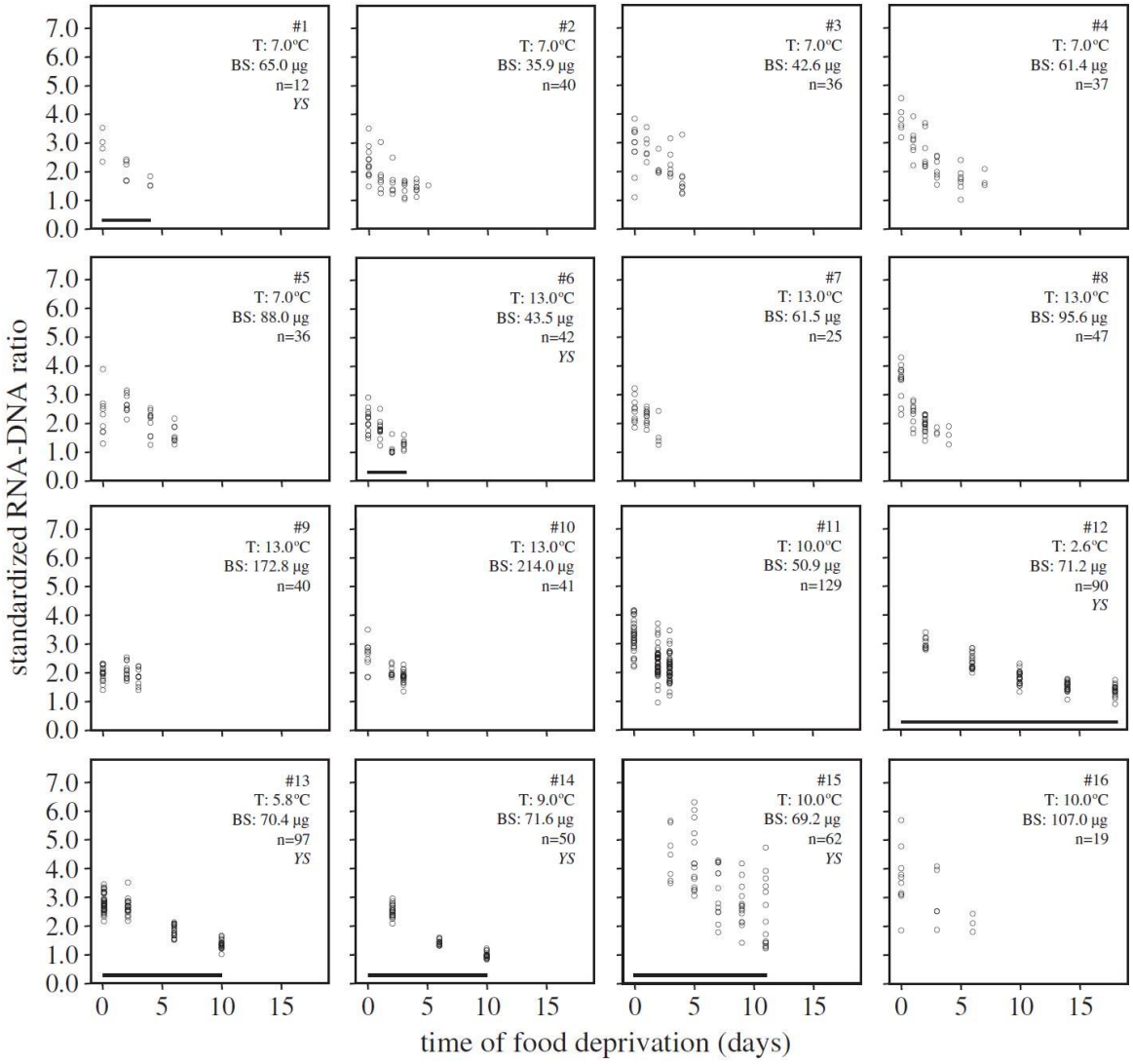

Figure II-2 Untransformed data overview for cod (*Gadus morhua*) food deprivation trials. Standardized RNA-DNA ratio of individuals (y axis) plotted against time of food deprivation (days; x axis). Black bars indicate the presence of endogenous yolk reserves. Samples were analyzed as whole body homogenates. T – temperature (°C), BS – body size/dry weight (µg), YS – yolk-sac stage. Panels are arranged by trial (#) numbers (Table 2).

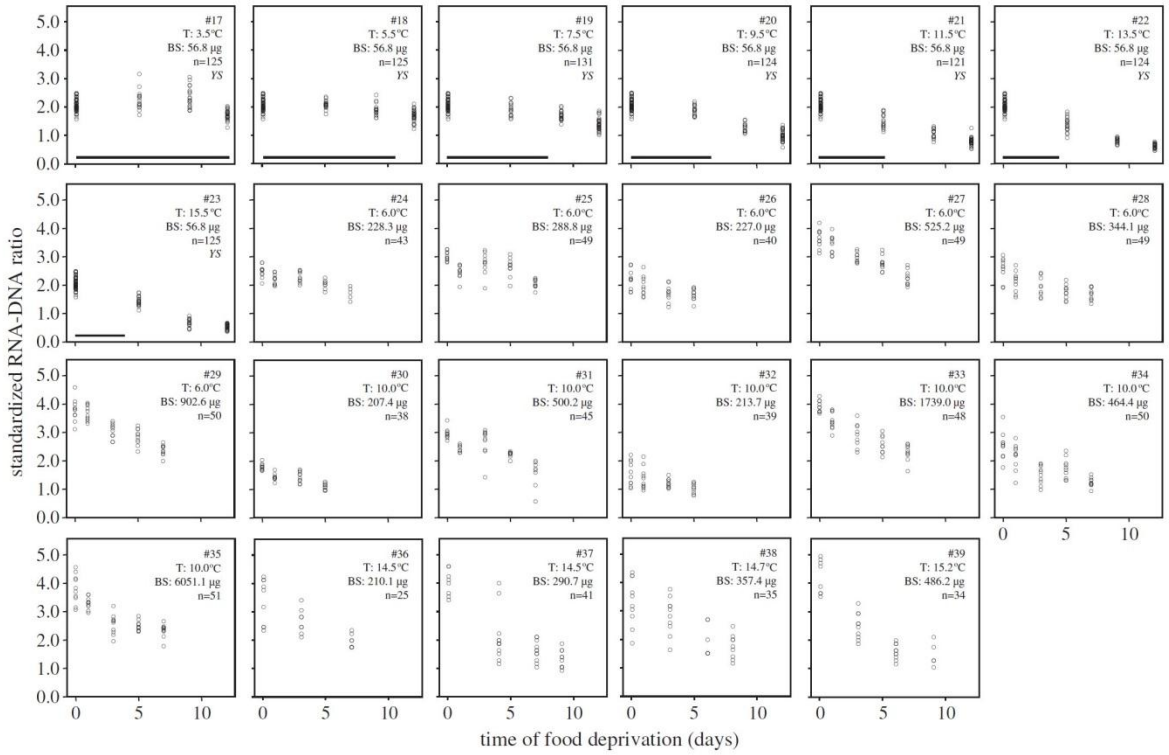

Figure II-3 Untransformed data overview for herring (*Clupea harengus*) food deprivation trials. Standardized RNA-DNA ratio of individuals (y axis) plotted against time of food deprivation (days; x axis). Black bars indicate the presence of endogenous yolk reserves. Samples were analyzed as whole body homogenates. T – temperature (°C), BS – body size/dry weight (µg), YS – yolk-sac stage. Panels are arranged by trial (#) numbers (Table 2).

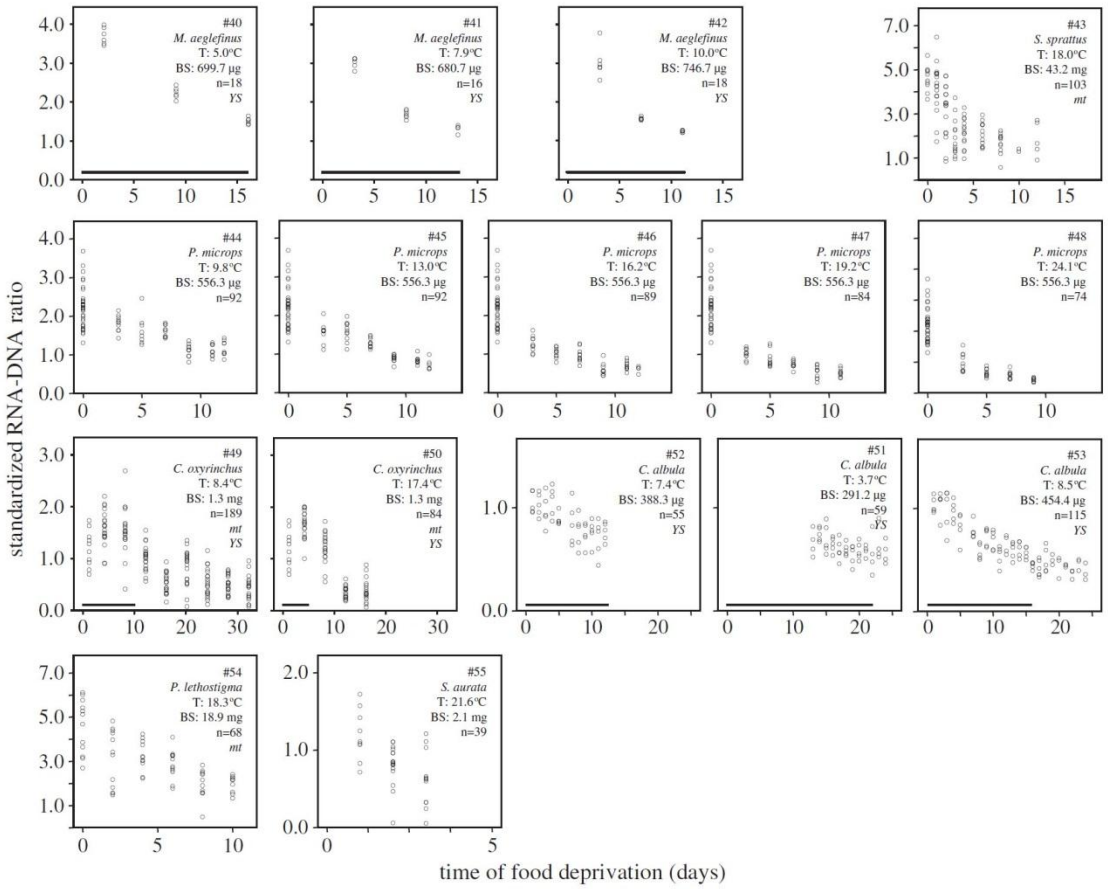

Figure II-4 Untransformed data overview for food deprivation trials in seven species. Standardized RNA-DNA ratio of individuals (y axis) plotted against time of food deprivation (days; x axis). Black bars indicate the presence of endogenous yolk reserves. Samples were either analyzed as muscle tissue (indicated, *mt*) or whole body (not indicated) homogenates. T – temperature (°C), BS – body size/dry weight (µg), YS – yolk-sac stage. Panels are arranged by trial (#) numbers (Table 2).

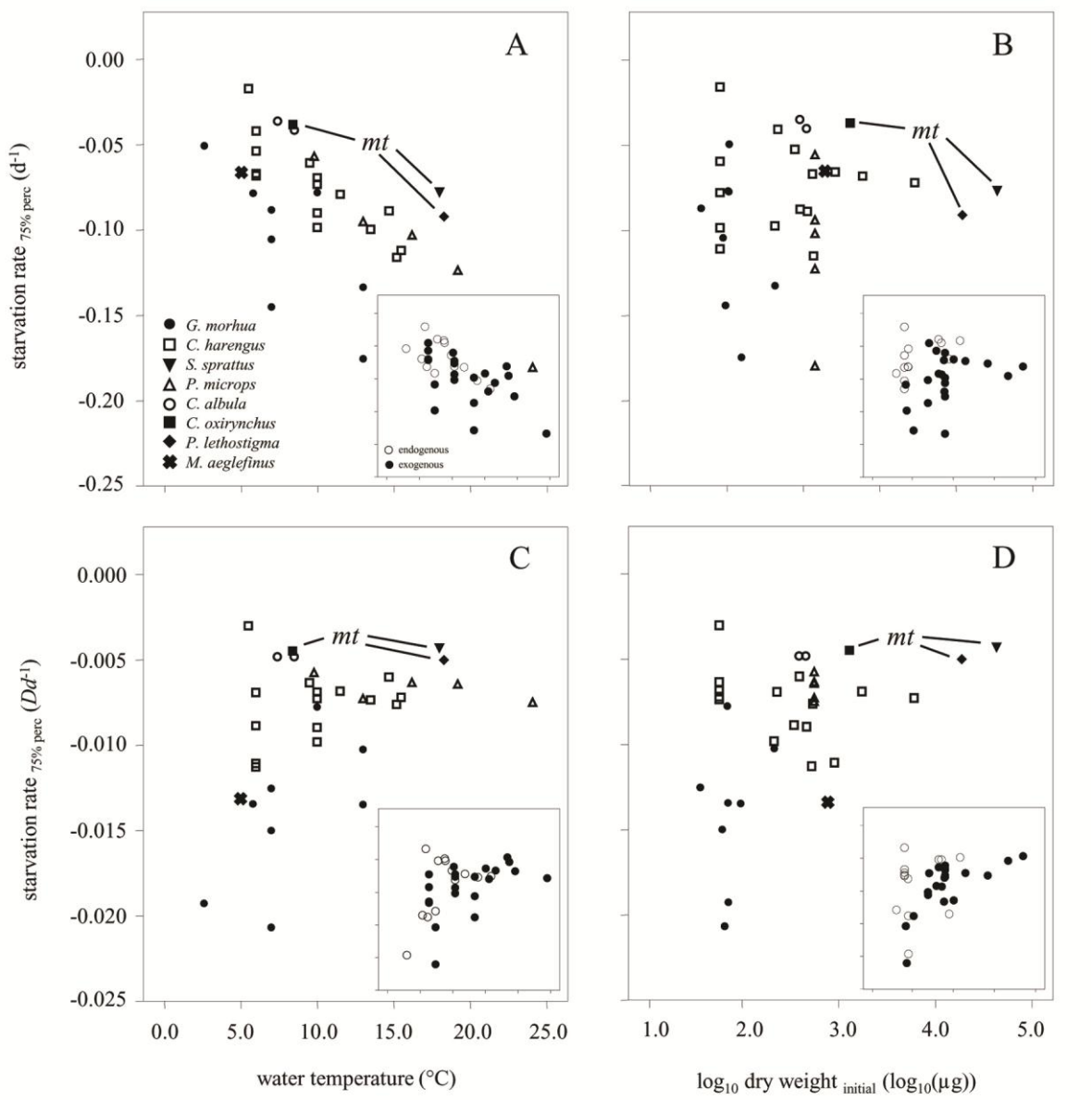

Figure II-5 Starvation rate (significant linear slope of 75th percentile $\log_e$-transformed *sRD* against time of food deprivation) before (d^{-1}; Panels A and B) and after (Dd^{-1}; Panels C and D) degree-day-transformation (y axis) plotted against water temperature (°C; x axis; Panels A and C) and $\log_{10}$-initial dry weight ($\log_{10}$(µg); x axis; Panels B and D). Symbols indicate species (see inlet figure); *mt* indicates muscle tissue *sRD* assays. Inserts: The same plots (same x- and y-axis) depicting endogenous yolk-sac (white fill) and exogenous (black fill) life stages.

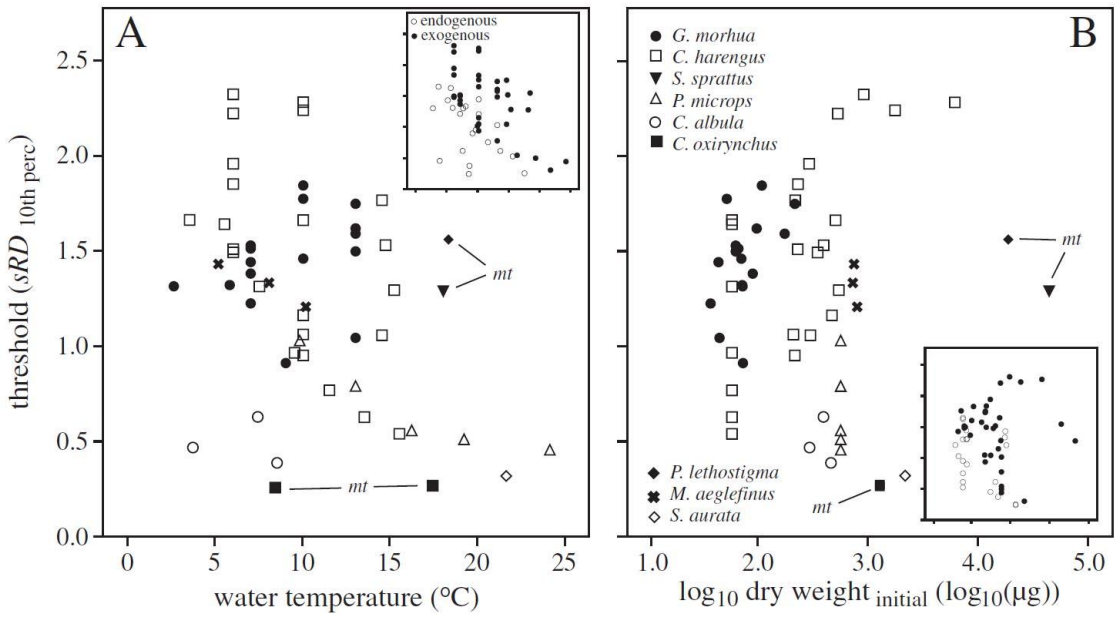

Figure II-6 Threshold *sRD* for each trial (y axis) plotted against water temperature (°C; x axis; Panel A) and $\log_{10}$-initial dry weight ($\log_{10}$(µg); x axis; Panel B). Threshold *sRD*. 10^{th} percentile values of *sRD* are assumed to represent the lowest possible biochemical condition sustaining life and therefore the edge of death. Symbols indicate species (see figure legend); *mt* indicates muscle tissue *sRD* assays. Inserts: The same plots (same x- and y-axis) with symbols indicating endogenous yolk-sac (white fill) and exogenous (black fill) life stages.

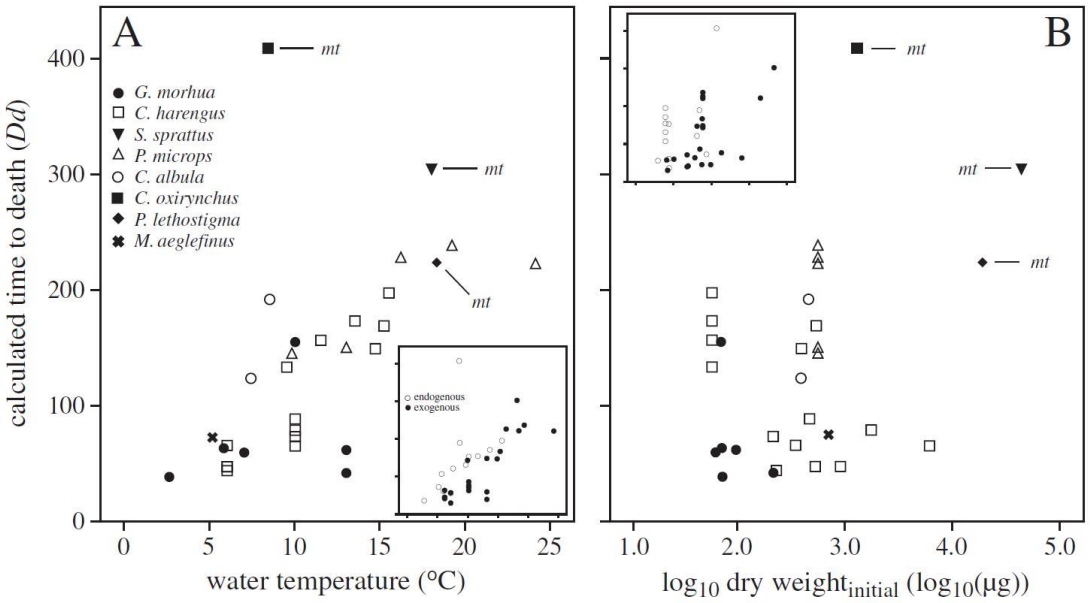

Figure II-7 Time to death (*Dd*) based on the percentile approach (y axis) plotted against temperature (°C; x axis; Panel A) and $\log_{10}$-initial dry weight ($\log_{10}$(µg) ; x axis; Panel B). Symbols indicate species (see figure legend); *mt* indicates muscle tissue *sRD* assays. Inserts: The same plots (same x- and y-axis) with symbols indicating endogenous yolk-sac (white fill) and exogenous (black fill) life stages.

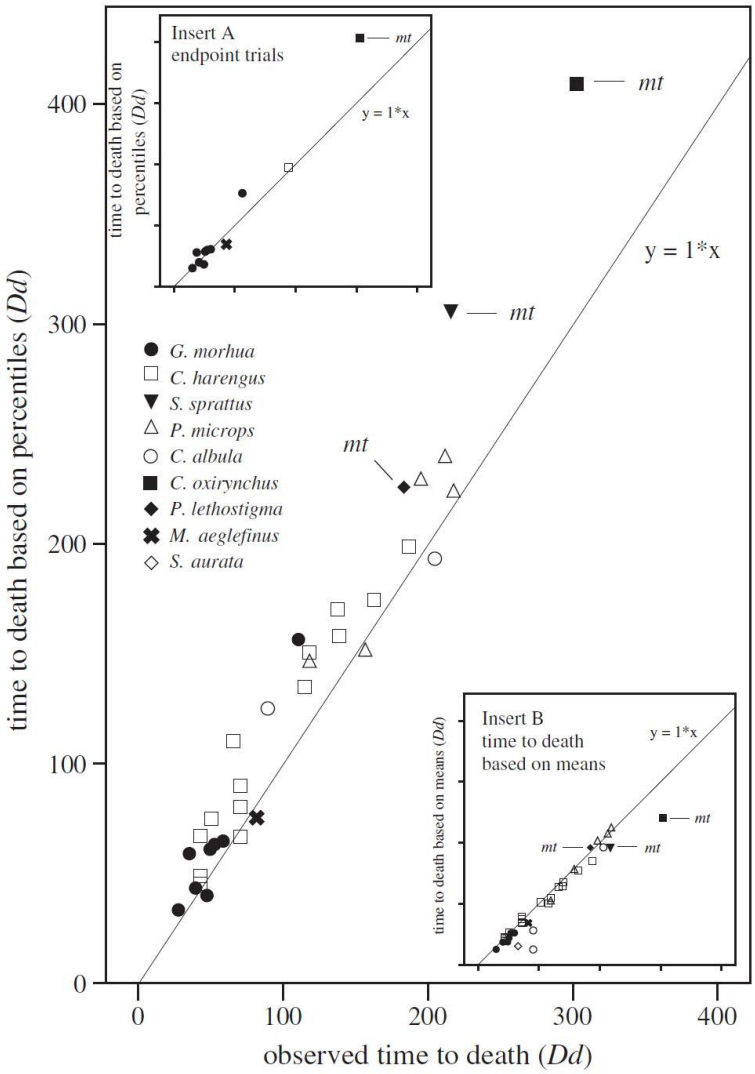

Figure II-8 Time to death (*Dd*) based on the percentile approach (y axis) plotted against observed time to death (*Dd*; x axis). Symbols indicate species (see figure legend); *mt* indicates muscle tissue *sRD* assays. One-on-one line (y = 1 x) for visual support; points to left of this line: time to death is underestimated and mortality not imminent; points to the right: time to death is overestimated and mortality has already occurred. Insert A: The same plot (same x- and y-axis) showing only endpoint trials, i.e. ending with 100 % larval mortality. Insert B: Time to death, based on means (*Dd*) (y axis) plotted against observed time to death (*Dd*) (x axis).

6. Tables

Table II-1 Overview of 15 laboratory-based food-deprivation experiments compiled in the current study. Given are the number of individual value pairs of body size and biochemical condition in each experiment (n), the number (#) of trials using different water temperatures (*T*, °C) and initial body sizes (*BS*). RNA and DNA standard curve slope ratios (dimensionless) and standardization factors SF_{pi} (dimensionless) were used for intercalibration of results (Caldarone et al., 2006) from different *RD* assays [1 – Caldarone et al. (2001), 2 – Clemmesen (1993), 3 – Suneetha et al. (1999), 4 – Westermann and Holt (1988), 5 – Wagner et al. (1998)]. Analyzed tissue types: *wb* – whole body, *mt* – muscle tissue preparations. References to each experiment are given in the last column.

Experiment ID	Species	n	# trials	# *T*	# initial *BS*	slope ratio	Standard. factor SF_{pi}	RD assay	Tissue type	Reference
A	*G. morhua*	161	5	1	5	0.81	0.34	1	*wb*	Meyer (unpublished)
B	*G. morhua*	195	5	1	5	0.75	0.31	1	*wb*	Meyer (unpublished)
C	*G. morhua*	129	1	1	1	0.79	0.33	1	*wb*	Meyer (unpublished)
D	*G. morhua*	237	3	3	1	2.60	1.08	1	*wb*	Caldarone et al. (2003)
E	*G. morhua*	80	2	1	2	1.89	0.79	1	*wb*	Meyer (unpublished)
F	*C. harengus*	875	7	7	1	2.20	0.92	2	*wb*	Harrer (2006)
G	*C. harengus*	551	12	2	12	2.20	0.92	3	*wb*	Folkvord et al. (2009)
H	*C. harengus*	135	4	1	4	2.03	0.85	2	*wb*	Clemmesen (1994)
I	*M. aeglefinus*	52	3	3	1	2.68	1.12	1	*wb*	Caldarone (2005)
J	*S. sprattus*	103	1	1	1	0.77	0.32	1	*mt*	Peck et al. (in prep)
K	*P. microps*	431	5	5	1	2.20	0.92	2	*wb*	Petereit (unpublished)
L	*C. oxyrinchus*	273	2	2	1	2.20	0.92	2	*mt*	Malzahn et al. (2003)
M	*C. albula*	213	3	3	3	2.20	0.92	2	*wb*	Petereit (unpublished)
N	*P. lethostigma*	68	1	1	1	3.90	1.63	4	*mt*	Faulk and Holt (2009)
O	*S. aurata*	39	1	1	1	5.50	2.29	5	*wb*	Faria et al. (2011)
Sum	**9**	**3542**	**55**	**33**	**40**					

Table II-2. Summarized results from food deprivation trials for all species in the present study: number of individuals (n), initial age (dph), yolk sac stage (*YS*), duration of the trial (d), water temperature (*T*, °C) and initial dry weight (DW_{ini}, µg), significance value *p* of temperature effect before (*T* effect d) and after (*T* effect *Dd*) degree-day normalization and dry weight effect (*DW* effect) on starvation time [tested within chosen experiments (indicated by horizontal lines), ANCOVA, significant difference in parameter estimates compared to reference trial (*ref.*)]. Significant differences are indicated by an asterisk (*). Starvation rates (Dd^{-1}, ±S.E.), normalized *sRD* start value (90th percentile *sRD*), *sRD* threshold (10th percentile *sRD*) and time to death (*Dd*) are given for those trials where significant regression lines could be fitted to 75th percentile data. *n.s.* – not significant, n.k. – not known.

Exp ID	Trial #	Species	n	Initial age (dph)	Duration (d)	*YS*	*T* (°C)	*T* effect d	*T* effect *Dd*	DW_{ini} (µg)	*DW* effect	Starvation rate (Dd^{-1})	±S.E.	90th percentile *sRD*	10th percentile *sRD*	Time to death (*Dd*)
A	1	*G. morhua*	40	8	5	+	7.0	-	-	35.9	<0.01*	-0.01251	0.00344	2.6	1.2	59.1
	2	*G. morhua*	36	12	4	-	7.0	-	-	42.6	0.59	*n.s.*	-	-	-	-
	3	*G. morhua*	12	17	4	-	7.0	-	-	65.0	0.38	-0.02065	0.00154	3.0	1.5	33.4
	4	*G. morhua*	37	17	7	-	7.0	-	-	61.4	0.08	-0.01498	0.00348	3.8	1.5	60.6
	5	*G. morhua*	36	25	6	-	7.0	-	-	88.0	*ref.*	*n.s.*	-	-	-	-
B	6	*G. morhua*	42	7	3	+	13.0	-	-	43.5	<0.01*	*n.s.*	-	-	-	-
	7	*G. morhua*	25	10	2	-	13.0	-	-	61.5	0.56	*n.s.*	-	-	-	-
	8	*G. morhua*	47	13	4	-	13.0	-	-	95.6	0.45	-0.01346	0.00329	3.8	1.6	62.9
	9	*G. morhua*	40	16	3	-	13.0	-	-	172.8	0.73	*n.s.*	-	-	-	-
	10	*G. morhua*	41	18	3	-	13.0	-	-	214.0	*ref.*	-0.01023	0.00077	2.7	1.8	42.9
C	11	*G. morhua*	129	9	3	-	10.0	-	-	50.9	-	*n.s.*	-	-	-	-
D	12	*G. morhua*	90	2	18	+	2.6	<0.01*	0.07	71.2	-	-0.01924	0.00167	2.8	1.3	39.5
	13	*G. morhua*	97	0	10	+	5.8	0.08	0.33	70.4	-	-0.01342	0.00141	3.1	1.3	64.3
	14	*G. morhua*	50	2	10	+	9.0	*ref.*	*ref.*	71.6	-	*n.s.*	-	-	-	-
E	15	*G. morhua*	62	1	11	+	10.0	-	-	69.2	<0.01*	-0.00774	0.00187	4.9	1.5	156.1
	16	*G. morhua*	19	15	6	-	10.0	-	-	107.0	*ref.*	*n.s.*	-	-	-	-

Table II–2. *continued.*

Exp ID	Trial #	Species	n	Initial age (dph)	Duration (d)	*YS*	*T* (°C)	*T* effect d	*T* effect *Dd*	DW_{ini} (μg)	*DW* effect	Starvation rate (Dd^{-1})	±S.E.	90th percentile *sRD*	10th percentile *sRD*	Time to death (*Dd*)
F	17	*C. harengus*	125	0	12	+	3.5	<0.01*	0.10	56.8	-	*n.s.*	-	-	-	-
	18	*C. harengus*	125	0	12	+	5.5	<0.01*	0.11	56.8	-	-0.00299	0.00054	2.3	1.6	110.3
	19	*C. harengus*	131	0	12	+	7.5	<0.01*	0.07	56.8	-	*n.s.*	-	-	-	-
	20	*C. harengus*	124	0	12	+	9.5	<0.05*	0.31	56.8	-	-0.00632	0.00133	2.3	1.0	134.3
	21	*C. harengus*	121	0	12	+	11.5	0.19	0.65	56.8	-	-0.00682	0.00090	2.3	0.8	157.6
	22	*C. harengus*	124	0	12	+	13.5	0.70	0.75	56.8	-	-0.00733	0.00068	2.3	0.6	174.2
	23	*C. harengus*	125	0	12	+	15.5	*ref.*	*ref.*	56.8	-	-0.00718	0.00097	2.3	0.5	198.5
G	24	*C. harengus*	43	14	7	-	6.0	-	-	228.3	<0.01*	-0.00689	0.00177	2.5	1.9	45.0
	25	*C. harengus*	49	14	7	-	6.0	-	-	288.8	<0.01*	*n.s.*	-	-	-	-
	26	*C. harengus*	40	28	5	-	6.0	-	-	227.0	<0.01*	*n.s.*	-	-	-	-
	27	*C. harengus*	49	28	7	-	6.0	-	-	525.2	0.17	-0.01125	0.00063	3.8	2.2	48.4
	28	*C. harengus*	49	42	7	-	6.0	-	-	344.1	<0.01*	-0.00884	0.00242	2.7	1.5	66.6
	29	*C. harengus*	50	42	7	-	6.0	-	-	902.6	*ref.*	-0.01105	0.00141	4.0	2.3	48.4
	30	*C. harengus*	38	14	5	-	10.0	-	-	207.4	<0.01*	*n.s.*	-	-	-	-
	31	*C. harengus*	45	14	7	-	10.0	-	-	500.2	0.10	*n.s.*	-	-	-	-
	32	*C. harengus*	39	28	5	-	10.0	-	-	213.7	<0.01*	-0.00979	0.00211	2.0	1.0	74.3
	33	*C. harengus*	48	28	7	-	10.0	-	-	1739.0	0.49	-0.00687	0.00032	3.9	2.2	79.9
	34	*C. harengus*	50	42	7	-	10.0	-	-	464.4	<0.01*	-0.00894	0.00170	2.6	1.2	89.6
	35	*C. harengus*	51	42	7	-	10.0	-	-	6051.1	*ref.*	-0.00725	0.00168	3.7	2.3	66.3
H	36	*C. harengus*	25	21	7	-	14.5	-	-	215.6	0.82	*n.s.*	-	-	-	-
	37	*C. harengus*	41	27	9	-	14.5	-	-	296.7	0.37	*n.s.*	-	-	-	-
	38	*C. harengus*	35	33	8	-	14.7	-	-	391.2	0.43	-0.00599	0.00076	3.8	1.5	150.3
	39	*C. harengus*	34	42	9	-	15.2	-	-	537.5	*ref.*	-0.00759	0.00158	4.7	1.3	170.0

Table II–2. *continued.*

Exp ID	Trial #	Species	n	Initial age (dph)	Duration (d)	*YS*	*T* (°C)	*T* effect d	*T* effect *Dd*	DW_{ini} (μg)	*DW* effect	Starvation rate (Dd^{-1})	±S.E.	90th percentile *sRD*	10th percentile *sRD*	Time to death (*Dd*)
I	40	*M. aeglefinus*	18	2	16	+	5.0	<0.05*	0.93	699.7	-	-0.01314	0.00093	3.9	1.5	75.9
	41	*M. aeglefinus*	16	3	13	+	7.9	0.25	0.94	680.7	-	n.s.	-	-	-	-
	42	*M. aeglefinus*	18	3	11	+	10.0	*ref.*	*ref.*	746.7	-	n.s.	-	-	-	-
J	43	*S. sprattus*	103	*n.k.*	12	-	18.0	-	-	43230.0	-	-0.00428	0.00132	4.8	1.3	305.3
K	44	*P. microps*	92	*n.k.*	12	-	9.8	<0.01*	<0.01*	556.3	-	-0.00573	0.00088	2.4	1.0	146.4
	45	*P. microps*	92	*n.k.*	12	-	13.0	<0.01*	0.07	556.3	-	-0.00726	0.00080	2.4	0.8	151.5
	46	*P. microps*	89	*n.k.*	12	-	16.2	<0.05*	0.36	556.3	-	-0.00632	0.00087	2.4	0.6	229.4
	47	*P. microps*	84	*n.k.*	11	-	19.2	0.24	0.90	556.3	-	-0.00640	0.00090	2.4	0.5	239.6
	48	*P. microps*	74	*n.k.*	9	-	24.1	*ref.*	*ref.*	556.3	-	-0.00746	0.00069	2.5	0.5	223.9
L	49	*C. oxyrinchus*	189	1	36	+	8.4	0.07	0.99	1291.0	-	-0.00447	0.00064	1.6	0.3	409.7
	50	*C. oxyrinchus*	84	1	16	+	17.4	*ref.*	*ref.*	1291.0	-	n.s.	-	-	-	-
M	51	*C. albula*	59	17	12	+	3.7	-	-	291.2	-	n.s.	-	-	-	-
	52	*C. albula*	55	5	12	+	7.4	<0.05*	0.16	388.3	-	-0.00480	0.00080	1.2	0.6	124.7
	53	*C. albula*	115	5	24	+	8.5	*ref.*	*ref.*	454.4	-	-0.00481	0.00039	1.0	0.4	192.8
N	54	*P. lethostigma*	68	51	10	-	18.3	-	-	18865.3	-	-0.00500	0.00033	4.8	1.6	224.8
O	55	*S. aurata*	39	35	3	-	21.6	-	-	2144.4	-	n.s.	-	-	-	-

Manuscript III:

III. Sublethal effects of Alizarin Complexone marking on Baltic cod (*Gadus morhua*) eggs and larvae

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Abstract

Standard, single-exposure Alizarin Complexone- (ALC) marking was conducted on early life stages of Baltic cod (*Gadus morhua* L.) to examine acute and chronic effects. Embryos and yolk sac larvae were marked using different concentrations of ALC (0, 50, 100 and 200 mg l^{-1}). Experiments included control groups for treatment and handling effects. In agreement with previous studies, long-lasting, distinct otolith marks were produced by immersion of yolk sac larvae in ≥50 mg l^{-1} ALC for 24 h. Mortality of eggs and larvae was low during the marking procedure. Hatching success of ALC marked embryos was significantly reduced and hatching was delayed with increasing ALC concentration. Growth rates of larvae through 21 *dph* (exogenous feeding stage) were significantly reduced in ALC marked fish compared to controls. Biochemical condition (RNA-DNA ratio) was not affected. Subtle changes in activity and metabolism were indicated by reduced first feeding success and yolk absorption rates. Our results reveal that batch marking of finfish early life stages using ALC can have not only significant, acute impacts on survival but, despite long-standing assumptions to the contrary, also sublethal effects on other vital parameters like growth. We discuss these acute and chronic effects and give recommendations for assessment of life stage- and species-specific ALC immersion marking procedures.

Keywords

Baltic cod; Alizarin Complexone; batch marking; chronic effects; stock enhancement

1. Introduction

Marking and identification techniques are an integral part of responsible stock enhancement and restocking programs (Blankenship and Leber, 1995; Lorenzen et al., 2010) and have been tested and employed in marine finfish and invertebrate species worldwide (e.g., Liu et al., 2009; Purcell and Blockmans, 2009). Immersion of finfish eggs, larvae or juveniles in chemical compounds such as Alizarin- and Oxytetracyclin-derivates that form fluorescent active complexes with calcified structures has proven to be cost effective and easily applied to large numbers of individuals (Babaluk and Craig, 1990; Nagięć et al., 1995; Pedersen and Carlsen, 1991; Rojas Beltran et al., 1995). Alizarin Complexone (ALC) yields a distinct, long-lasting mark on otoliths, allowing reliable identification of specimens captured after release and has been applied on a number of marine fish species including Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*) and gilthead sea bream (*Sparus aurata*) (e.g., Blom et al., 1994; Sanchez-Lamadrid, 2001; Støttrup et al., 2002).

Although most studies optimizing ALC marking protocols for stock enhancement of finfish and aquatic invertebrates only measure survival as a proximate endpoint, the ultimate conclusion drawn from the literature is that ALC has no persistent adverse effect on the marked organism (Blom et al., 1994; Tsukamoto, 1988; Tsukamoto et al., 1989). Some evidence supports this assertion when ALC is used in older, juvenile finfish (e.g., Baumann et al., 2005; Liu et al., 2009), but few studies have systematically examined the potential persistent impacts of ALC marking. This is surprising since these sublethal effects could seriously undermine stocking success. Measurements of rates of metabolism and somatic growth, biochemical indices of condition (e.g., RNA-DNA ratio) and behavioural characteristics are known to reflect the overall condition of marine fish larvae (Ferron and Leggett, 1994), yield a function-based understanding of fish welfare (Fraser, 1999; Huntingford et al., 2006) and would provide useful indications of sublethal, chronic effects. Furthermore, the interplay of these proximate measures can provide insight on the ultimate, underlying patho-physiological processes caused by ALC exposure.

The aim of the present study was to establish ALC marking protocols for embryos and yolk sac larvae of Baltic cod and to investigate how marking and handling procedures affected these early life stages. Proximate effect parameters were assessed on a continuum from acute (survival, hatching) to chronic (yolk absorption, growth rates, RNA-DNA ratio, and feeding incidence). This research was conducted as part of a project aimed at restocking Eastern Baltic cod by releasing first feeding yolk-sac larvae (Støttrup et al., 2008a). Therefore, the priority of the present research was to develop techniques that had the lowest possible impacts on the survival, growth and condition of cod pre-recruit life stages.

2. Material and methods

2.1. General experimental conditions

Baltic cod eggs and larvae were produced by broodstock fish caught in the eastern Baltic that were maintained at a commercial cod hatchery (Bornholm's Lakseklækkeri, Nexø, Bornholm, Denmark) for at least six months prior to spawning. Eggs were collected from spontaneous

group spawning, disinfected and incubated in slightly aerated, flow-through (20 l h^{-1}), conical 80 l incubators, using methods described by Støttrup et al. (2008a), until the start of experiments.

Marking procedures described below used aqueous solutions of Alizarin Complexone (ALC; CAS-number: 3952-78-1; buffered with 1 M KOH) prepared in artificial saltwater immediately prior to the start of the marking procedure. During the 24 h marking procedure, salinity (year 2006, 19±0.1 psu, mean±range; year 2008, 15±0.1 psu) and temperature (7±0.2 °C) were similar to incubation conditions. High oxygen saturation (93±5.0 %) levels and stable pH (8.3±0.3) were maintained. Given are means and ranges, respectively.

2.2. Effect of ALC concentration

A first experiment investigated the influence of different ALC concentrations on embryo survival and hatching success (year 2006). Eggs spawned on July 2nd were removed one day prior to expected hatching and immersed for 24 h at either 0, 50, 100 or 200 mg l^{-1} ALC concentration in replicated (n=2), slightly aerated 1 l marking containers each containing 110 eggs. After marking, eggs were transferred to 1.5 l beakers (n=2) and were incubated for 7 days until all eggs were either hatched or embryos were dead. Hatched larvae were counted and removed each day.

Larval survival was assessed in early stage yolk sac larvae that were marked on day 3 post hatch (*dph;* spawned July 20th) using the same procedure as described above. Triplicate marking containers were used for each concentration and stocking density during marking was 50 larvae l^{-1}. After marking, larvae were loaded into 1.5 l beakers (n=3) randomly arranged in a temperature controlled waterbath and were held for 9 days. Dead individuals were counted and removed each day.

2.3. Effect of ALC marking procedure

A second experiment investigated the handling-effect of ALC marking procedure on larval growth and condition (year 2008). Cod larvae used for this experiment stemmed from one spawning event (June 11th) and measured (mean ± standard deviation, SD) 4.2±0.2 mm standard length (*SL*), 60.1±9.7 µg freeze-dried weight (*DW*) and had a yolk-sac area (*YSA*) of 1.46(±0.19) mm^2 at hatch. Yolk sac larvae (3 *dph*) were distributed among three treatment groups: a) larvae marked with 0 mg l^{-1} (handling group) or b) 50 mg l^{-1} ALC (marking group) in 25 l marking containers (100 larvae l^{-1} stocking density, 24 h exposure time, slightly aerated, 7±0.2 °C, pH 8.3±0.3, mean ± range) or c) larvae that were not manipulated in any way (control group). Control group larvae were left under pristine conditions in egg/larval incubators. After 24 h, 1500 larvae of each treatment group were divided evenly into 3 groups and transferred to triplicate 25 l rearing tanks (20 larvae l^{-1}). Water in the tanks was slightly aerated and mixed using airlift pumps separated from the larvae with a 320 µm mesh-sieve tank partition. Within 12 h after loading, water temperature was increased from 7.0 to 10.0±0.2 °C (mean ± range). *Nannochloropsis* sp. (10^6 cells ml^{-1}) was added to each rearing tank and, from day 5 post hatch, larvae were fed daily rations of calanoid copepod (*Acartia tonsa)* nauplii (>1.5 ml^{-1}). Samples for larval growth and condition were taken from each tank every third day from 5 to 20 *dph*. At each sampling, 10 larvae were anaesthetized with 50 mg l^{-1} MS222, photographed under a stereo microscope, rinsed with distilled water and stored at -

80 °C prior to freeze-drying and RNA-DNA ratio (*RD*) analysis. Larval *SL* and *YSA* was measured with an image analysis software tool (ImageJ 1.41o, Wayne Rasband) to the nearest 0.1 mm and 0.1 mm^2, respectively, and *DW* to the nearest 0.1 µg (SE2 Ultra Micro, Sartorius). The growth experiment was terminated after 16 days (day 20 post hatch). The number of surviving larvae per tank was counted on this day.

Incidence of first feeding was assessed 24 h after marking (day 1 post exposure, *dpe*; 5 *dph*) using naïve larvae offered *A. tonsa* nauplii at a prey-concentration of 1 ml^{-1} for the first time. After 4 h feeding time, 10 fish were randomly sampled from each rearing tank and gut content inspected using a stereo microscope.

Survival as a proximate consequence of marking procedure was assessed in yolk sac larvae that were transferred to 3 replicate survival tanks (50 larvae per tank) per treatment directly after marking. Tanks contained 4 l of slightly aerated water and no food was added. Larval survival (whole tank count) and morphometrics (*SL* and *YSA* of 20 larvae per tank) were assessed on 5 *dpe* (9 *dph*).

2.4. RNA-DNA ratio analysis

The RNA-DNA ratio of whole, individual larvae was analyzed using a fluorescent-dye, microplate assay, modified after Caldarone et al. (2001). In short, freeze-dried larvae were homogenized in 1 % Sarcosil-Tris-EDTA-buffer using an ultrasonic disruptor, diluted with Tris-EDTA-buffer and two aliquots were mixed with Ethidiumbromide (EB) in a 96-well plate. Fluorescence was measured at 520/605 nm (excitation/emission) in a microplate fluorometer (Xenius XC, SAFAS) and the average of the two aliquots was used. Subsequent addition of specific restriction enzymes (R 6513 and D 4263, Sigma Aldrich) eliminated RNA and DNA from the samples. Concentrations were determined based on calibration curves using highly-purified 18S + 28S rRNA from calf liver and calf thymus DNA (R 0889 and D 4764, Sigma Aldrich). A standardization factor (SF_{Pi} = 0.33±0.05, mean ± SE) for inter-laboratory comparison of RNA-DNA values was calculated based on the RNA and DNA standard curve slope ratios of all nucleic acid assays performed in this study (Caldarone et al., 2006).

2.5. Data analysis

The time of 50 % maximum hatching success of eggs marked with different ALC concentrations was estimated by fitting third order polynomial regressions to tank-wise, daily cumulative hatching success data. Yolk absorption rates and *DW*-specific growth rates (% d^{-1}) were derived from the exponential regression model parameter g (instantaneous growth rate, d^{-1}) fitted to replicate tank-wise mean values versus time and calculated using the formula: $SGR = 100*(e^g - 1)$. Absolute *SL*-growth rates (mm d^{-1}) were derived from linear regression models fitted to replicate tank-wise mean values of *SL* versus time. Percentage data were arcsine transformed [arcsine $(\%/100)^{0.5}$] prior to statistical testing. Treatment-related differences in *SL*- and *DW*-growth in exogenously feeding larvae (growth-experiment) were tested using Multivariate Analysis of Covariance (MANCOVA), using age (*dph*) as covariate. *DW* data were ln-transformed prior to MANCOVA. One-way ANOVA and subsequent post-hoc tests (SNK procedure) were used to test for differences in survival, hatching success, feeding incidence and yolk absorption rates. Kendall's W non-parametric test for concordance

between ranks was used to discriminate treatment effects of ALC marking procedure across different effect parameters. Statistical significance was set at alpha = 0.05. If not stated otherwise, all means (± SE) are based on replicate tanks. All analyses were performed using SPSS (v18, SPSS Inc.).

3. Results

3.1. Effect of ALC concentration

Hatching was significantly affected by ALC concentration (Fig. 1, Table 1). Cumulative hatching success at 200 mg l^{-1} was significantly lower (39.5±5.0 %, mean ± SE, used throughout the remainder of the manuscript) than the control group (0 mg l^{-1}: 88.0±2.0 %) and the 50 mg l^{-1} group (78.0±5.0 %), but not significantly different from 100 mg l^{-1} (57.0±12.0 %) (ANOVA, $F(3,4)=10.4$, $p=0.023$). Please note that cumulative hatching success is equal to embryo survival, because all unhatched embryos died in their egg shells. Larval survival after hatch was not assessed. In the 0 mg l^{-1} ALC control group, 50 % maximum hatch was reached within 4.8±0.5 h after exposure and was significantly delayed to 38.4±9.6, 50.4±4.8 and 81.6±2.4 h after marking in 50, 100 and 200 mg l^{-1} concentration groups (ANOVA, $F(3,4)=32.3$, $p=0.003$).

Yolk sac larval survival during 24 h ALC exposure and the following 48 h was high in all treatment groups (>96 %, Fig. 2). Subsequent survival was highly variable among replicates and no significant treatment-related difference (multiple ANOVAs, Bonferroni-corrected $p>0.05$) was found on any day (Fig. 2). Average survival on day 5 post exposure was 30.6±18.7, 6.0±3.1, 36.6±11.8 and 17.2±13.1 % in 0, 50, 100 and 200 mg l^{-1} treatment groups, respectively, and was not significantly correlated to ALC concentration (Table 1).

3.2. Effect of ALC marking procedure

Yolk sac larval survival was significantly affected by ALC marking procedure. Larvae from the 50 mg l^{-1} ALC marked group had a significantly lower survival (28.0±3.1 %) than handled (50.0±7.6 %) and un-handled control (58.0±3.5 %) groups (ANOVA, $F(2,6)=9.3$, $p=0.014$).

Yolk absorption rates, as a proxy for catabolic metabolism in feeding and food-deprived yolk sac larvae (Table 1), were not significantly affected (feeding yolk sac larvae: $F(2,6)=1.61$, $p=0.276$; food-depleted yolk sac larvae: $F(2,6)=0.99$, $p=0.426$). However, the lowest yolk absorption rates were observed in marked larvae.

Incidence of first feeding, a proxy for larval foraging activity, was not significantly affected ($F(3,8)=1.30$, $p=0.341$). Successful first feeding was 60.0±0.0 % in the control group, 43.0±9.0 % in 50 mg l^{-1} ALC marking group and 47.0±9.0 % in handling group (Table 1).

Growth rates in *SL* and *DW* were calculated for the time period starting after cessation of mixotrophic feeding (11 *dph*) until the last day of successful sampling (20 *dph* for n=2, day 14 for n=1 rearing tank in each treatment) (Table 1). There was a significant effect of ALC marking procedure on *SL* and *DW* growth (MANCOVA, Pillai's trace statistic, $V=0.40$, $F(4,52)=3.2$, $p=0.020$). Subsequent univariate ANCOVAs on the outcome variables revealed significant effects for both *SL* ($F(2,26)=3.3$, $p=0.038$) and *DW* ($F(2,26)=6.1$, $p=0.007$). Post-

hoc contrasts for both variables showed that the marking group was significantly different from the handling group, but not significantly different from the control.

Survival could not be assessed during the experiment because the larvae could not be clearly observed in the green water rearing tanks. Sampling corrected survival, assessed on the last day of the experiment (16 *dpe*; 20 *dph*), was low in all replicate tanks and ranged in average from 1.1 % to 9.6 % (Table 1). In three tanks (one tank of each treatment), mortality had reached 100 % before the last day of sampling. These 3 tanks also exhibited the lowest growth increment since the previous two sampling occasions, indicating a link between low tank-survival and poor growth-performance.

Biochemical condition (RNA-DNA ratio) was not significantly different among treatment groups over time (Fig. 3). RNA-DNA ratios were initially high (1.4±0.1 µg μg^{-1}) at 4 *dph* and slightly decreased to 1.00±0.04, 1.10±0.03 and 1.22±0.05 µg μg^{-1} in marked, handled and control group respectively on 11 *dph* (end of mixotrophic feeding stage). A moderately poor state of condition (across replicates average of 1.09 µg μg^{-1}) was maintained until the end of the experiment on 20 *dph*.

Kendall's W significantly discriminated the three treatment levels (Kendall's W=0.755, $\chi^2(2)=10.57$, p=0.005). All proximate effect variables parameterized in this experiment were equally included based on their treatment average judging ranks (Table 1). Pair-wise post-hoc comparisons revealed that measurements made on handling (p=0.003) and control group (p=0.008) larvae were both significantly different from marking group, but not from one another (p=0.789).

4. Discussion

In contrast to the results of previous studies conducted on Atlantic cod (Blom et al., 1994; Svåsand, 1995), our work indicated that Alizarin Complexone (ALC) marking of Baltic cod embryos and yolk sac larvae was associated with acute as well as chronic effects. In the following sections, we will relate our findings on the response of different proximate effect parameters to ALC marking procedures and give recommendations for their assessment.

A literature review of studies applying Alizarin marking to finfish early life stages and invertebrates indicated that only 46 of 75 laboratory studies (61 %) assessed potential deleterious effects (ASFA database search, February 2011). In those studies, comparison of the survival of treated and untreated (control) groups was common. However, only 14 studies (19%) assessed sublethal effects (Table 2). The majority of those studies compared size-at-age of marked and unmarked fish or invertebrates (8 references). Growth or calcification rates were assessed in 6 studies (8%) and only one study investigated growth and growth variability using a sufficient duration of time to detect subtle growth retardation and recovery in a slow-growing species, the green sea urchin *Strongylocentrotus droebachiensis* (Ellers and Johnson, 2009). Studies on the effects of marking should quantify both acute (e.g., survival) and chronic (e.g., growth rates) effect parameters, especially where the aim is to release marked individuals that would recruit and enhance the local population.

4.1. Acute Effects of ALC marking

In the present study, hatching success of Baltic cod embryos was significantly affected by exposure to the lowest ALC concentration (50 mg l^{-1}) and a negative correlation to hatching success was found over the full range of concentrations from 0 to 200 mg l^{-1}. Blom et al. (1994) marked Atlantic cod eggs in a similar developmental stage and reported no effect on hatching and high survival rates (~96 to 99 %) within 24 h after exposure, irrespective of utilized ALC concentration (up to 200 mg l^{-1}). It can be speculated that ALC impacts on hatching success found in our study may be due to the specific characteristics of eggs produced by cod in the Baltic Sea. Compared to conspecifics in marine environments, cod in the brackish Baltic Sea produces eggs that have a larger diameter and a thinner chorion (Nissling et al., 1994a) that may allow higher effective doses of ALC to reach embryos as opposed to embryos of conspecifics from other (marine) populations.

Although embryos from all treatment groups survived the first 24 to 48 h after exposure, many were incapable of hatching. This caused a significant delay in peak hatch (time of 50 % hatch) by up to 3 days in the highest concentration group. Two patho-physiological mechanisms for a similar delay in hatching success caused by aquatic pollutants were suggested by von Westernhagen (1988). Either a reduction in pH caused by the polluting agent may inhibit hatching enzyme activity or reduced movements of lethargic embryos may lead to insufficient dispersal of the hatching enzyme in the perivitelline fluid and ultimately hamper emersion from the eggshell. Although the consequences of hatching delay has been studied, it may affect larval quality and thus their long-term viability.

Marking of 3 *dph* larvae yielded satisfying results in terms of survival and marking success and is our recommended protocol for restocking efforts with Baltic cod yolk sac larvae (Støttrup et al., 2008a). Distinct otolith marks were apparent after immersion in 50 mg l^{-1} ALC for 24 h and could be successfully recognized up to 74 *dph* (S. Meyer, unpublished data). In this respect, our results agree well with those of other studies conducted on cod (Blom et al., 1994) and other species such as turbot (Iglesias and Rodríguez-Ojea, 1997) and striped bass (Secor et al., 1995).

None of the ALC concentrations had a significant effect on yolk sac larval survival, indicating that 200 mg*l^{-1} was well below the acute mortality threshold for this species and life stage. It has to be noted that the larval batch used in this trial exhibited a low overall survival (less than ~37 %) on day 5 after exposure (9 *dph*) which is well before endogenous reserves were exhausted (S. Meyer, unpublished data) and could thus not be attributed to starvation mortality. Poor larval quality has therefore probably superimposed the sought after effect of ALC concentration. Another batch of yolk sac larvae of the same age was used in the trial investigating the effect of marking procedure and handling stress. In this case, survival at day 5 after exposure was overall higher (in average between 28 and 58 %) and was indeed significantly affected by ALC marking. This trial was conducted at a higher water temperature and larvae were therefore already closer to the point of irreversible starvation when survival was assessed. The consequently higher susceptibility to starvation-induced mortality has potentially exacerbated the negative ALC treatment effect.

4.2. Chronic Effects of ALC marking

The present study suggests that using a "standard" marking protocol (in terms of ALC concentration and immersion time) on marine fish early life stages may result in a number of sublethal effects related to either handling and/or chemical stress. In our work with Baltic cod yolk sac larvae, the ALC marking protocol had a tendency to affect feeding incidence and yolk absorption rates. First-feeding success was highest (60 %) in control group and lower (43 %) in larvae exposed to 50 mg l^{-1} ALC. Even this seemingly subtle difference in first-feeding, not significant under our experimental conditions, could represent a fitness handicap for released larvae in the field by reducing in the window of opportunity (Overton et al., 2010).

An indication for a second chronic effect was found in yolk sac absorption rates in both well-fed and food-depleted larvae. Firstly, the increased yolk absorption rates in the handling group (compared to the control) can be potentially attributed to physical stress through (micro-) oxygen depletion and mechanical handling stress, both mechanisms known to cause increased blood cortisol and corticosteroid concentrations in (larval) cod and other teleosts (Herbert and Steffensen, 2005; King and Berlinsky, 2006) and therefore potentially increasing metabolic rates. Secondly, the relatively lower yolk absorption rates in the ALC treated larvae are speculated to be a result from an interference of ALC with physiological processes (e.g., cell respiration rates, protein biosynthesis), counteracting the aforementioned stress-induced changes.

The most important finding of this study was that ALC marking was related to significantly reduced growth, a chronic effect not previously found in studies employing ALC marking on finfish early life stages (see Table 2) and assumed not to exist when ALC is applied to aquatic organisms in general. It is also important to note that the growth rate differences and size variability observed here may not accurately reflect the magnitude of potential differences since larval growth rates were relatively low. Relatively small prey (copepod nauplii) were used throughout the experiment which were likely sub-optimal for growth of larger larvae (Puvanendran et al., 2004; Rowlands et al., 2008). This appears reasonable since RNA-DNA ratios, which are highly correlated to short-term (1 to 3 days) growth in fish larvae (Buckley et al., 2008), were generally low during the latter half of the trial. Visual inspection of the 75th percentile of the sampling population did support the assertion of diverging sub-populations (Folkvord et al., 2009) in all treatments and suggests that in the marking group tanks, some individuals might even have recovered from marking stress. A recovery period of 1 month post exposure to ALC for growth rates of a slow-growing sea urchin species was reported by Ellers and Johnson (2009). The authors repeatedly measured wet weight of urchins and analyzed the residuals of size-specific growth rate functions to overcome the shrouding effects of increasing intrinsic growth variability (Ellers and Johnson, 2009). When exactly the marked individuals in our study might have recovered from the sublethal growth-retarding effect and if they were growing at rates comparable to the controls cannot be answered here.

Naturally, any reduction in growth rates of marked and released larvae would diminish chances of survival by extending the duration of time when larvae are within stages that are particularly vulnerable to predation and nutritional deficiencies (Houde, 2008). It appears that Baltic cod larvae in our study ingested ALC during the marking period, likely via drinking (Mangor-Jensen and Adoff, 1987), since fluorescent light microscopy detected ALC in both

gut epithelia and liver directly after the marking procedure through 9 *dph* (S. Meyer, personal observation). The underlying patho-physiological processes involved in reduced growth may, therefore, be related to reduced digestion and/or resorption capacities although this remains to be examined.

The combined analysis of all proximate effect parameters of the ALC marking procedure experiment integrates the previously described findings. Using eight different parameters on a continuum from acute (survival) to chronic (yolk absorption, growth, and feeding incidence), we could show that ALC marking has a significant effect on the early life stages of marine fish larvae and that this effect cannot be attributed solely to handling stress.

4.3. Conclusions and Recommendations

The results of this study indicated that exposing 3 *dph* Baltic cod larvae to 50 mg l^{-1} ALC for 24 h is the preferred marking procedure for restocking efforts using releases of yolk sac larvae (Støttrup et al., 2008b). However, survival and growth of larvae were negatively affected by ALC treatment, possibly because Baltic cod larvae are more sensitive to ALC than marine conspecifics, since our results were in contrast to those obtained with marking similar stages of Atlantic cod (Blom et al., 1994; Svåsand, 1995). We recommend that marking efficiency and longevity of lower concentrations should be assessed. Future experiments with finfish larvae should quantify both acute (e.g., survival) and chronic (e.g., hatching success, first feeding, growth during exogenous feeding) effects and utilize a handling control when optimizing ALC marking protocols for the target species and life stage.

Acknowledgements

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5. Figures

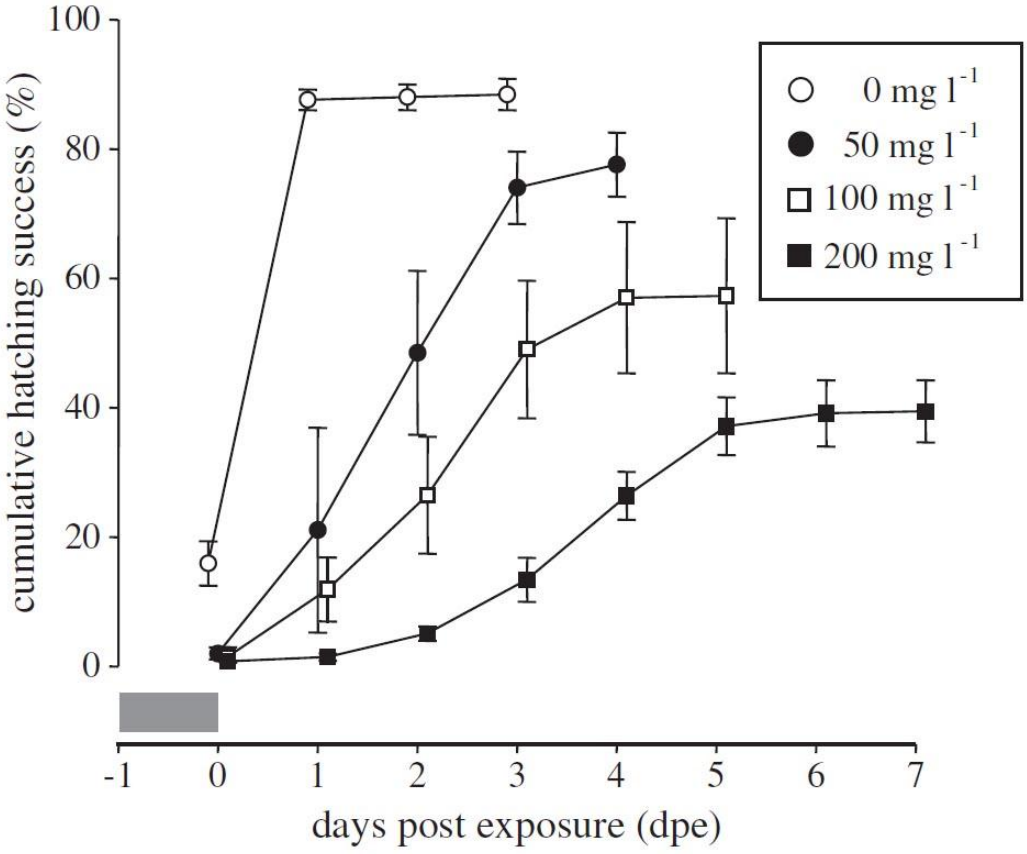

Figure III-1 Cumulative hatching success (%) of Baltic cod (*Gadus morhua*) embryos versus time (days) after a 24 h exposure to ALC. Symbols indicate replicate tank-wise mean ± SE for nominal ALC concentrations: 0 (open circles), 50 (black circles), 100 (open squares) and 200 mg l^{-1} (black squares). Symbols were slightly shifted along the abscissa to improve visual clarity. Grey bars indicate relative time of ALC marking procedure.

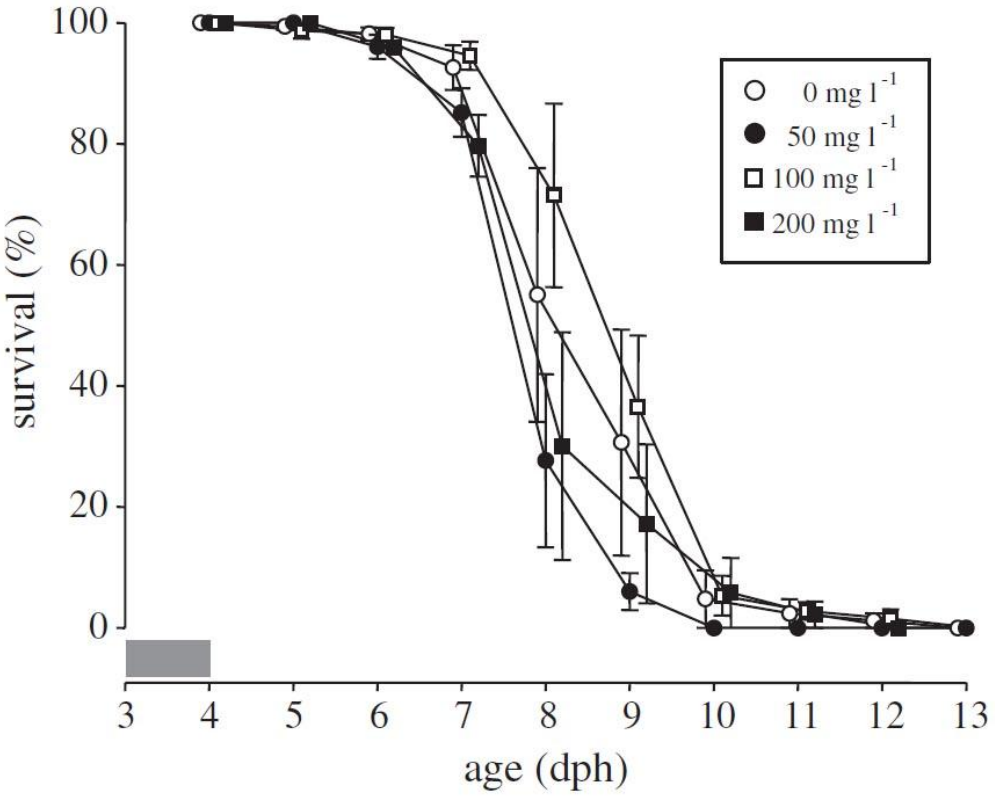

Figure III-2 Survival (%) of Baltic cod (*Gadus morhua*) yolk sac larvae versus age (days post hatch, *dph*) after a 24 h exposure to ALC. Symbols indicate replicate tank wise mean ± SE for nominal ALC concentrations: 0 (open circles), 50 (black circles), 100 (open squares) and 200 mg l^{-1} (black squares). Symbols were slightly shifted along the abscissa to improve visual clarity. Grey bars indicate relative time of ALC marking procedure.

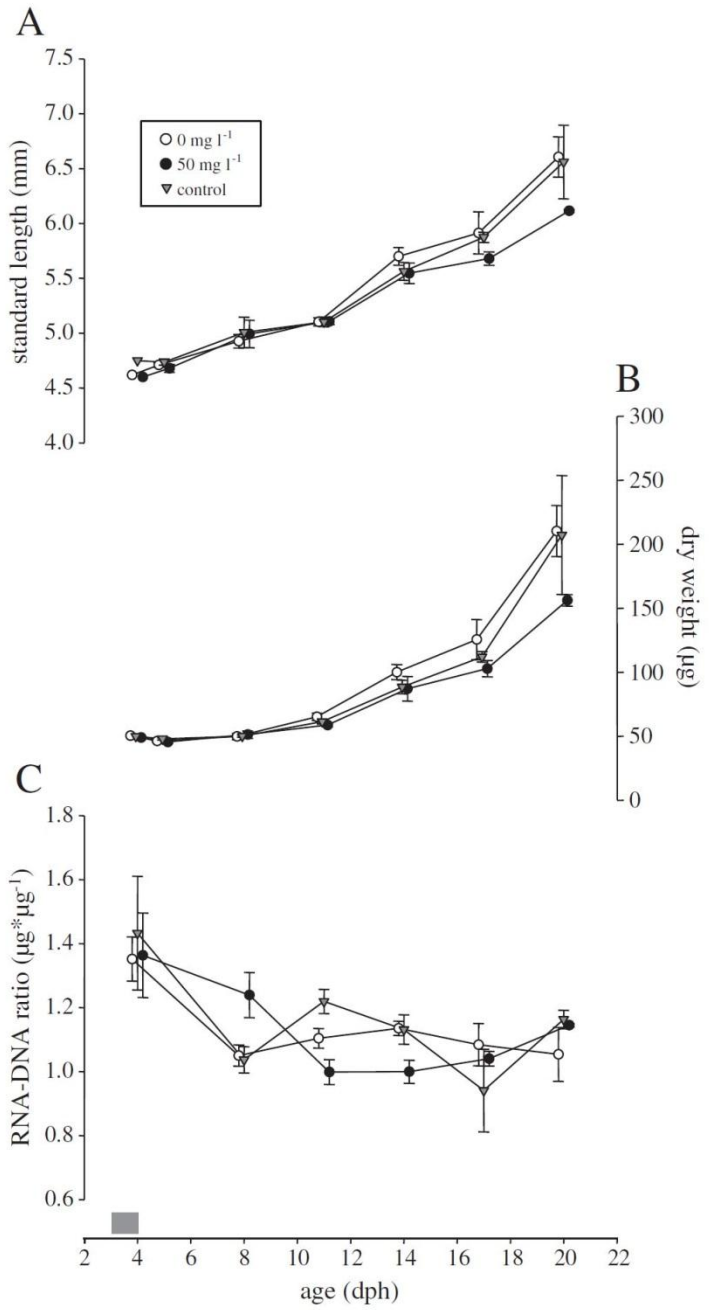

Figure III-3 A: standard length (mm), B: dry weight (µg) and C: RNA-DNA ratio (µg µg^{-1}) of Baltic cod (*Gadus morhua*) larvae versus age (days post hatch, *dph*). Symbols indicate replicate tank-wise mean ± SE of marking procedures: marked with 50 mg l^{-1} ALC (black circles), handled and exposed to 0 mg l^{-1} ALC (white circles) and a control group (grey triangles) that was neither exposed to ALC nor handled. Symbols were shifted slightly along the abscissa to improve visual clarity. Grey bars indicates relative time of ALC marking procedure.

6. Tables

Table III-1 Effect of Alizarin Complexone (ALC) concentration and marking procedure on Baltic cod (*Gadus morhua*) embryos and larvae. Given are replicate tank wise mean ± SE values for selected acute and chronic effect parameters used in the experiments of this study. Different superscripts indicate significant differences (ANOVA, $p \leq 0.05$). R_{KW} indicates treatment average relative judging rank used for Kendall W test.

Effect of ALC concentration	0 mg l^{-1}	50 mg l^{-1}	100 mg l^{-1}	200 mg l^{-1}
Embryos marked 1 day prior to expected hatch				
Maximum hatching success (%)	88.0±2.0 ^{a}	78.0±5.0 ^{a}	57.0±12.0 ^{a,b}	39.5±5.0 ^{b}
Time of 50 % HS_{max} (*hpe*)	4.8±0.5 ^{a}	38.4±9.6 ^{b}	50.4±4.8 ^{b}	81.6±2.4 ^{c}
Yolk sac larvae marked on day 3 post hatch				
Survival, 5 dpe (%)	30.6±18.7	6.0±3.1	36.6±11.8	17.2±13.1

Effect of ALC-marking procedure	handling (0 mg l^{-1})	R_{KW}	marking (50 mg l^{-1})	R_{KW}	unhandled control	R_{KW}
Yolk sac larvae marked on day 3 post hatch						
Survival, 5 *dpe* (%)	50.0±7.6 ^{a}	(2)	28.0±3.1 ^{b}	(3)	58.0±3.5 ^{a}	(1)
Survival, 16 *dpe* (%)	4.8±3.5	(2)	1.1±1.1	(3)	9.6±4.9	(1)
Yolk absorption rate, feeding (% d^{-1})	38.8±2.7	(1)	30.1±4.8	(3)	33.9±2.2	(2)
Yolk absorption rate, food-deprived (% d^{-1})	38.9±2.4	(1)	33.8±2.6	(3)	36.9±2.8	(2)
First feeding incidence, 1 *dpe* (%)	47.0±9.0	(2)	43.0±9.0	(3)	60.0±0.0	(1)
Growth rate $_{SL}$ (mm d^{-1})	0.16±0.01 ^{a}*	(1)	0.10±0.01 ^{b}*	(3)	0.16±0.02 ^{a,b}*	(2)
Specific growth rate $_{DW}$ (% d^{-1})	12.9±0.7 ^{a}*	(1)	9.2±1.3 ^{b}*	(3)	12.5±1.7 ^{a,b}*	(2)

HS_{max}: maximum hatching success; *hpe*: hours post exposure; *dpe*: days post exposure; *SL*: standard length, *DW*: dry weight. * multivariate (*SL, DW*) analysis MANCOVA.

Table III-2 Literature review of studies assessing sublethal effects of Alizarin-derivates (Alizarin Complexone, ALC; Alizarin Red S, ARS) on early life stages of finfish or aquatic invertebrates. Studies assessed marking methodology relevant parameters (concentration, exposure duration, life stage, and others) in comparison to untreated control groups.

Species	marked life stage	Alizarin derivate	study type	survival	sublethal effects		Reference
Finfish							
Anguilla anguilla	glass eel	ARS	L	n.s.	growth:	n.s.	(Simon et al., 2009)
Coregonus albula	larva	ARS	F, L	n.s.	size-at-age:	n.s.	(Eckmann et al., 1998)
Coregonus lavaretus	larva	ALC	F	n.s.	"	n.s.	(Hoeglund and Wahlberg, 1997)
Cyprinus carpio	juvenile	ARS	L	n.s.	growth:	n.s.	(Meunier and Boivin, 1978)
Gadus morhua	egg	ALC	F, L	n.s.	n.a.	-	(Blom et al., 1994) & (Svåsand, 1995)
		ARS	L	(-)*	n.a.	-	"
	larvae	ALC	L	n.s.	n.a.	-	"
		ARS	L	(-)*	n.a.	-	"
	juvenile	ALC	L	n.s.	size-at-age:	n.s.	"
					disease suscept.:	(-)*	
					condition factor:	(-)*	
		ARS	L	n.s.	n.a.	-	"
Oncorhynchus mykiss	juvenile	ARS	L	n.s.	growth:	n.s.	(Meunier and Boivin, 1978)
Paralichthys olivaceus	juvenile	ALC	L	n.s.	size-at-age:	n.s.	(Liu et al., 2009)
		ARS	L	n.s.	"		"
		ALC	L	n.a.	"	(-)*	(Isshiki and Katayama, 2007)
Psetta maxima	juvenile	ALC	L	n.s.	"	n.s.	(Lagardere et al., 2000)
		ARS	L	n.s.	"	n.s.	"
Salmo trutta	larva	ARS	F, L	(-)*	"	n.s.	(Baer and Rosch, 2008)
Takifugu rubripes	egg	ALC	L	n.s.	growth:	n.s.	(Matsumura, 2005)
	larva	ALC	L	n.s.	"	n.s.	"
	juvenile	ALC	L	n.s.	"	n.s.	"

Table III-2 *continued.*

Aquatic invertebrates							(Lagardere et al., 2000)
Diploria strigosa, coral	12 cm Ø	ARS	L	n.a.	calcification rate:	(-)*	(Dodge et al., 1984)
Mercenaria	larva	ARS	L	(-)*	growth:	(-)*	(Hidu and Hanks, 1968)
mercenaria, clam	juvenile	ARS	L	n.a.	"	(-)*	"
Mya arenaria, clam	juvenile	ARS	L	n.a.	"	n.s.	"
Strongylocentrotus droebachiensis, urchin	4-44 mm Ø	ALC	L	n.s.	"	(-)*	(Ellers and Johnson, 2009)

Abbreviations: F, field study (mesocosms, release-recapture); L, laboratory study (controlled biotic and abiotic parameters); Ø, diameter; n.a., not assessed; n.s., not significant; (-)*, significant negative effect

Literature for Table III-2

(Baer and Rosch, 2008; Blom et al., 1994; Dodge et al., 1984; Eckmann et al., 1998; Ellers and Johnson, 2009; Hidu and Hanks, 1968; Hoeglund and Wahlberg, 1997; Isshiki and Katayama, 2007; Lagardere et al., 2000; Liu et al., 2009; Matsumura, 2005; Meunier and Boivin, 1978; Simon et al., 2009; Svåsand, 1995)

Manuscript IV:

IV. Role of heterotrophic protists in first feeding by cod (*Gadus morhua*) larvae

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Abstract

The present study evaluated whether the presence of autotrophic phytoplankton and/or heterotrophic dinoflagellates (*Oxyrrhis marina*) influenced first-feeding success and early larval growth and development of Baltic cod *Gadus morhua* L. Newly hatched cod larvae were maintained in salt water ('clear water', CW), greened water using Nannochloropsis sp. (NA) or a combination of *Nannochloropsis* sp. and O. marina (NO) from 2 to 11 d post-hatch. On each day, larvae from each group were placed into feeding tanks and provided potential prey (nauplii of the calanoid copepod *Acartia tonsa*). Feeding performance was measured both as the percentage incidence of feeding and gut fullness index. The onset of first feeding, point of no return and the magnitude of feeding were derived from the feeding incidence. The study clearly demonstrated precocious, exogenous feeding by cod larvae in the presence of *Nannochloropsis* sp. and O. marina by 1.2 and 2.2 d (10°C), respectively. Gut fullness was also significantly improved in the presence of both phytoplankton and protists over the entire window of opportunity (defined as the period between onset of first feeding and point of no return). However, the nutritional capacity of these unicellular organisms was not sufficient to alter the somatic growth characteristics in NA and NO groups relative to starving yolk-sac larvae maintained in only salt water (CW group). These findings expand the current understanding of the degree of interaction between fish larvae and unicellular plankton communities and indicate a lack of nutritional benefits from feeding on components of the pelagic microbial loop but a clear benefit in terms of 'priming' first-feeding capabilities.

Keywords

Protists, Marine fish, First feeding, Larvae, Cod, *Oxyrrhis marina*, *Nannochloropsis* sp., Survival, Growth

1. Introduction

Understanding the factors that contribute to recruitment variability has been at the heart of marine fisheries research since Hjort (1914) first devised the 'critical period hypothesis'. Rates of survival of early life stages of marine fish species are thought to be highly sensitive to variability in prey field characteristics (abundance, composition) and/or hydrographic factors (e.g. water temperature and currents) and even small changes in mortality and growth rates of early life stages can cause order-of-magnitude differences in year-class success (Houde 2008). A particularly vulnerable period during early life is the transition from endogenous to exogenous feeding, as larvae need to successfully initiate feeding before they cross the 'point of no return' (PNR; sensu Blaxter & Hempel 1963). Clearly, it is important to understand the processes influencing temporal and spatial match-mismatch dynamics between larvae and suitable prey (both in terms of prey size and quality) since these processes govern the probability of successful first feeding and early survival of larval fish (Cushing 1975, Yúfera & Darias 2007, Houde 2008).

It has long been believed that larval marine fish are part of a linear food chain where autotrophic phyto-plankton is fed on by heterotrophic zooplankton that, in turn, is preyed upon by fish larvae. Analyses of larval fish gut contents and feeding behaviour has resulted in the claim that herbivorous, crustacean zooplankton (copepods) is the preferred prey of young marine fish larvae (Munk 1997, Pepin & Penney 2000). However, recently there has been some evidence to suggest a potential flexibility in the feeding behavior of fish larvae (Pepin & Dower 2007). In particular, more focus has been given to representatives of the planktonic microbial loop. For example, marine fish larvae have been reported to directly feed on heterotrophic protists (Lasker 1975, Scura & Jerde 1977, Fukami et al. 1999), organisms that can be highly abundant in marine environments during specific time periods (Tamigneaux et al. 1997, Hansen & Jensen 2000, Sommer et al. 2002, Ptacnik 2003). However, it is still largely unknown if indirect (incidental) or direct ingestion of algae and heterotrophic protists provides any nutritional and/or energetic value for marine fish larvae.

Evidence exists from marine fish larviculture that microalgae can increase the survival and condition of young, first-feeding larvae; hence 'green water' techniques have been utilised in rearing protocols for decades (Howell 1979, Skiftesvik et al. 2003). For example, yolk-sac Atlantic cod Gadus morhua L. larvae have been reported to ingest phytoplankton either passively or through active filter feeding prior to 'first feeding' on copepod nauplii (Ellertsen et al. 1980, van der Meeren 1991). Changes in the lipid composition that occurred in first-feeding cod larvae reared in the presence of microalgae (van der Meeren et al. 2007) were thought to provide nutritional benefits that delayed the onset of fasting symptoms in unfed larvae uch as gut epithelia degeneration, hepatocyte degeneration and cholestasis (Kjørsvik et al. 1991, Diaz et al. 1998, Maurizi 2000). Thus, ingestion of autotrophic phytoplankton (and perhaps the heterotrophic protists that feed on those algae) may represent an alternative (nutritional) pathway that increases starvation resistance and the probability of survival in marine fish larvae in variable feeding environments.

The present laboratory study was designed to answer 2 questions. First, is first-feeding success of marine fish larvae (in this case Baltic cod larvae) on metazooplankton increased when larvae have been reared in the presence of autotrophic phytoplankton (Nannochloropsis sp.) and/or a heterotrophic dinoflagellate (Oxyrrhis marina)? Second, do larvae receive direct nutritional benefits from these microbial loop components as assessed via early growth? Answering these 2 questions will help assess the potential role played by algae and heterotrophic protists in the early feeding, survival and growth of marine fish larvae.

2. Material and methods

2.1. Production of yolk-sac larvae

The larvae used in the experiment were hatched from eggs collected from captive Eastern Baltic broodstock cod collected in March 2006 east of Bornholm (55° 03' 00''N, 15° 11' 50'' E). The egg batch was collected on 16 May 2008 from spontaneous group spawning within the broodstock tank. A total of 600 ml of eggs (90% fertilisation) was gently collected from the water surface via a PVC pipe into a 500 μm mesh bag. The eggs were subsequently disinfected using 400 mg l–1 glutaraldehyde solution for 10 min before being transferred to a 100 l cylindrical, black, PVC tank, where they were incubated within a recirculation system at 7°C and a salinity of 16.0 using artificial salt water (Tropic Marin®). The surface light intensity was maintained at 15 lux (DVM1300, Velleman®). The natural light regime occurring at the time of spawning was used (15h dark: 9h light). Only eggs hatching within a 24 h period (between 84 and 93 degree-days after collection) were used in experiments. These larvae were maintained for an additional 24 h prior to the start of the experiment.

2.2. Experimental set-up

The experiment was conducted within a controlled-temperature room at 10.0 ± 0.2°C (mean ± range). At 2 days post-hatch (dph), 1200 larvae were transferred to 1 of 3 black, square, 60 l holding tanks, representing 3 treatment groups. Each tank was filled with artificial salt water (0.1 μm filtered, salinity 16.0). Larvae were maintained in either salt water ('clear water', CW), in salt water containing the eustigmatophycean *Nannochloropsis* sp. (NA) (1.5 × 106 to 2.0 × 106 cells ml–1, size range 2.0 to 3.4 μm), or in a tank containing both Nannochloropsis sp. and the heterotrophic dinoflagellate Oxyrrhis marina Dujardin (NO) (1000 cells ml–1, size range 13.3 to 16.0 μm). The concentration of these organisms in larval tanks was monitored during the experimental period. *Nannochloropsis* sp. was maintained at the same concentrations in both the NO and NA treatments; decreased concentrations due to feeding by *O. marina* were offset by supplemental additions of Nannochloropsis sp. Gentle aeration (4 mm diameter glass capillaries, individual air bubbles) was applied to maintain steady water circulation and high oxygen concentrations (>10 mg l–1). All tanks received constant (24 h) light. The NO and NA treatments were maintained at constant light at ~400 lux (underwater reading of 180 lux), to ensure sufficient light energy for *Nannochloropsis* sp. to maintain positive growth rates. A lower surface light intensity (10 lux, 5 lux underwater reading) was used in the CW treatment to account for the higher light penetration in that tank due to the absence of algae.

At 2 dph, 1200 larvae were randomly loaded into each of the holding tanks (1 holding tank treatment–1). For each of the 3 treatment holding tanks, triplicate 5 l plastic aquaria (each containing 4 l salt water) were allowed to acclimatise to experimental conditions 14 h before the daily experimentation began. The experimental tanks contained the same algal and light conditions as in the associated holding tanks. The experimental tanks remained static, with no air supplementation. The entire experiment was conducted at 10 ± 0.2°C. In order to maintain these temperature conditions, holding tanks and experimental tanks were placed in temperature-controlled water baths. The temperature was controlled by computer (Aqua Medic, T Computer) and monitored throughout the experiment (TLog64-USB, Hygrosens; resolution 0.06°C). The salinity was 16.0 ± 0.1 measured at the beginning and end of the experiment (WTW cond315i).

Prey used in daily feeding trials were 2 d old nauplii of the calanoid copepod Acartia tonsa that had been fed the cryptophycean Rhodomonas baltica Karsten (mean cell size: 7.6 μm). Both A. tonsa and R. baltica were from cultured strains produced by DTU Aqua (Technical University of Denmark) and reared according to methods reported by Peck & Holste (2006). The red algal pigment in the guts of the otherwise transparent copepod nauplii acted as a natural marker and aided in the detection of nauplii in the guts of feeding larval cod. To ensure food was non-limiting, concentrations of 1 nauplii ml^{-1} were employed.

The experiment was conducted for 9 d, through the PNR and after the exhaustion of yolk-sac reserves. Starting at 2 dph, 60 larvae were removed each day from each holding tank and 20 larvae were placed into each of 3 experimental tanks. Food items were added to the experimental tanks prior to larval loading. The larvae were then allowed to feed for 4 h ± 10 min, after which most of the water was removed and the tanks were placed on ice to chill the remaining water (~1 l) and larvae to a low temperature to greatly minimise any further digestion and/or feeding. Each larva was viewed under a dissecting microscope (50×) for the presence of food (both Acartia tonsa and algae) in the gut and subsequently digitally photographed at 12× magnification (Leica DFC). Ten of these larvae were rinsed with distilled water to remove excess salt, placed in 1.5 ml micro-centrifuge vials and stored at –80°C (ultra freezer) until further processing. Any mortalities and deformed or moribund fish were noted. Mortality was noted as a lack of heartbeat. On the day of 50% hatching (0 dph), 20 additional larvae were photographed and stored for dry mass analysis.

2.3. Measurements

The standard length (SL, ± 0.1 mm), myotome height (MH, ± 0.1 mm) and yolk-sac area (YSA, ± 0.01 mm2) of each larva was measured using image analysis software (Image J, version 1.40g, freeware, Wayne Rasband, NIH, USA). Myotome height was used as a proxy for condition, reflecting energy allocation to body mass rather than axial length. The larvae were freeze-dried (Christ-Alpha, 16 h, 0.2 millibars) and their freeze-dried mass (DM, ± 0.1 μg) was measured using a digital microbalance (Sartorius 1773 MP8).

In larvae with food in the gut, the magnitude of feeding was assessed using a simple gut fullness index (GFI; van der Meeren et al. 2007) that employed scores of 1 (<6 intact copepods and/or remnants in the gut), 2 (>6 clearly distinguished copepods in the gut but a

gut that was not distended) or 3 (fully distended gut packed with prey). For each replicate tank, GFI was calculated using the formula:

GFI = (N1 + 2N2 + 3N3) × (N1 + N2 + N3)–1 (1)

where N1 to N3 represent the number of larvae with gut fullness scores 1 to 3, respectively. A mean GFI score for each treatment was calculated based upon the mean scores from each of the 3 replicate tanks.

2.4. Data analysis

The mean (± SD) incidence of first feeding (FI) was calculated for each treatment on each day based on the percentages of larvae containing food in the gut in each of the 3 replicate tanks. These treatment mean values (n = 3 tanks) were the unit of measure used for all statistical analyses. A 3-parameter log-normal regression was fitted to the data for each treatment:

(2) $$FI = FI_{\mathrm{MAX}} \cdot \mathrm{e}^{-0.5 \cdot \left(\frac{\ln\left(\frac{age}{t_{FI\,\mathrm{MAX}}}\right)}{b} \right)^2}$$

where FIMAX is a parameter estimating maximum feeding incidence, age = larval age in dph, tFI MAX is a parameter representing the age at maximum mean FI, and b is a constant. Although data were collected on a daily basis, using this regression allowed us to more clearly estimate the point of first feeding, FI50 (the larval age when 50% of the larvae had food in the gut), the larval age and percentage of maximum incidence of feeding (FIMAX) and the larval age at the PNR (the larval age when the incidence of first feeding had reduced to ≤50%, PNR50). The window of opportunity (WOO, the timeframe in which feeding incidence was >50%) could be calculated as PNR50 – FI50. In addition to this, the magnitude of feeding during the WOO could also be estimated (based on the integral of the curve above FI50).

Mean larval FI (%, arcsine-transformed), SL (mm), DM (μg), MH (mm), YSA (mm2) and GFI were compared among treatments (n = 3 tanks treatment–1) on each day of sampling using a 1-way ANOVA followed by pair-wise comparisons using a Tukey test (Sigma Stat, version 2.0, SPSS).

3. Results

3.1. Incidence of first feeding

Feeding occurred at a younger age in the NA and NO treatments compared to the CW treatment (Fig. 1). Successful first feeding (>50% mean FI) was initiated by larvae at an age of 4 dph in NA and NO treatments compared to 6 dph for larvae in the CW treatment (calculated FI50 = 3.96, 3.37 and 5.57 dph for NA, NO and CW, respectively; Table 1). The point of maximum feeding (FIMAX), as calculated by nonlinear regression, was reached 1.0 and 1.3 d earlier in larvae from NA and NO treatments compared to larvae from the CW treatment. However, there was no difference in the PNR50 (8.2, 8.3 and 8.9 d for larvae in CW, NA and NO treatments, respectively). Therefore, precocious exogenous feeding by NA

and NO larvae extended their WOO compared to CW larvae. The mean magnitude of first feeding (within the 4 h measurement period) by yolk-sac larvae in NA and NO treatments was respectively 1.4 and 2.4 times greater than that observed for larvae in the CW treatment.

3.2. Gut fullness

The addition of algae in the water increased larval gut fullness (Fig. 2). The cod larvae in NA and NO treatments exhibited a high GFI at 3 to 10 dph compared to CW larvae, which were moderate feeders(significant difference at 4, 5, 7 and 9 dph; Fig. 2D). Overall, there was no significant difference in GFI between NA and NO treatments except at 10 dph, when the index of the latter was greater than the former.

3.3. Growth and development

Larval SL increased from 4.4 to 4.6 mm by 5 dph and then remained more or less unchanged, whereas DM steadily decreased from 68.7 to 27.9 to 28.9 μg from the start until the end of the experiment (Fig. 3). Yolk-sac area also decreased throughout the experiment (from 1.39 to 0.015–0.027 mm2) and yolk absorption rates, as calculated from exponential regression lines, were –0.451, –0.442 and –0.441 d–1 in the NO, NA and CW treatments, respectively. Myotome heights were between 0.21 and 0.24 mm and changed little throughout the experiment. For each of these 4 measures (SL, DM, YSA and MH), the vast majority of daily treatment comparisons was not significantly different except in 4 instances: (1) SL at 10 dph (NA > CW), (2) DM at 10 dph (CW > NO and NA), (3) YSA at 4 dph (NA > NO and CW) and (4) MH at 9 dph (CW > NO and NA).

4. Discussion

The present study clearly demonstrated precocious and more intense exogenous feeding by the larvae of a marine fish species in the presence of autotrophic phytoplankton (Nannochloropsis sp.) and heterotrophic dinoflagellates (Oxyrrhis marina). Young larvae in our CW, NA and NO treatments started first feeding at 6, 4 and 3 dph, had 11, 42 and 60% yolk reserves remaining on that day, and reached a peak of 68, 75 and 95% first feeding on 7, 6 and 5 dph, respectively. The presence of autotrophic phytoplankton and heterotrophic protists, therefore, corresponded to a substantial increase in first-feeding success and expansion of the WOO for first feeding. Despite these benefits, our experiment also revealed that the nutritional value of these protists was not great enough to alter the (macroscopic) growth characteristics of yolk-sac larvae relative to conspecifics maintained in pure salt water.

The results of the present study agree well with those of previous research reporting beneficial effects of rearing marine fish larvae in the presence of microalgae including (1) earlier first feeding (Kentouri 1985, Naas et al. 1992, Maurizi 2000), (2) reduction of metabolic stress and prolonged survival of unfed larvae (reviewed in Muller-Feuga et al. 2003) and (3) dramatic improvement of early survival in marine fish species considered problematic to rear (Naas et al. 1992, Reitan et al. 1997). After first feeding, the presence of algae may also (4) increase rates of food consumption and/or growth (Øie et al. 1997, Bengtson et al. 1999, van der Meeren et al. 2007). Previous work by van der Meeren et al. (2007) indicated increased gut filling at 3 dph (7 to 8°C) in cod larvae exposed to microalgae, which agrees with the results

obtained in the present study. However, the present study indicates that this improved gut filling is sustained throughout the mixed feeding stage until PNR is reached. The mechanism responsible for our GFI findings is not known but could involve either intrinsic and/or extrinsic (environmental) factors. For example, different combinations of light intensity and algal concentration may enhance visual contrast, improving prey capture in larval fish (Boehlert & Morgan 1985, Naas et al. 1992). It is important to note that the differences in feeding intensity observed among treatments in the present study cannot be attributed to the small differences in light intensity (175 lux) experienced by larvae in CW compared to NA and NO groups. No significant differences in first feeding by cod larvae were observed over a larger range in light intensities (~50 to 690 lux) in the presence or absence of microalgae (van der Meeren et al. 2007). The difference in feeding intensity between our NA and NO groups (which were at the same light and algal densities) supports the overriding effect of algal characteristics over light intensity in this particular study.

This stimulation of early feeding could be linked to the drinking phase, an important preliminary phase that maintains osmotic balance in the developing larvae as well as instigating absorption of dissolved organic material and ingestion of some small particulate matter (Muller-Feuga et al. 2003). During the drinking phase, cod have been reported to ingest dinoflagellates either passively (via drinking) or actively (by filter feeding) (Ellertsen et al. 1980, van der Meeren 1991), and evidence of some nutritional benefit from imbibing algae has been observed, including changes in phospholipid composition and triglycerol content in first-feeding larval cod attributed to the presence of microalgae (Isochrysis sp.) (van der Meeren et al. 2007). After first feeding, cod larvae have been shown to feed on small protozoans but this appears to be more energetically costly compared to feeding on larger copepod nauplii (Hunt von Herbing et al. 2001). Thus, our finding of no direct (macroscopic) nutritional benefit, such as decreased yolk/energy utilisation or improvement in larval condition indices (dry mass- and myotome heightat- age) among larvae in any feeding treatment, may not be surprising. Regardless of a lack of direct growth benefits, stimulation of first feeding by microalgae and heterotrophic protists may be particularly important for the survival of cod and perhaps in other marine fish species that have a relatively brief WOO. Larvae in the CW treatment started to first-feed (reached FI50) at 60 degree-days post-hatch (ddph = °C·dph), and at 63 ddph, unfed cod larvae exhibited morphological changes in gut villi detrimental to digestive and absorp tive capacity (Kjørsvik et al. 1991). Furthermore, the earlier feeding and increased GFI when feeding would have likely caused NO and NA larvae to exhibit more rapid growth compared to CW larvae after first feeding, but this was not tested in the present study.

Although direct nutritional benefits of algae and heterotrophic protists to larvae were lacking in the present study, their presence appeared to ‘prime’ biochemical and/or developmental systems that, in turn, promoted first feeding. During early development, larval fish cannot synthesise phospholipids (Bell et al. 2003) and must acquire these through their diet. Algae have been demonstrated to stimulate the production of digestive enzymes such as amylase and trypsin, characteristically the first enzymes to be recorded in early marine fish ontogeny (Cahu et al. 1998). It was hypothesized by Cahu et al. (1998) and other authors (Støttrup 1994) that the large amounts of free amino acids contained in algae could be responsible for

the observed stimulation in the production of trypsin. In the wild, free amino acids are often at concentrations <10–7 M (Braven et al. 1984) but can occur at higher concentrations in areas of high phytoplankton production (Williams & Poulet 1986). Therefore, intuitively, one could link these dissolved free amino acids to the stimulation of feeding of fish larvae in the wild. This seems reasonable since concentrations of algae and protists used in the present laboratory study were of the same magnitude as those occurring in situ (e.g. Arndt 1991, Tamigneaux et al. 1997, Hansen & Jensen 2000) at the locations and during times of the year when first-feeding cod larvae would be expected to occur. Naturally, other benefits from microalgae and protozooplankton such as improvement in larval microbial gut flora (Skjermo & Vadstein 1993) should not be dismissed.

The results of the present study highlight the need to revisit the importance of autotrophic phytoplankton and heterotrophic protists in the early survival and growth of marine fish larvae. Previous studies indicated that the larvae of some marine fish species routinely ingest phytoplankton (e.g. Northern anchovy Engraulis mordax feeding on dinoflagellates) which appeared to offer some nutritional benefit (e.g. Scura & Jerde 1977) and was thought to be important for early survival and recruitment success (Lasker 1975). Our results clearly indicated that cod obtained no nutritional (growth) benefit from ingesting algae and heterotrophic protists (protozooplankton) prior to first feeding. However, the presence of adequate amounts of protozooplankton may be critical to the survival and growth of cod by acting to prime first-feeding capabilities of this species, altering the window of opportunity for successful firstfeed and, in turn, influencing the match-mismatch dynamics between first-feeding cod and their macrozooplankton prey.

5. Figures

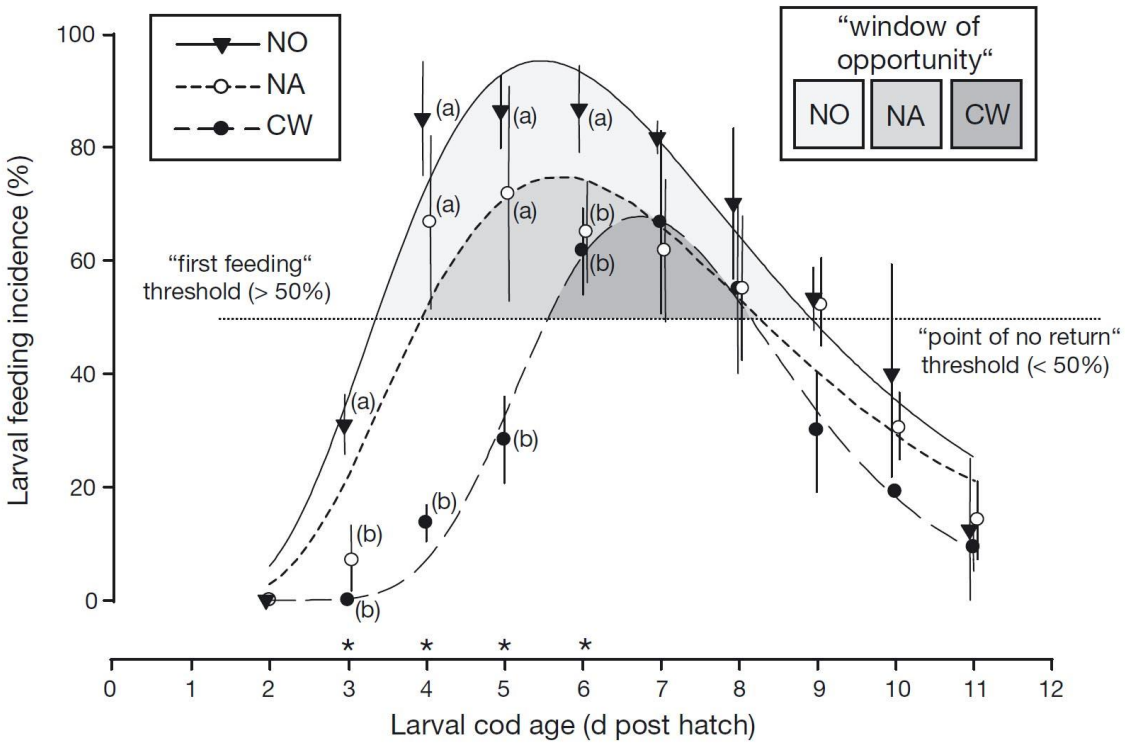

Figure IV-1 Mean feeding incidence (FI) in yolk-sac larvae versus larval age in the presence (NA) or absence (CW) of algae (Nannochloropsis sp.) or in the presence of algae and Oxyrrhis marina (NO). Significant differences in FI were found among treatments at ages 3 to 6 d post-hatch. #Days with significant differences among treatment values. Different letters (a, b) denote significant differences (ANOVA, Tukey post hoc test, $p \leq 0.05$, n = 3 replicates treatment–1). The 'window of opportunity' and magnitude of FI are also indicated (shaded areas) calculated from regression equations (Eq. 2 in 'Data analysis' under 'Materials and methods'; see Table 1). Error bars indicate SD (n = 3).

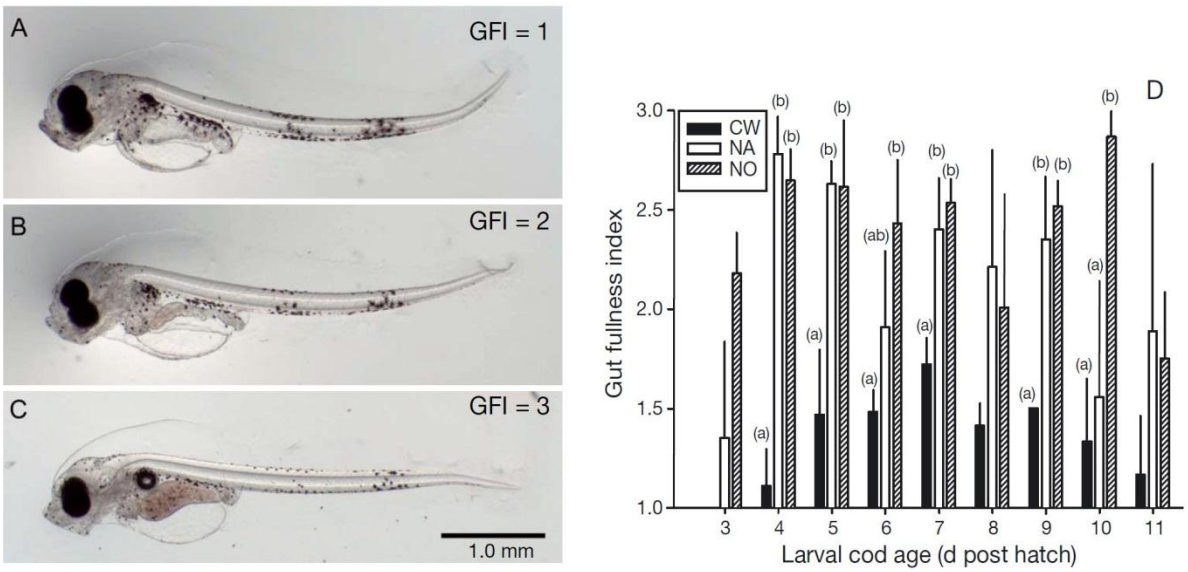

Figure IV-2 (A–C) Example of gut fullness index (GFI) scores in first-feeding larvae, as typed either '1' (<6 intact copepods and/or remnants in the gut), '2' (>6 clearly distinguished copepods in the gut but a gut that was not distended) or '3' (fully distended gut, packed with prey). Photos taken at 12× magnification. (D) Mean GFI scores in yolk-sac larvae versus age for larvae reared in the presence (NA) or absence (CW) of algae (Nannochloropsis sp.) or in the presence of algae and Oxyrrhis marina (NO). On a given sampling day, significant differences in mean GFI are indicated with different letters (ANOVA, Tukey post hoc test, $p < 0.05$). No significant differences among treatments were found at 3, 8 and 11 d post-hatch. Error bars indicate SD (n = 3 tanks).

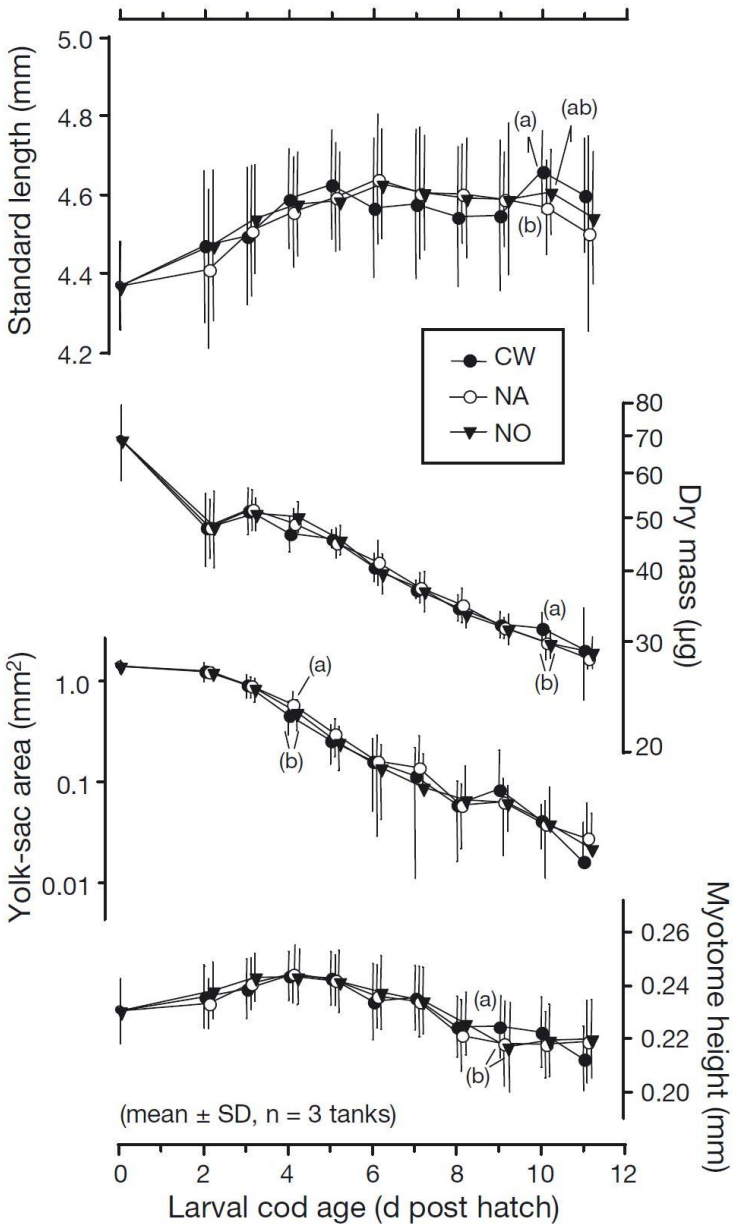

Figure IV-3 Mean standard length, freeze-dried mass, yolk-sac area and myotome height versus age for yolk-sac larval cod reared in the presence (NA) or absence (CW) of algae (Nannochloropsis sp.) or in the presence of algae and Oxyrrhis marina (NO). On a given sampling day, different letters denote significant differences (ANOVA, Tukey post hoc test, $p < 0.05$, n = 3 replicate tanks treatment–1). Error bars indicate SD (n = 3).

6. Tables

Estimates	Treatment		
	CW	NA	NO
***FI* parameter estimates (Eq. 2)**			
FI_{MAX} (%)	67.72 ± 2.43	74.62 ± 6.40	95.13 ± 5.36
b	0.24 ± 0.01	0.41 ± 0.05	0.43 ± 0.03
$t_{FI\ MAX}$ (age at FI_{MAX}) (dph)	6.73 ± 0.07	5.71 ± 0.24	5.48 ± 0.16
Statistics (Eq. 2)			
Observations (n)	10	10	10
Adjusted r^2	0.91	0.85	0.93
p	<0.01	<0.01	<0.01
Calculated first-feeding estimates			
t_{50} (first age at FI_{50}) (dph)	5.57	3.96	3.37
PNR_{50} (dph)	8.15	8.26	8.91
WOO ($PNR_{50} - FI_{50}$) (dph)	2.58	4.3	5.54
Relative feeding magnitude[a]	1.0	1.4	2.4

[a]Calculated as the relative change in the area under the *FI* curve above 50% *FI* between t_{50} and PNR_{50} for the 3 treatments (shaded areas in Fig. 1)

Table IV-1 Summary information for feeding incidence (FI) by yolk-sac cod larvae reared at 10°C in only seawater (clear water, CW) and in seawater including Nannochloropsis sp. (NA) and both Nannochloropsis sp. and Oxyrrhis marina (NO). Parameter estimates and statistics for Eq. (2) (see 'Data analysis' under 'Materials and methods') are provided: maximum mean feeding incidence (FIMAX), slope (b) and age of larvae at FIMAX. Calculated values include the age of larvae at 50% feeding incidence (t50), the point of no return (PNR50), window of opportunity (WOO) and the relative magnitude of feeding (see Fig. 1). FI was expressed as the mean of 3 replicate tanks. dph: days post-hatch

Literature

(Arndt, 1991; Bell et al., 2003; Bengtson et al., 1999; Blaxter and Hempel, 1963; Boehlert and Morgan, 1985; Braven et al., 1984; Cahu et al., 1998; Cushing, 1975; Diaz et al., 1998; Ellertsen et al., 1980; Fukami et al., 1999; Hansen and Jensen, 2000; Hjort, 1914b; Houde, 2008; Howell, 1979; Hunt von Herbing et al., 2001; Kentouri, 1985; Kjørsvik et al., 1991; Lasker, 1975; Maurizi, 2000; Muller-Feuga et al., 2003; Munk, 1997; Naas et al., 1992; Øie et al., 1997; Peck and Holste, 2006; Pepin and Penney, 2000; Pepin and Dower, 2007; Ptacnik, 2003; Reitan et al., 1997; Scura and Jerde, 1977; Skiftesvik et al., 2003; Skjermo and Vadstein, 1993; Sommer et al., 2002; Støttrup, 1994; Tamigneaux et al., 1997; van der Meeren et al., 2007; Williams and Poulet, 1986; Yufera and Darias, 2007)

Discussion

The fragile early life stages of marine fish larvae have attracted much scientific attention ever since their relevance for recruitment success and fisheries had been realized. Cod (*Gadus morhua*) has not only become a model species for biological oceanography, but this species also justifies its use in historic and modern fisheries research by its unmatched economic importance. A wealth of knowledge on the biology of the species was accumulated in the last centuries and still new discoveries are to be made. The present work is a contribution to the biological understanding on one of the minor sized populations of cod, located in the eastern part of the Baltic Sea.

This more-or-less genetically distinct population is currently considered one of the more productive fisheries stocks and has undergone severe fluctuations in the last decades. The unique hydrographic conditions of its brackish habitat have pushed the stock, and the related fisheries industry, to the borders of its economic extinction and the same environmental factors brought the population back to life. This work was carried out in the context of two research projects: UNCOVER ("Understanding the mechanisms of stock recovery", EU-FP 6, Contract number 022717) and RESTOCK ("The production of Baltic cod larvae for restocking in the eastern Baltic", Financial Instrument for Fisheries Guidance, FIFG). The former sought to understand the mechanisms of stock recovery and the latter sought to restock the eastern Baltic cod population by the release of newly hatched yolk sac larvae. Both projects had a common sense on the importance of in-depth understanding of the effects of key environmental factors on cod larval growth and condition. The mandate to conduct this work was therefore very clear: controlled laboratory experiments with eastern Baltic cod larvae are needed.

The experiments were conducted on the island of Bornholm, the pearl of the Baltic, at the western boundary of the population's distribution range. Two years of intense laboratory experimental seasons were needed to gather enough data to present this thesis. The author has spent 13 months on the island, during the summer-time spawning season, and has intensely worked with the broodstock and egg and larval material provided by Bornholm's Lakseklækkeri, Nexø, Denmark. Sample processing and data analysis was conducted at the Institute of Hydrobiology and Fisheries Science, University Hamburg and at DTU Aqua, Copenhagen.

The experiments were grouped in two thematic areas. The first block of experiments, mainly conducted in the year 2007, investigated the effects of food and temperature on larval cod vital rates (growth and condition) and the resulting implications for fisheries science. The second group of experiments in the year 2008 investigated the effects of Alizarin Complexone marking, a method of pivotal importance for the assessment of stocking success, and innovative "green water" techniques to enhance larval fitness in aquaculture. The results of these experiments are collated in this thesis and can be found as peer-reviewed scientific publications (three published manuscripts; one manuscript draft).

1. Implications for fisheries science

1.1. Growth – condition - survival

The effects of temperature and feeding levels, ranging from ad libitum to food depletion, on the early larval stages, i.e. from the onset of exogenous feeding until the late pre-metamorphic stage, were successfully parameterized in a number of growth and condition models with high practical relevance for applied fisheries field research and larval IBM modeling (Manuscript I).

Abiotic and biotic factors affect the growth and survival of marine fish larvae. Examining these factors under controlled laboratory conditions allows us to disentangle the processes affecting recruitment dynamics in field populations. We examined the effects of three different ambient water temperatures (7, 10, 13 °C, *T*) and feeding levels, ranging from food depletion to ad libitum feeding, on growth (*G*) in standard length and dry weight as well as biochemical condition (RNA-DNA ratio, *sRD*) and otolith size of the larvae of Eastern Baltic cod. Larvae were reared up to 32 days post hatch and grew up to standard lengths of 7.0 mm and dry weights (*DW*) of 297 µg. A series of models were developed, relating the aforementioned metrics to each other (length – weight, body size – otolith size, size-at-age, *G-sRD-T*) to provide condition-based growth and survival proxies for applications in field process studies. Although observed growth rates (*DW*-specific growth rates ranging from 4.7 to 22.1 % d^{-1}) of Eastern Baltic cod were somewhat lower than those previously reported for other, oceanic Atlantic cod populations at the same temperatures, morphological and biochemical condition indices were comparable. Various generalized linear models were parameterized to estimate recent growth from *sRD*, but all of these models failed to correctly estimate the negative growth rates observed for food-depleted larvae. On the other hand, comparably simple length – weight models were able to correctly identify food-depleted larvae and otolith size models could be used to estimate size and age. This study is the first to successfully rear exogenously feeding Eastern Baltic cod larvae in the laboratory and our growth and condition metrics will be useful when applied to field-caught larvae and for the correct parameterization of feeding and growth models for the larvae of this population.

The current results show that the interaction of *T* and *sRD* is less relevant than the actual temporal perspective of growth rate back-calculation. Not surprisingly, the best results will be achieved when a longer time period of growth and constant abiotic and biotic conditions are integrated. This situation is, apparently, limited to the controlled conditions of the laboratory. In turn, the practical consequence for field application of *sRD* is that in apparent situations of high experienced environmental fluctuation, it might be favorable to use a less retrospective model with lower predictive capacity to gain a more recent estimate of *G* or condition. Otolith size suggests itself as a useful and simple to assess longitudinal estimator of integrated larval growth history. The inherent growth variability of individuals within the same environment requires that growth histories be examined on field-caught individuals, e.g. by investigating growth trajectories from otoliths, and not merely instantaneous estimates of growth, like RNA-DNA ratio. The metrics provided in this study will help to resolve the role of growth – survival – condition relationships in recruitment dynamics of Eastern Baltic cod larvae and to get a bit closer to the Holy Grail of fisheries science (Houde, 2008).

1.2. Starvation induced mortality

The susceptibility of fish larvae to starvation and the modulating effects of temperature and body size on key parameters "on the edge of death" in eight different species has been investigated in a meta-analysis of larval RNA-DNA ratio and body mass changes throughout the course of food depletion under controlled experimental conditions (Manuscript II).

Gaining reliable estimates of how long fish early life stages can survive without feeding and how starvation rate and time until death are influenced by body size, temperature and species is critical to understanding processes controlling mortality in the sea. The present study is an across-species analysis of starvation-induced changes in biochemical condition in early life stages of nine marine and freshwater fishes. Data were compiled on changes in body size (dry weight, *DW*) and biochemical condition (standardized RNA-DNA ratio, *sRD*) throughout the course of starvation of yolk-sac and feeding larvae and juveniles in the laboratory. In all cases, the mean biochemical condition of groups decreased exponentially with starvation time, regardless of initial condition and endogenous yolk reserves. A starvation rate for individuals was estimated from discrete 75^{th} percentiles of sampled populations versus time (degree-days, *Dd*). The 10^{th} percentile of *sRD* successfully approximated the lowest, life-stage-specific biochemical condition (the edge of death). Temperature could explain 59 % of the variability in time to death whereas *DW* had no effect. Species and life-stage-specific differences in starvation parameters suggest selective adaptation to food deprivation. Previously published, interspecific functions predicting the relationship between growth rate and *sRD* in feeding fish larvae do not apply to individuals experiencing prolonged food deprivation. Starvation rate, edge of death, and time to death are viable proxies for the physiological processes under food deprivation of individual fish pre-recruits in the laboratory and provide useful metrics for research on the role of starvation in the sea.

Our analysis has shown that starvation-induced changes in condition in pre-recruit life stages of fishes can be described by a common function when temperature effects, life stage and species-specific differences are taken into account. Starvation rates were normalized with respect to temperature by expressing the duration of food deprivation on a degree-day basis. We fitted functional models to discrete percentiles of biochemical condition (standardized RNA-DNA ratio) in sampled populations to derive an estimate for starvation rates and mortality thresholds of biochemical condition on the level of an individual. Within narrow ranges of body sizes and life stages, we were able to quantify key aspects of starvation (initial condition, rate of decrease in condition, mortality threshold, and time to death) based on data from controlled laboratory trials. Although the selective loss of individuals in poor condition will undoubtedly differ between the laboratory and sea, our analysis represents a step towards a tailored condition index that takes into account species-, stage- and time frame-specific attributes of starvation (Suthers, 1998). Additional research is needed to address gaps in our knowledge of how different life stages and/or species are able to cope with periods of prey deprivation and how frequently cohorts of fish experience life "on the edge of death".

2. Implications for aquaculture

2.1. Fluorescent marking of larval otoliths

The necessity for fluorescence marking of the earliest life stages of eastern Baltic egg and yolk sac larvae, in the context of the RESTOCK project, has triggered a study to investigate the sublethal effects of Alizarin Complexone exposure on larval and embryonic survival and larval growth and condition, and has resulted in recommendations for future stock enhancement efforts to assess and subsequently avoid effects of growth retardation and reduced larval fitness (Manuscript III).

Standard, single-exposure Alizarin Complexone- (ALC) marking was conducted on early life stages of Baltic cod (*Gadus morhua* L.) to examine acute and chronic effects. Embryos and yolk sac larvae were marked using different concentrations of ALC (0, 50, 100 and 200 mg l^{-1}). Experiments included control groups for treatment and handling effects. In agreement with previous studies, long-lasting, distinct otolith marks were produced by immersion of yolk sac larvae in ≥50 mg l^{-1} ALC for 24 h. Mortality of eggs and larvae was low during the marking procedure. Hatching success of ALC marked embryos was significantly reduced and hatching was delayed with increasing ALC concentration. Growth rates of larvae through 21 *dph* (exogenous feeding stage) were significantly reduced in ALC marked fish compared to controls. Biochemical condition (RNA-DNA ratio) was not affected. Subtle changes in activity and metabolism were indicated by reduced first feeding success and yolk absorption rates. Our results reveal that batch marking of finfish early life stages using ALC can have not only significant, acute impacts on survival but, despite long-standing assumptions to the contrary, also sublethal effects on other vital parameters like growth. We discuss these acute and chronic effects and give recommendations for assessment of life stage- and species-specific ALC immersion marking procedures.

The results of this study indicated that exposing 3 *dph* Baltic cod larvae to 50 mg l^{-1} ALC for 24 h is the preferred marking procedure for restocking efforts using releases of yolk sac larvae (Støttrup et al., 2008b). However, survival and growth of larvae were negatively affected by ALC treatment, possibly because Baltic cod larvae are more sensitive to ALC than marine conspecifics, since our results were in contrast to those obtained with marking similar stages of Atlantic cod (Blom et al., 1994; Svåsand, 1995). We recommend that marking efficiency and longevity of lower concentrations should be assessed. Future experiments with finfish larvae should quantify both acute (e.g., survival) and chronic (e.g., hatching success, first feeding, growth during exogenous feeding) effects and utilize a handling control when optimizing ALC marking protocols for the target species and life stage.

2.2. Green water techniques

The (personal) conclusion that larval cod needs “green water” and the presence of a heterotrophic prey organism like *Oxyrrhis marina* has stipulated a study on the relevance of a heterotrophic microbial loop component for first feeding Eastern Baltic cod yolk sac larvae and came in turn back to the conclusion that the stimulatory effects of a complex green water environment can surpassingly improve larval culture performance (Manuscript IV).

The present study evaluated whether the presence of autotrophic phytoplankton and/or heterotrophic dinoflagellates (*Oxyrrhis marina*) influenced first-feeding success and early larval growth and development of Baltic cod *Gadus morhua* L. Newly hatched cod larvae were maintained in salt water ('clear water', CW), greened water using Nannochloropsis sp. (NA) or a combination of *Nannochloropsis* sp. and O. marina (NO) from 2 to 11 d post-hatch. On each day, larvae from each group were placed into feeding tanks and provided potential prey (nauplii of the calanoid copepod *Acartia tonsa*). Feeding performance was measured both as the percentage incidence of feeding and gut fullness index. The onset of first feeding, point of no return and the magnitude of feeding were derived from the feeding incidence. The study clearly demonstrated precocious, exogenous feeding by cod larvae in the presence of *Nannochloropsis* sp. and O. marina by 1.2 and 2.2 d (10°C), respectively. Gut fullness was also significantly improved in the presence of both phytoplankton and protists over the entire window of opportunity (defined as the period between onset of first feeding and point of no return). However, the nutritional capacity of these unicellular organisms was not sufficient to alter the somatic growth characteristics in NA and NO groups relative to starving yolk-sac larvae maintained in only salt water (CW group). These findings expand the current understanding of the degree of interaction between fish larvae and unicellular plankton communities and indicate a lack of nutritional benefits from feeding on components of the pelagic microbial loop but a clear benefit in terms of 'priming' first-feeding capabilities.

The results of the present study highlight the need to revisit the importance of autotrophic phytoplankton and heterotrophic protists in the early survival and growth of marine fish larvae. Previous studies indicated that the larvae of some marine fish species routinely ingest phytoplankton (e.g. Northern anchovy *Engraulis mordax* feeding on dinoflagellates) which appeared to offer some nutritional benefit (e.g., Scura and Jerde, 1977) and was thought to be important for early survival and recruitment success (Lasker, 1975). Our results clearly indicated that cod obtained no nutritional (growth) benefit from ingesting algae and heterotrophic protists (protozooplankton) prior to first feeding. However, the presence of adequate amounts of protozooplankton may be critical to the survival and growth of cod by acting to prime first-feeding capabilities of this species, altering the window of opportunity for successful first feed and, in turn, influencing the match-mismatch dynamics between first-feeding cod and their macrozooplankton prey.

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A. Appendix

A.I. Co-author manuscript

(Skovgaard et al., 2010)

Skovgaard, A., S. Meyer, Overton, J. L., Støttrup, J., Buchmann, K. (2010). "Ribosomal RNA gene sequences confirm that protistan endoparasite of larval cod *Gadus morhua* is *Ichthyodinium* sp." Diseases of Aquatic Organisms 88(2): 161-167.

Abstract:

An enigmatic protistan endoparasite found in eggs and larvae of cod Gadus morhua and turbot Psetta maxima was isolated from Baltic cod larvae, and DNA was extracted for sequencing of the parasite's small Subunit ribosomal RNA (SSU rRNA) gene. The endoparasite has previously been suggested to be related to Ichthyodinium chabelardi, a dinoflagellate-like protist that parasitizes yolk sacs of embryos and larvae of a variety of fish species. Comparison of a 1535 bp long fragment of the SSU rRNA gene of the cod endoparasite showed absolute identify with I. chabelardi, demonstrating that the 2 parasites are very closely related, if not identical. This finding is discussed in relation to some morphological differences that appear to exist. between I. chabelardi and the cod endoparasite.

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DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published January 25

NOTE

Ribosomal RNA gene sequences confirm that protistan endoparasite of larval cod *Gadus morhua* is *Ichthyodinium* sp.

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ABSTRACT: An enigmatic protistan endoparasite found in eggs and larvae of cod *Gadus morhua* and turbot *Psetta maxima* was isolated from Baltic cod larvae, and DNA was extracted for sequencing of the parasite's small subunit ribosomal RNA (SSU rRNA) gene. The endoparasite has previously been suggested to be related to *Ichthyodinium chabelardi*, a dinoflagellate-like protist that parasitizes yolk sacs of embryos and larvae of a variety of fish species. Comparison of a 1535 bp long fragment of the SSU rRNA gene of the cod endoparasite showed absolute identity with *I. chabelardi*, demonstrating that the 2 parasites are very closely related, if not identical. This finding is discussed in relation to some morphological differences that appear to exist between *I. chabelardi* and the cod endoparasite.

KEY WORDS: Fish egg · Yolk sac parasite · Endoparasite · *Gadus morhua* · *Ichthyodinium*

INTRODUCTION

Eggs and larvae of Atlantic cod *Gadua morhua* from the Baltic Sea are hosts to a protistan endoparasite that is located in the yolk sac of embryos and newly hatched larvae (Buchmann et al. 1993). Prevalence of the protistan endoparasite was as high as 25 to 88% in eggs from wild cod caught in Danish waters (Buchmann et al. 1993, Pedersen et al. 1993, Pedersen & Køie 1994), and the parasite is, therefore, thought to be of significant ecological importance. Mortality caused by infection of this parasite has not been investigated in much detail, but infection has been suggested to be lethal (Pedersen 1993) since the parasite seems to be associated with elevated mortality (Pedersen & Køie 1994). An indistinguishable protistan parasite exists in embryos and larvae of turbot *Psetta maxima* (as *Scophthalmus maximus*; Pedersen 1993) from the western part of the Baltic Sea. Eggs of Atlantic cod and turbot are buoyant (i.e. pelagic). The demersal eggs of herring *Clupea harengus* in the Baltic Sea, on the other hand, are not susceptible to infection by this parasite (Pedersen & Køie 1994).

The protistan endoparasite of cod and turbot can typically be detected as large spheres (25 to 80 µm) in the yolk sac of embryos (Pedersen 1993, Pedersen et al. 1993). Mononucleate stages are spherical with a smooth surface, whereas larger, multinucleate plasmodia have more irregular surfaces. The larger, multinucleate stages are thought to produce smaller mononucleate cells through budding, but the actual development of parasite stages has not been investigated (Pedersen 1993). The parasites seem to disappear from the yolk sac before the end of the yolk sac stage, presumably because parasite cells enter the vascular system and/or the tissue of the larvae (Pedersen

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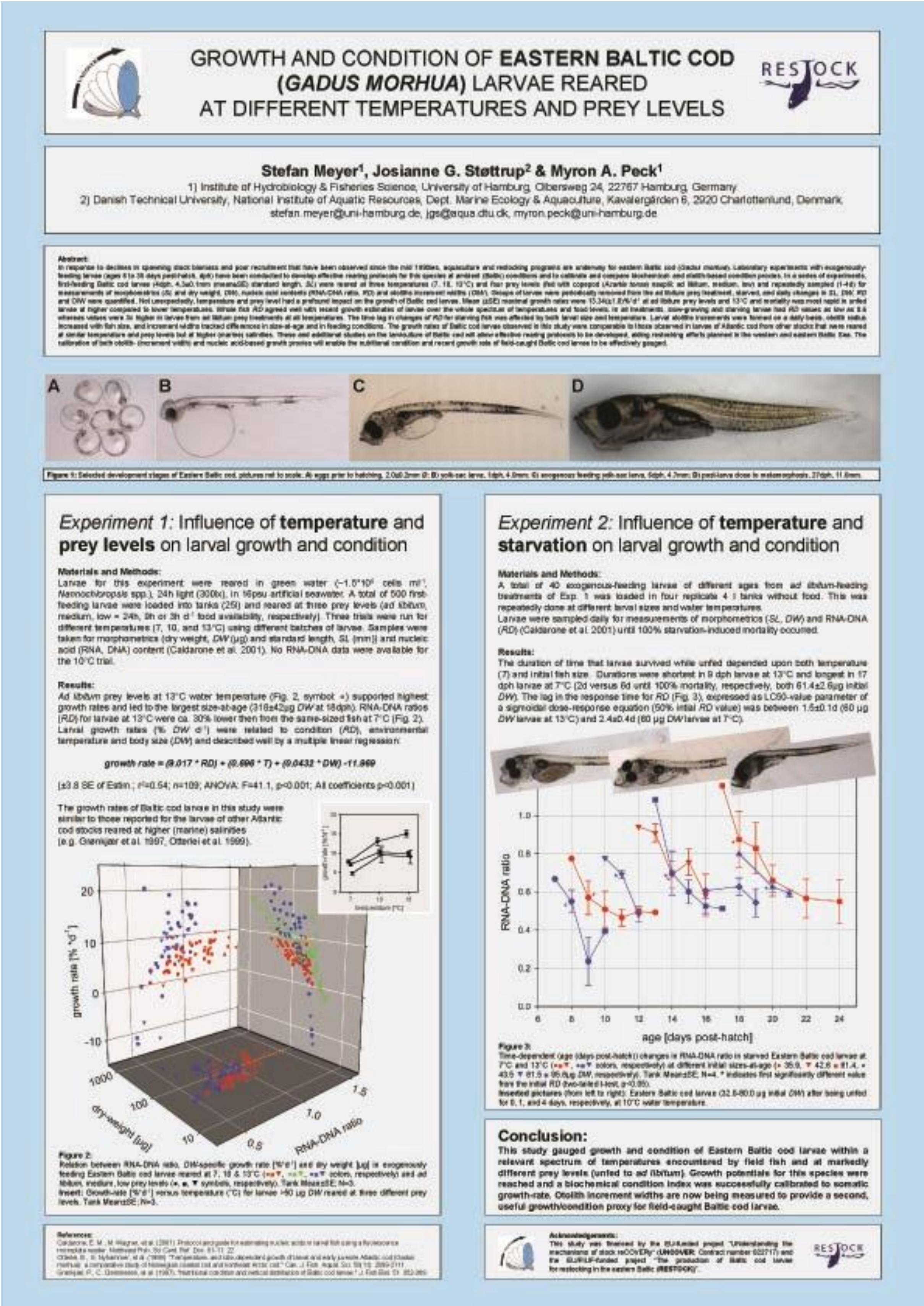
A.II. Poster LFC 2008, Kiel



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A.III. Poster Youmares 2010, Hamburg

Green is good, protists are better!

Baltic cod (*Gadus morhua*) larvae reared in green water with heterotrophic protist *Oxyrrhis marina*

Stefan Meyer*, Julia L. Overton, Josianne G. Støttrup & Myron A. Peck

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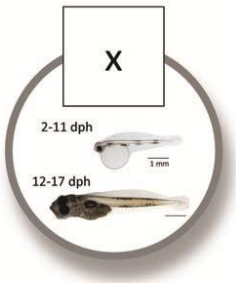
ABSTRACT: Green water techniques are known for their multiple beneficial effects on marine finfish larvae. Besides a nutritional effect, it has been hypothesized that the presence of unicellular organisms enhances visual contrast and stimulates foraging behavior. We tested this hypothesis by comparing Baltic cod (*Gadus morhua*) yolk sac and post yolk sac larvae raised in different green water types containing either only autotrophic microalgae (*Nannochloropsis* sp.) or a mixture with heterotrophic protists (*Oxyrrhis marina*) and a clear water control. Characteristics of larval behavior, growth and condition were assessed at the onset of first feeding in naïve yolk sac (2-11dph) and in older, exogenously feeding (12-17dph) larvae until the point of no return. Maximum observed first feeding success was higher in both green water types compared to control. The point of 50% first feeding was reached earlier in the mixed green water than in any other treatment. Point of no return, i.e. when first feeding success decreased to <50%, was not significantly affected by treatment. In exogenously feeding larvae, survival in both green waters was high during 3 days of food depletion and larvae exhibited positive growth rates when being re-fed. In control group, survival during starvation was low and no larvae survived the period of re-feeding. RNA-DNA ratios reflected periods of starvation and re-feeding in post yolk sac larvae. This study confirmed that utilizing green water has a beneficial effect on the larviculture of Baltic cod by stimulating foraging behavior and starvation resistance. Green water conditioned Baltic cod yolk sac larvae had an increased first feeding success and yolk sac and post yolk sac larvae showed increased growth and survival rates near the point of no return. The presence of the heterotrophic protist *O. marina*, in combination with the green water microalgae *Nannochloropsis* sp. had a stimulative effect on larval behavior, i.e. increased activity level and foraging success. Post yolk sac larvae from both green water treatments exhibited an increased resistance to starvation. In summary, the two green water types applied in this study substantially increased the success of larviculture of Baltic cod and are highly recommended over clear water rearing for this species.

Treatments



clear water
16.0 psu artificial salt water
0.1 µm filtered
10°C water temperature
stagnant batch culture

green water
same as clear water
Nannochloropsis spec. (10^6 #/ml)
- commonly used in cod aquaculture
- shading and probiotic effect
- high fatty acid (EPA) content

green water with *Oxyrrhis*
same as green water
Oxyrrhis marina (10^3 #/ml)
- heterotrophic (eats algae)
- *de novo* synthesis of essential FA
- vivid swimmer
- visible to cod larvae

Conclusion

high mortality
late first feeding (5.6 dph)
point-of-no-return unaffected

clear water

high mortality
low condition
premature point-of-no-return

early first feeding (4.0 dph)
vivid larval activity
yolk absorption rates unaffected
- no nutritious effect

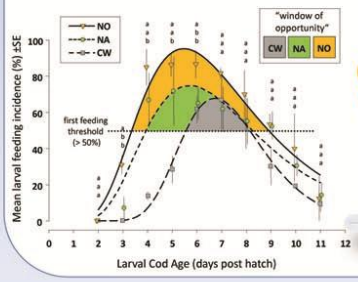
green water

good growth and survival rates
increased starvation resistance
well prepared for exogenous feeding

early first feeding (3.4 dph)
highest feeding incidence
greatest „window of opportunity"
no nutritious effect

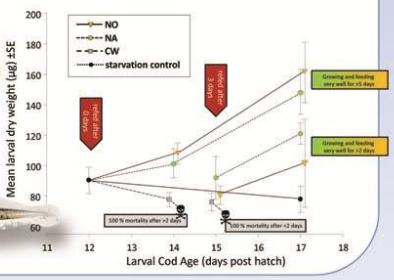
green water + *Oxyrrhis*

same positive effects as green water
same growth rates
no nutritious effect from *Oxyrrhis*



I ♥ green water with *Oxyrrhis*!

Oxyrrhis is good for me when I am young



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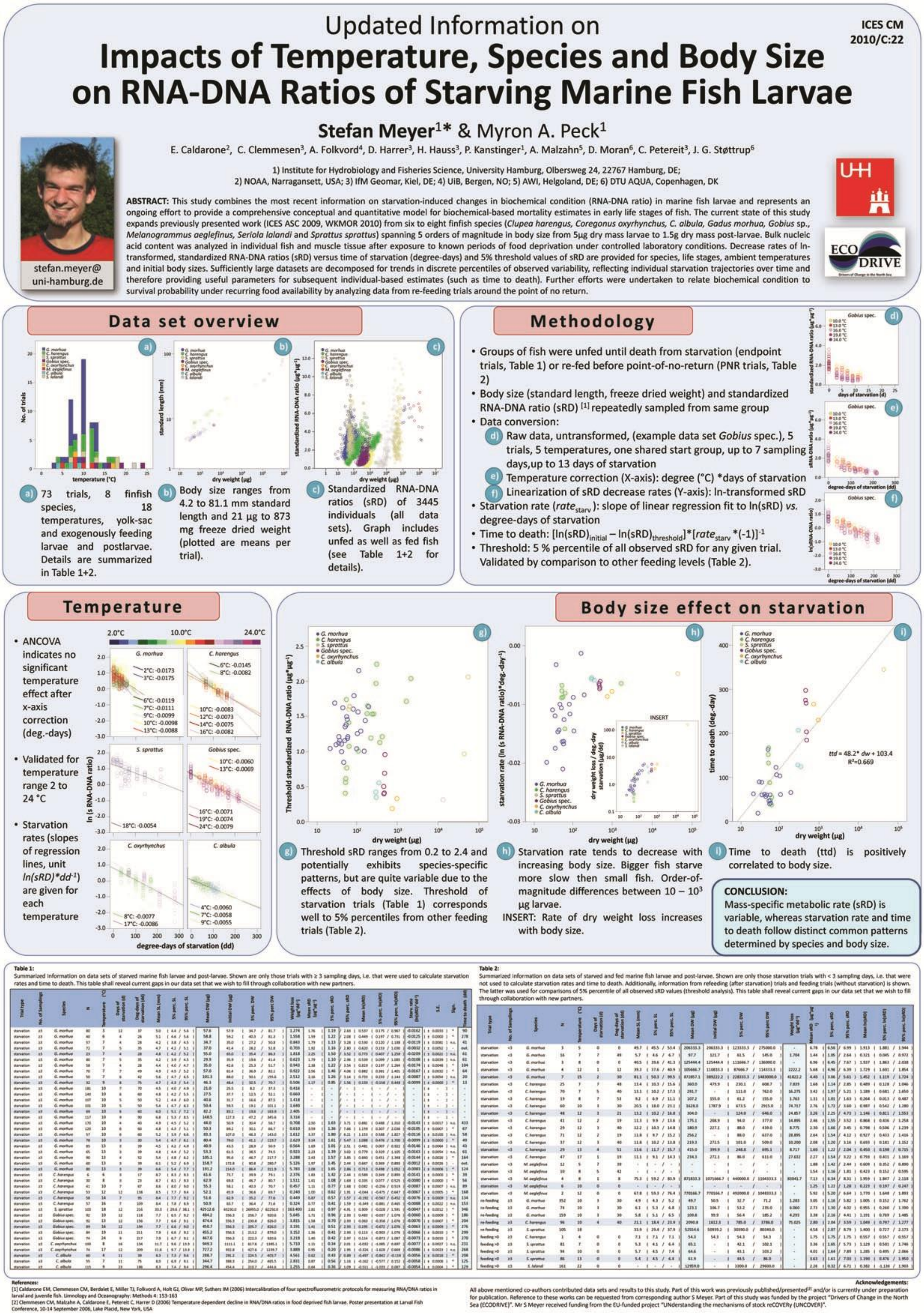

Updated Information on
Impacts of Temperature, Species and Body Size on RNA-DNA Ratios of Starving Marine Fish Larvae
ICES CM 2010/C:22
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ABSTRACT: This study combines the most recent information on starvation-induced changes in biochemical condition (RNA-DNA ratio) in marine fish larvae and represents an ongoing effort to provide a comprehensive conceptual and quantitative model for biochemical-based mortality estimates in early life stages of fish. The current state of this study expands previously presented work (ICES ASC 2009, WKMOR 2010) from six to eight finfish species (Clupea harengus, Coregonus oxyrhynchus, C. albula, Gadus morhua, Gobius sp., Melanogrammus aeglefinus, Seriola lalandi and Sprattus sprattus) spanning 5 orders of magnitude in body size from 5µg dry mass larvae to 1.5g dry mass post-larvae. Bulk nucleic acid content was analyzed in individual fish and muscle tissue after exposure to known periods of food deprivation under controlled laboratory conditions. Decrease rates of ln-transformed, standardized RNA-DNA ratios (sRD) versus time of starvation (degree-days) and 5% threshold values of sRD are provided for species, life stages, ambient temperatures and initial body sizes. Sufficiently large datasets are decomposed for trends in discrete percentiles of observed variability, reflecting individual starvation trajectories over time and therefore providing useful parameters for subsequent individual-based estimates (such as time to death). Further efforts were undertaken to relate biochemical condition to survival probability under recurring food availability by analyzing data from re-feeding trials around the point of no return.
Data set overview
a) 73 trials, 8 finfish species, 18 temperatures, yolk-sac and exogenously feeding larvae and postlarvae. Details are summarized in Table 1+2.
b) Body size ranges from 4.2 to 81.1 mm standard length and 21 µg to 873 mg freeze dried weight (plotted are means per trial).
c) Standardized RNA-DNA ratios (sRD) of 3445 individuals (all data sets). Graph includes unfed as well as fed fish (see Table 1+2 for details).
Methodology
• Groups of fish were unfed until death from starvation (endpoint trials, Table 1) or re-fed before point-of-no-return (PNR trials, Table 2)
• Body size (standard length, freeze dried weight) and standardized RNA-DNA ratio (sRD) [1] repeatedly sampled from same group
• Data conversion:
d) Raw data, untransformed, (example data set Gobius spec.), 5 trials, 5 temperatures, one shared start group, up to 7 sampling days, up to 13 days of starvation
e) Temperature correction (X-axis): degree (°C) *days of starvation
f) Linearization of sRD decrease rates (Y-axis): ln-transformed sRD
• Starvation rate (rate_starv): slope of linear regression fit to ln(sRD) vs. degree-days of starvation
• Time to death: [ln(sRD)_initial – ln(sRD)_threshold]*[rate_starv *(-1)]^-1
• Threshold: 5 % percentile of all observed sRD for any given trial. Validated by comparison to other feeding levels (Table 2).
Temperature
• ANCOVA indicates no significant temperature effect after x-axis correction (deg.-days)
• Validated for temperature range 2 to 24 °C
• Starvation rates (slopes of regression lines, unit ln(sRD)*dd-1) are given for each temperature
2.0°C
10.0°C
24.0°C
G. morhua
C. harengus
S. sprattus
Gobius spec.
C. oxyrhynchus
C. albula
2°C: -0.0173
3°C: -0.0175
6°C: -0.0119
7°C: -0.0111
9°C: -0.0099
10°C: -0.0098
13°C: -0.0088
6°C: -0.0145
8°C: -0.0082
10°C: -0.0083
12°C: -0.0073
14°C: -0.0075
16°C: -0.0082
18°C: -0.0054
10°C: -0.0060
13°C: -0.0069
16°C: -0.0071
19°C: -0.0074
24°C: -0.0079
8°C: -0.0077
17°C: -0.0086
4°C: -0.0060
7°C: -0.0058
9°C: -0.0055
ln (s RNA-DNA ratio)
degree-days of starvation (dd)
Body size effect on starvation
g) Threshold sRD ranges from 0.2 to 2.4 and potentially exhibits species-specific patterns, but are quite variable due to the effects of body size. Threshold of starvation trials (Table 1) corresponds well to 5% percentiles from other feeding trials (Table 2).
h) Starvation rate tends to decrease with increasing body size. Bigger fish starve more slow then small fish. Order-of-magnitude differences between 10 – 10^3 µg larvae.
INSERT: Rate of dry weight loss increases with body size.
i) Time to death (ttd) is positively correlated to body size.
ttd = 48.2* dw + 103.4
R²=0.669
Threshold standardized RNA-DNA ratio (µg*µg-1)
starvation rate (ln (s RNA-DNA ratio)*deg-day-1)
time to death (deg.-day)
dry weight (µg)
CONCLUSION:
Mass-specific metabolic rate (sRD) is variable, whereas starvation rate and time to death follow distinct common patterns determined by species and body size.
Table 1:
Summarized information on data sets of starved marine fish larvae and post-larvae. Shown are only those trials with ≥ 3 sampling days, i.e. that were used to calculate starvation rates and time to death. This table shall reveal current gaps in our data set that we wish to fill through collaboration with new partners.
Table 2:
Summarized information on data sets of starved and fed marine fish larvae and post-larvae. Shown are only those starvation trials with < 3 sampling days, i.e. that were not used to calculate starvation rates and time to death. Additionally, information from refeeding (after starvation) trials and feeding trials (without starvation) is shown. The latter was used for comparisons of 5% percentile of all observed sRD values (threshold analysis). This table shall reveal current gaps in our data set that we wish to fill through collaboration with new partners.
References:
[1] Caldarone EM, Clemmesen CM, Berdalet E, Miller TJ, Folkvord A, Holt GJ, Olivar MP, Suthers IM (2006) Intercalibration of four spectrofluorometric protocols for measuring RNA/DNA ratios in larval and juvenile fish. Limnology and Oceanography: Methods 4: 153-163
[2] Clemmesen CM, Malzahn A, Caldarone E, Petereit C, Harrer D (2006) Temperature dependent decline in RNA/DNA ratios in food deprived fish larvae. Poster presentation at Larval Fish Conference, 10-14 September 2006, Lake Placid, New York, USA
Acknowledgements:
All above mentioned co-authors contributed data sets and results to this study. Part of this work was previously published/presented[2] and/or is currently under preparation for publication. Reference to these works can be requested from corresponding author S Meyer. Part of this study was funded by the project "Drivers of Change in the North Sea (ECODRIVE)". Mr S Meyer received funding from the EU-funded project "UNderstanding the mechanisms of stock reCOVERy (UNCOVER)".

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GROWTH AND CONDITION OF EASTERN BALTIC COD (*GADUS MORHUA*) LARVAE REARED UNDER DIFFERENT TEMPERATURES, SALINITIES, PHOTOPERIODS AND FOOD LEVELS

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Introduction

The declining eastern Baltic cod stock biomass and commercial landings (ICES, 2005; Köster et al., 2005a), together with the high demand for a well-renown local product, urge the aquaculture sector to produce Baltic cod (*Gadus morhua*) for growout- or restocking purposes. New species-specific rearing protocols for eastern Baltic cod have to be developed to initiate the process of domestication. The current knowledge on Atlantic cod production needs to be re-assessed, especially regarding the peculiar abiotic and biotic factors that this species encounters within the Baltic Sea. Some of the most important abiotic factors include temperature, salinity, photoperiod which interact together with the level of food resources to impact the vital rates of early life stages of all fish species.

Material and Methods

In a series of separate experiments, Baltic cod yolk-sack larvae (4 days-post-hatch (dph), 4.3±0.1mm (mean±SE) standard length, SL) produced by an captive broodstock at Bornholm's Lakseklækkeri (Nexø, Denmark) were transferred to 25 l tanks at stocking densities between 20 and 25 larvae*l^{-1} for rearing within different treatments including four salinities (7, 12, 17, 30psu), four photoperiods (10, 14, 18, 22h), three temperatures (7, 10, 13°C) and/or four food levels (ad libidum, medium, low, starving). Fish were fed copepod (*Acartia tonsa*) nauplii and were reared (in baseline situations) at 17psu, within green-water in well-mixed tanks.

Samples for larval morphometrics (SL and dry weight, DW), biochemistry (RNA-DNA ratio) and otoliths (increment analyses) were taken every 1-4 days. In some experiments, fish in well-fed tanks were starved and sampled daily. Experiments were terminated after 16-33dph. All treatments were run in triplicate (abiotic treatments and food levels) or quadruplicate (starved).

Results

Salinity was positively correlated to survival at 16dph. No significant effect on growth rate (in dry weight) was observed but mean(±SE) growth rates in tanks were variable (from -4.9(±2.9) to 9.73(±2.3)%*d^{-1}). Although photoperiod duration did not significantly influence larval

survival or growth rate growth rates tended to be higher (12.7(±4.0)%*d^{-1}) at the longest (22h) photoperiod. Temperature and food level had the most profound impacts on the growth of post-yolk-sack (ages from 6 and 35dph) larval Baltic cod. In short, higher temperatures supported higher growth rates (up to 13.34(±1.0)%*d^{-1}) at ad libidum feeding levels (Figure 1) and more rapid mortality when food was deprived compared to lower temperatures.

Whole fish RNA-DNA ratio (RD) agreed well with recent growth estimates of larvae over the whole spectrum of abiotic and biotic factors. Slow-growing and starving larvae in all treatments had low RD values as low as 0.6, whereas RD values of up to 1.8 were found in larvae from the salinity and low temperature and ad-libidum feeding treatments. The time lag in change of RD for starving fish was affected both by initial size-at-age and temperature.

Larval otoliths radius increased with fish size and increments were formed on a daily base, coding a high resolution growth estimate information.

Discussion

The results of this study implicate a high potential for successful larval rearing in intensive aquaculture systems. The observed growth rates at long photoperiods (24h light) and high temperatures (13°C) under ad libidum feeding conditions are comparable to growth rates of other Atlantic cod stocks reared under similar conditions (Otterlei et al., 1999). In the face of upcoming Baltic cod culture and restocking efforts in the western and eastern Baltic sea (e.g. Støttrup et al., 2008b) the authors of this study conclude, that the responsible utilization of this species to the collective good has just started.

VIABILITY OF BALTIC COD (*GADUS MORHUA*) EGGS AND LARVAE AFTER MARKING WITH ALIZARIN COMPLEXONE

Stefan Meyer*[,a], Sune R. Sørensen[b], Myron A. Peck[a] and Josianne G. Støttrup[b]

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[b] Section for Coastal Ecology, National Institute for Aquatic Resources (DTU Aqua), The Technical University of Denmark, Charlottenlund Castle, DK-2920 Charlottenlund, Denmark

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Introduction

Mark-recapture studies are an important component of fisheries research and the evaluation of stock enhancement efforts (Støttrup and Sparrevohn, 2007). Alizarin Complexone (*AC*) is one of the most frequently used marking agents for finfish larvae and successful protocols have been previously described for a variety of species (Svåsand et al., 2000). *AC* is a fluorescent dye binding with the protein matrix of otoliths deposited during the marking procedure. Fish with marked otoliths can be identified with the help of an UV-microscope.

This study was performed to evaluate the effect of different *AC* marking procedures on egg and larval viability of Baltic cod as part of Danish restocking project for this species (EU/FIUF-funded project "The production of Baltic cod larvae for restocking in the eastern Baltic", RESTOCK).

Material and Methods

In a first set of experiments, we investigated the effect of different *AC* concentrations on viability. Cod eggs (1 day prior to hatch) and larvae (3 days post hatch, *dph*) were exposed to one of four concentrations of *AC* (0, 50, 100 and 200mg*l^{-1}) for 24h and hatching success and/or survival were assessed daily for a maximum of 11 days. The success of fluorescent marking of otoliths was assessed in larvae to 50dph.

In a second set of experiments, we assessed the effect of the marking procedure on viability (growth and condition) of larvae. Cod larvae (3dph) were exposed to 0mg*l^{-1} (handling control), 50mg*l^{-1} *AC* or were not exposed (control, directly transferred to rearing tanks). Fish were reared in green water (10°C, 15PSU) under *ad libitum* feeding conditions for up to 20dph. Incidence of first feeding was assessed at 5dph. Samples for larval growth (standard length, *SL* and dry weight, *DW*) and biochemical condition (RNA-DNA ratio, *RD*) were taken every 3 days. Survival and starvation resistance (i.e. numbers of larvae surviving until the end of the observation period) were assessed in unfed yolk-sack and post-yolk-sack larvae (4dph and 9dph initial age, respectively).

Results

AC concentrations ≥50mg*l^{-1} were found to have a detrimental effect on egg survival and hatching success. The effect was highest in the 200mg*l^{-1} treatment group where survival (50%) and hatching success (30%) were lower than in the 0mg*l^{-1} group (90% and 90%, respectively). Hatching was delayed in all treatment groups (200mg*l^{-1}: 5 days, compared to 0mg*l^{-1}). Larval mortality was low in all groups up to day 3 after marking. Marking of 3dph larvae with 50mg*l^{-1} *AC* was found to have the lowest impact on viability and still giving a strong, long-lasting (min. 50dph) fluorescent mark in the otoliths.

Compared to larvae treated with 0mg*l^{-1} (handling control) and larvae in the control group (direct rearing), marking procedure employing 50mg*l^{-1} *AC* concentration caused higher mortalities (72±3%, assessed on 9dph) and lower first feeding success (43±9%, assessed on 5dph) Increases in larval *SL* and *DW* were similar in all treatment groups (Figure 1). Although, length- and weight-specific growth rates were not significantly different among the three treatment groups, growth rates were lowest in larvae treated with 50mg*l^{-1} (2.0±0.1%*d^{-1} and 11.2±0.4%*d^{-1}), followed by 0mg*l^{-1} (2.3±0.5%*d^{-1} and 11.2±2.2%*d^{-1}) and the control group (2.7±0.3%*d^{-1} and 12.8±1.9%*d^{-1}). Larval condition (*RD*) was high in 5dph yolk-sack larvae (1.4µg*µg^{-1}) and low in post yolk-sack larvae (0.9-1.2 µg*µg^{-1}, 8-20dph) irrespective of treatment. Starvation resistance in post yolk-sack larvae (9-13dph) was not affected by marking procedure.

Discussion

Of all tested *AC* concentrations in this study, 50mg*l^{-1} had the lowest effect on cod larval viability. Marking of eggs was found to be unsuitable. Observed impacts of the marking procedure on larval viability can be attributed to increased stress related to chemical exposure and handling. These findings stand in contrast to results and marking protocols suggested for other Atlantic cod stocks (Blom et al., 1994; Svåsand, 1995). Implications for stock enhancement efforts and fisheries studies are discussed.

A.VII. Abstract ICES ASC 2009, Berlin

ICES CM 2009/T

Live and let die – condition indices and starvation-induced mortality thresholds during the early life of marine fish

Stefan Meyer, Helena M. Hauss, Philip Kanstinger, Myron A. Peck, Josianne G. Støttrup

A number of techniques allow one to estimate the prevalence of starvation among individuals of early life stage marine fish in the wild. Highly sensitive nucleic-acid and enzyme assays and image analysis of otolith microstructure are well documented and established methods. Recent inter-calibration studies between laboratory and field material led to enhanced understanding of recruitment mechanisms. In this presentation, we discuss the results of a suite of laboratory experiments calibrating otolith- (increment widths) and biochemical-based (nucleic acid ratios) condition proxies in larval stages of Baltic cod and sprat and larvae and juveniles of Atlantic herring that were reared under controlled laboratory conditions using different levels of abiotic (temperature, salinity, photoperiod) and biotic (prey levels) factors. Special emphasis is placed on quantifying temporal and inter-individual variability in these condition proxies during starvation and comparing results obtained for larval and juvenile conspecifics. Threshold-levels for irreversible starvation and rates of degradation can be approximated by means of biochemical condition-indices and otolith increments. We provide an inter-specific comparison to assess potential differences in prey requirements (leading to different life history strategies) and, thus, unsuitable habitats (those potentially leading to irreversible starvation and mortality) of early life stages of these three species.

Keywords: Baltic cod, Baltic sprat, North Sea herring, larvae and juveniles, starvation, condition, RNA/DNA ratio, otolith increments.

Contact author: Stefan Meyer, Institute for Hydrobiology and Fisheries Science, University Hamburg, Olbersweg 24, 22767 Hamburg, Germany [tel: +49 40 42838 6643; e-mail: stefan.meyer@uni-hamburg.de].

A.VIII. Abstract, vTI Rostock, 2010, Rostock

„Leben und sterben lassen - Kondition und Wachstum von Fischlarven an der Schwelle zum Tode.“

Dienstag, den 19.01.2010, 14:00 Uhr,

Konferenzraum, vTI OSF

Stefan Meyer

Universität Hamburg, Institut für Hydrobiologie und Fischerei

Wachstumsraten und Überlebenswahrscheinlichkeiten von marinen Fischlarven können durch eine Reihe von Konditionsindizes abgeschätzt werden. Die morphologische Kondition (Längen-Gewicht-Verhältnis) und die biochemische Kondition (RNA-DNA-Verhältnis) stellen jedoch nur Momentaufnahmen eines Individuums dar, die nichts über die Richtung einer Konditionsänderung aussagen.

Diese Indizes wurden bei genährten und hungernden Larven sowie Juvenile von Dorsch, Sprott und Hering untersucht. Die in Laborversuchen ermittelten Ergebnisse werden im Vortrag verglichen, um gemeinsame, artübergreifende Muster in der Konditionsänderung an der Schwelle zum Hungertod aufzudecken.

A.IX. Abstract WKMOR, Aberdeen

On the Edge of Death: Impacts of Temperature, Species and Body Size on RNA-DNA Ratios of Starving Marine Fish Larvae

In this study, we combined datasets from 50 laboratory trials examining the impact of food-deprivation on changes in RNA-DNA ratios of marine fish early life stages. The analysis included 3156 individuals of six temperate marine finfish species (*Gobius* spec., *Clupea harengus, Coregonus oxyrhynchus, Gadus morhua, Melanogrammus aeglefinus* and *Sprattus sprattus*) that spanned 3.5 orders of magnitude in body size from 20 µg dry mass larvae to 90 mg dry mass post-larvae. Changes in standard length, dry mass and individual-based standardized RNA-DNA-ratio (sRD) were assessed versus degree-days of starvation (dd_starv) to quantitatively compare (and develop a conceptual model for) the effects of temperature, species and/or body-size on starvation trajectories. During starvation, ln(sRD) of food-deprived individuals decreased linearly with time and slopes compared well (for all species at similar body sizes) among temperatures when time was expressed in dd. The largest differences in slopes were related to body size (-5.4 versus $-10.9*10^3$ ln(sRD)*dd_starv^{-1} for 35 mm post larvae and 5-10 mm larvae, respectively). Temporal resistance against food deprivation (i.e. time to death), was strongly impacted by initial condition and body mass but not by water temperature when expressed in dd. Changes in the 90% percentile of condition with dd_starv were compared across all trials to assess the potential highest magnitude of changes in sRD that could be expected from the highest ranking individuals within each trial. The conceptual model provides criteria to judge the risk of mortality of an individual due to starvation given measurements of sRD.

Session II. Laboratory studies: aquaculture, mesocosms, and small-scale interactions.

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GREEN IS GOOD, PROTISTS ARE BETTER! BALTIC COD (*GADUS MORHUA*) LARVAE REARED IN GREEN WATER WITH HETEROTROPHIC PROTIST *OXYRRHIS MARINA*

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Introduction

Green water techniques, using various species of unicellular microalgae, are known for their multiple beneficial effects on marine finfish larvae. In Atlantic cod, essential fatty acids (here: DHA) were transferred from algae to larvae through their rotifer-prey, a food item otherwise depleted in this nutritious compound (van der Meeren et al., 2007). Besides the nutritional effect, it has been hypothesized that the presence of unicellular organisms enhances visual contrast and stimulates foraging behavior. We tested this hypothesis by comparing Baltic cod (*Gadus morhua*) yolk sac and post yolk sac larvae raised in different green water types containing autotrophic microalgae and heterotrophic protists and a clear water control. Characteristics of larval behavior, growth and condition were assessed at the onset of first feeding and throughout the mixed feeding period in newly-hatched larvae and until the point of no return in temporarily food-deprived, older larvae.

Material and Methods

Newly-hatched yolk sac (2-11dph) and older, exogenously feeding (12-17dph) Baltic cod larvae were reared in either clear water (*CW*) or green water using *Nannochloropsis* sp. (*NA*, 1.5 to $2.0*10^6$ cells*ml^{-1}, size range 2.0 to 3.4µm) or *Nannochloropsis* sp. and *Oxyrrhis marina* (*NO*, likewise and 1000 cells*ml^{-1}, size range 13.3 to 16.0µm) at 10.0±0.2ºC and 16.0±0.1psu (mean±range) water temperature and salinity, respectively. In Trial 1 naïve yolk sac larvae (2 to 11dph) from the three rearing treatments were exposed to defined concentrations of *Acartia tonsa* nauplii (1*ml^{-1}) on a daily base and first feeding success and gut fullness (1: <6 nauplii, 2: >6 nauplii, 3: fully distended gut) were assessed 4h after initial food exposure. Trial 2 and 3 applied a similar methodology using first feeding yolk sac (Trial 2, 3-11dph) and older post yolk sac (Trial 3, ad libitum feeding up to 12dph, raised in *NA*-green water) larvae. In Trial 2, larvae were first introduced to food on day 3, 5, 7, 9 or 11dph and were held under ad libitum conditions for 5 days. In Trial 3, older larvae were food deprived for 3 days and were then re-fed for 2 days (ad libitum). Samples for growth (standard-length, freeze-dried weight, yolk sac area) and biochemical condition (RNA-DNA ratio) were taken at 2 to 3 day intervals.

Results

In Trial 1, mean (±SE) maximum observed first feeding success was higher in *NO* (86.7±4.4%, 6dph) and *NA* (71.7±10.9%, 5dph) rearing group compared to *CW* (66.7±9.3%, 7dph). The point of 50% first feeding was reached earlier in *NO* (3.4dph) than in any other treatment (*NA*: 4.0dph, *CW*: 5.6 dph), as calculated from non-linear regression curves fitted to feeding incidence (see Figure 1). Point of no return, i.e. when first feeding success decreased to <50%, was not significantly affected by treatment (*CW*: 8.2, *NA*: 8.3, *NO*: 8.9dph). Window of opportunity (product of time and magnitude of feeding) in *NO* was 2.4 times bigger than in *CW* and 1.4 times *NA*. Average gut fullness index was higher in *NO* (1.8-2.9) and *NA* (1.4-2.8) than in *CW* (1.1-1.7) on every day of the observation period. In Trial 2, yolk sac larval survival was high and not affected by treatment when food was introduced on 3 or 5dph. Pre-conditioned yolk sac larvae, first introduced to food on 7dph (initial dry weight 57.2±1.5µg), survived for 5 days (end of observation period). During this time, *NA* group larvae increased in dry weight (65.7±8.4µg), whereas *NO* larvae maintained their weight (53.0±7.3µg) and *CW* larvae lost weight (41.8±1.1µg) due to continued yolk absorption. When food was introduced on 9dph, no *CW* larvae survived longer than 3 days, whereas *NO* and *NA* larvae survived up to 5 days. Offering food 2 days later did not sustain larvae any longer than 3 days, irrespective of treatment. In Trial 3, survival in *NO* and *NA* green water was high during 3 days of food depletion and larvae exhibited positive growth rates when being re-fed. In *CW* control group, survival during starvation was low and no larvae survived the period of re-feeding. RNA-DNA ratios reflected periods of starvation and re-feeding in post yolk sac larvae.

Discussion

This study confirmed that utilizing green water has a beneficial effect on the larviculture of Baltic cod by stimulating foraging behavior and starvation resistance. Green water conditioned Baltic cod yolk sac larvae had an increased first feeding success and yolk sac and post yolk sac larvae showed increased growth and survival rates near the point of no return. The presence of the heterotrophic protist *O. marina*, in combination with the green water microalgae *Nannochloropsis* sp. (*NO* treatment group), stimulated the highest and earliest feeding incidence in yolk sac larvae during the first six days after hatch, indicating a stimulative effect on larval behavior, i.e. increased activity level and foraging success. Characteristics of short-term (up to 5 days) growth and biochemical condition in feeding larvae were positively affected by the presence of autotrophic microalgae and heterotrophic protists, highlighting their relevance during periods of food deprivation. Yolk sac and post yolk sac larvae from *NO* and *NA* treatment exhibited an increased resistance to starvation (assessed as biochemical condition and survival) and an extended window of opportunity. In summary, the two green water types applied in this study substantially increased the success of larviculture of Baltic cod and are highly recommended over clear water rearing for this species.

A.XI. RNA-DNA work protocol

Detailed protocol and working instructions for RNA/DNA analysis for cod larvae
IHF, University Hamburg, Germany, June 2010, modified by Stefan Meyer, stefan.meyer@uni-hamburg.de

- **Sample preparation**
  - Remove 1 control-homogenate (e.g. cod yolk sac larvae "H, dry", ~600 µg dry bio mass) and 64 samples from -80 °C freezer
  - Add 100 µL 1 % STEB to freeze dried samples and "H, dry"
  - Add 1 small spoon of glass beads (mixture of small and medium sized beads) to every vial
- Sample homogenisation
  - Use shaking mill Retsch MM400
  - Load vials into pre-cooled racks (4°C, 10 in each)
  - Shake (1 min/ 30 $*sec^{-1}$)
  - Centrifuge samples, to get rid of the foam (small Eppendorf lab-bench centrifuge, 10 sec)
- **Turn on photometer (SAFAS, Xenius XC)**
- **Turn on Centrifuge, cool it down (1 °C/ 4800 rpm/ 20 min/ quick start and stop)**
- Nucleic acid extraction
  - Dilute samples by adding 900 µL Tris-EDTA-buffer (5 mM Tris, 0.5mM EDTA, pH 7.5) to samples and homogenates (dilution factor 1:10, final volume 1.0 mL)
  - Vortex (15 min/ strength 7)
  - Centrifuge (1 °C/ 4800 rpm/ 20 min/ quick start and stop)
- Nucleic acid standards **(during centrifuging)**
  - Prepare 12 empty caps in rack
  - 1 RNA and 1 DNA standard-cap from freezer, thaw it and load after the following scheme:

RNA	µL stock	µL of 0,1 % STEB
R-1	10	300
R-2	20	300
R-3	55	300
R-4	80	240
R-5	110	240
R-6	110	200

DNA	µL stock	µL of 0,1 % STEB
D-1	5	300
D-2	10	300
D-3	20	300
D-4	30	300
D-5	40	300
D-6	60	240

- **Microplate loading (see figure)**
  - label 2 multiwell-plates (NUNC, black, F96) (1 + 2)
  - Add 75 µL of RNA (R-1 to R-6) and DNA (D-1 to D-6) (2 wells each)
  - Add 75 µL of 0,1 % STEB (4 wells STEB)
  - move sample-vials from centrifuge to cool-rack
  - Add 75 µL of control-homogenate (4 wells H)
  - Add 75 µL of sample-supernatant (S-1 to S-32; 2 each)
- **Freeze samples (-80°C) or keep in fridge (4°C) for subsequent Protein analysis**
- Introducing the fluorophore (dyeing), total fluorescence reading
  - Prepare Ethidium-bromide:
    - 32 µL Ethidium stock (1 mg*L^{-1} stock solution)
    - 16 mL Tris-EDTA buffer
  - Add 75 µL EB to each well (1200 µL electro-multi pipette)
  - Cover plates (foil) and shake (5 min/ 25 °C/ 700 rpm)
  - 1st reading, (520 nm excitation/ 605 nm emission/ 500 V PMT/ 5 readings per well/ 0.1 mm distance/ 0.5 sec)
- Eliminating RNA
  - Thaw 2 caps RNase, pour in a tray
  - Add 10 µL RNase to each well (200 µL electro-multi pipette)
  - Cover plates (foil) and shake (20 min/ 25 °C/ 700 rpm)
  - 2nd reading (as above)
- Eliminating DNA
  - Thaw 1 cap DNase
  - Prepare working solution DNase (in tray):
    - 660 µL 0,1 M $MgCl_2$
    - 660 µL 0,08 M $CaCl_2$
    - 450 µL DNase
    - 1230 µL Tris-EDTA
  - Add 10 µL DNase to each well
  - Cover plates and shake (60 min/ 37 °C/ 700 rpm)
  - Remove from shaker and let cool to room-temperature for 30 min
  - 3rd reading (as above)
- **Export data to Excel-sheet template**

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