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Impact of global change on the development and survival of Atlantic herring (*Clupea harengus*) : a multi-stressors approach

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Impact des changements globaux sur le développement et la survie des larves de hareng de l'Atlantique (Clupea harengus): une approche multi-stress

Abstract

Global changes are leading to multiple modifications of the environment. One crucial question today is to determine the vulnerability of species to these changes. In particular, they may jeopardise fish populations, which may have deleterious consequences for ecosystems and humans. The objective of this thesis was to assess the potential of herring (*Clupea harengus*) larvae to cope with a modified environment. The combination of direct abiotic effects, ocean warming and acidification, and an indirect effect, with different dietary content of nutrients and essential fatty acids, was tested. The survival potential of the larvae was assessed using several measures at different levels of biological organisation to identify the presence of potential compensatory mechanisms. At the whole organism level, the growth rate remained constant at all temperatures, but on the contrary the development rate (transition to the next larval stage) was accelerated with increasing temperature. This resulted in smaller individuals for the same developmental stage, at the end of the larval period, under global change treatment. The majority of physiological measures were little affected by warming, acidification and food quality, revealing a strong acclimation potential in herring larvae. Nevertheless, the analyses show that warming and acidification constitute a stressful environment for the individuals, since at the molecular level an important stimulation of genes involved in Heat Shock Protein production was measured. At the biochemical level, the fatty acid profiles also reveal a potential anti-inflammatory response. This means that the larvae are able to face environmental modifications, but not without energetic consequences. The smaller size of the individuals in the warmer, more acidic environment make them more vulnerable to predation mortality, and thus could lead to reduced recruitment in herring population. This phenomenon could be exacerbated by taking into account other anthropogenic pressures on the organisms, as well as the variability of prey availability in the natural environment. It is therefore in the study of these other combined aspects that future studies should focus, in order to predict more precisely the fate of herring larvae.

Résumé

Les changements globaux induisent de multiples modifications de l'environnement. Une question cruciale aujourd'hui est de déterminer la vulnérabilité des espèces faces à ces changements. Ils risquent notamment de mettre en péril les populations de poissons, ce qui peut avoir des conséquences délétères pour les écosystèmes et les hommes. L'objectif de cette thèse était d'évaluer le potentiel des larves de hareng (*Clupea harengus*) à résister et se maintenir dans un environnement modifié par les changements globaux. La combinaison d'effets abiotiques directs, le réchauffement et l'acidification des océans, et d'un effet indirect, avec différents apports alimentaires en nutriments et acide gras essentiels, a été testée. Le potentiel de survie des larves a été évalué en utilisant plusieurs mesures à différents niveaux d'organisation biologique, afin d'identifier la présence de potentiels mécanismes de compensation. À l'échelle de l'organisme entier, le taux de croissance est resté constant à toutes les températures, mais au contraire le taux de développement (passage au stade larvaire suivant) a été accéléré avec l'augmentation de la température. Il en a résulté des individus plus petits, dans les conditions de changements globaux, pour un même stade de développement à la fin de la période larvaire. La majorité des mesures physiologiques ont été

peu perturbées par le réchauffement, l'acidification et la qualité alimentaire, révélant un fort potential d'acclimatation chez les larves de harengs. Néanmoins, les analyses montrent que le réchauffement et l'acidification constituent un environnement stressant pour les individus, puisqu'au niveau moléculaire une importante stimulation de gènes impliqués dans la production d'Heat Shock Protein a été mesurée. Au niveau de la composition biochimique des individus, les profils en acide gras révèlent aussi une potentielle réponse anti-inflammatoire. Cela signifie donc que les larves sont en mesure de lutter pour se maintenir dans le nouvel environnement, mais non sans conséquences énergétiques. La plus petite taille des individus en milieu plus chaud et acidifié les rend plus vulnérables à la mortalité par prédation, et donc pourrait entrainer une diminution du recrutement pour le hareng. Ce phénomène pourrait être exacerbé en prenant en compte les autres pressions anthropiques qui pèsent sur les organismes, ainsi que la variabilité de la disponibilité en proies dans l'environnement naturel. C'est donc dans l'étude de ces autres aspects combinés que devront se tourner les futures études, pour prédire plus précisément le devenir des larves de hareng.

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«Marier le devoir et le plaisir est

l’apanage de ceux qui ont

trouvé leur voie»

Erylis à Ewilan – L’œil d’Otolep

Les Mondes d’Ewilan – Pierre Bottero

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List of abbreviations/acronyms

AB	Alcian Blue
ALA	alpha linoleic acid
aminP	Aaminopeptidase N
AP	alkaline phosphatase
ARA	arachidonic acid
ARA	Arachidonic acid
BB	brush borders
CA	Correspondence Analsyis
CDD	Cumulative Deree Days
CHOL	cholesterol
CME	Coopérative Maritime Etaploise
DD	degree day
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
dph	day post hatch
DW	Dry Weight
EFA	Essential Fatty Acid
EFD	Elliptic Fourier Descriptors
EPA	eicosapentaenoic
FA	Fatty Acid
FAME	Fatty acid methylesther
FFA	Free Fatty Acid
GDD	Growing degree days
GHG	GreenHouse Gas
HSP	Heat Shock Protein
ICES	International Council for the Exploration of the Sea
IPCC	Intergovernemental Panel for Climate Change
Leu-Ala	leucine-alanine peptidase
MUFA	mono-unsaturated fatty acids
NL	Neutral Lipid
OA	Ocean Acidification
OW	Ocean Warming
OWA	Ocean Warming and Acidification
PAS	Periodic acid-Schiff
PCA	Principal compenents analysis
PL	Polar Lipid
ppm	parts per million
PUFA	Polyunsaturated fatty acids
RCP	Representative Common Pathway
SFA	Saturated Fatty Acid
SSP	Shared Socioeconomic Pathways
TA	Total Alkalinity
TAG	triglycerids
TL	Total length
TLi	Total Lipid

General Introduction

Marine life in an era of global change

From climate to global change: direct abiotic effects

Since the industrial revolution at the end of the 18th century, gas emission and accumulation in the atmosphere has been steadily increasing, particularly under the form of CO_2, a GreenHouse Gas (GHG). Before the industrial era, the concentration of CO_2 was around 278 ppm (Siegenthaler et al., 2017), and is now above 400 ppm (Mauna Loa Observatory, NOAA), leading to a rapid climate warming. Anthropic activities are without a doubt responsible for the actual climate change (Cook et al., 2016), differing from natural climate variations by the rapid rate of changes. Anthropic impacts on the Earth are multiple and identified in all part of the globe, to such an extent that our era start to be referred as The Anthropocene (Lewis and Maslin, 2015), and climate change to now be considered as global change.

Changes in atmospheric conditions drive physical ocean properties, which, in turn, influence biological processes of marine life (Boyd et al., 2018; IPCC, 2019). In addition to warming through heat absorption, the ocean plays a key buffer role in global warming by removing CO_2 from the atmosphere (Cao et al., 2009), being a net sink for anthropogenic carbon release. However, the increase in CO_2 dissolution in the ocean produces a disturbance of the carbonate system in the seawater. Atmospheric CO_2 is absorbed in the water and reacts to form carbonic acid (H_2CO_3) that dissociates in bicarbonate ($HCO3^-$) and hydrogen ions (H^+), and elevated H^+ concentrations decrease the ocean pH (Caldeira and Wickett, 2005). Thus an indivisible effect of ocean warming, mainly due to CO_2 gas emissions, is ocean acidification (Doney et al., 2009). The Intergovernmental Panel for Climate Change (IPCC) has developed different scenarios of ocean warming and acidification predicted for 2100, depending on the global GHG emissions

and their subsequent concentration in the atmosphere (IPCC, 2014). The worst-case scenario, named RCP 8.5 (Representative Common Pathway) or “business as usual”, if nothing is done to reduce anthropic emissions of GHG, predicted an average increase of 3°C of the sea surface temperature and a pH drop by 0.4 units, for the end of the century. The names of the scenarios have changed in the last IPCC report (IPCC, 2021), the trajectories are now called SSP (Shared Socioeconomic Pathways), however the previous nomenclature will be used (RCP) in this manuscript as it was the one used at the beginning of my PhD.

Direct abiotic modifications of the environment related to global change are already visible in the biosphere (Parmesan, 2006; Poloczanska et al., 2013), and have for example led to event of mass mortality (Harley, 2008; Jones et al., 2018) or geographic shifts in species distribution (Azzurro et al., 2019; Harley and Paine, 2009). For ectotherms, temperature is an important threat as the metabolism is based on temperature-dependent chemical reactions (Monaco and Helmuth, 2011; Nilsson and Lefevre, 2016). Temperature is influencing animal’s physiology and define a thermal window in which life is possible, and a smaller range where fitness is optimum (Pörtner and Farrell, 2008). Individual’s tolerance to temperature depends on the organism specialization and stage of life cycle, for example stenotherm organisms have a narrower window than eurytherm organisms (Hochachka and Somero, 2002). The vulnerability of individuals in the face of ocean warming is then depending on the limit of their thermal windows (Pörtner and Peck, 2010). Conversely, ocean acidification and the disturbance of the carbonate system may be particularly important for calcifying organisms, but increasing pCO_2 (partial pressure of CO_2) may also influence acid-base balance mechanisms (Portner, 2004). Direct effects of global change could challenge marine life at all level of biological organisation, and potentially negatively affect the fitness of marine organisms (Bellard et al., 2012).

Biological interactions: indirect biotic effects

Direct effects of global change could affect species performance and subsequent survival, but also species interactions (Tylianakis et al., 2008). Modifications at different levels of the trophic chain could propagate along it and lead to an alteration of food web functioning via modifications of trophic interactions and energy transfer (Rosenblatt and Schmitz, 2016). Disturbance in species interactions can affect the entire community vulnerability to climate change and is now highlighted as an important factor shaping ecological communities (Boukal et al., 2019; Murphy et al., 2020).

Individual species responses toward global change are firstly behavioral and physiological, with changes in geographic distribution (Chen et al., 2011), morphology (Sheridan and Bickford, 2011), phenology and timing of key seasonal events (Sydeman and Bograd, 2009). At the basis of the food chain, primary productivity drives recruitment success of higher trophic levels (Murphy et al., 2020). A change in recruitment could lead to reorganization of community assemblages with potential changes in ecosystem functioning (Albouy et al., 2014). Climate change can also alter trophic interaction through modifications of the resource quality (Rosenblatt and Schmitz, 2016). Primary production and phytoplankton nutritional quality is mainly support by nutrients availability in the environment, which supply in the marine system are disturbed due to human activities (Berhe et al., 2018; Peñuelas et al., 2013). In addition, phytoplankton cells are the main producer of omega-3 and omega-6 polyunsaturated fatty acids (PUFA), which are critical structural and functional components incorporated in animal tissues, involved in key physiological processes (Dalsgaard et al., 2003). Higher trophic level have limited *de novo* synthesis capacity of these PUFA and are consequently highly dependent of primary producer's composition for their growth and

development (Sargent et al., 2003; Tocher, 2003). Warming could affect phytoplankton physiology and alter their ratio of saturated fatty acid (SFA) in comparison to PUFA, decreasing PUFA quantities (Hixson and Arts, 2016), by homeoviscous adaptation (Sinensky, 1974). The increase in SFA in response to warming environment is done to maintain membranes fluidity and functioning in all ectotherms (Guschina and Harwood, 2009). Such a change in the biochemistry of phytoplankton could result in less fatty acids availability for higher trophic levels and compromise their development and survival. Species will face multiple modifications of their environment, abiotic and biotic ones. Their survival and dynamic will be challenged directly by individual behavioral and physiological response toward the abiotic changes, and these responses will also depend and be modulated by trophic interactions. As species development and survival are threatened by human-induced modifications of the environment, acceleration of species loss jeopardize ecosystems health and services, with potential negative feedback on humans well-being (Bergstrom et al., 2021; Worm and Duffy, 2003).

Understand and predict the consequences on marine life

The multiple changes within the ecosystem caused by climate change could have detrimental or beneficial consequences on organisms, resulting in future winners and losers (Fabricius et al., 2011; Fulton, 2011; Lam et al., 2016; Somero, 2010). Understanding and predicting the species vulnerability toward these changes is necessary to implement adaptive measures and support actions reducing anthropic impacts on marine life (Pereira et al., 2010). Determining whether populations could persist at their actual level or not is one of the major goal of climate change research (Munday et al., 2013). Organisms can respond to environmental changes at

different levels of organisation, space, and time, with or without interaction, through migration, acclimation and adaptation (Bellard et al., 2012). For mobile species, poleward migration can be induced by climate change to stay within animal optimum of their thermal window (Hochachka and Somero, 2002), like it has been seen for several fish species of the North Sea (Perry et al., 2005). At a longer-term scale, adaptation via natural selection could be a mechanism for persistence of a species in the new environmental conditions, but the adaptation rate might not be fast enough to keep up with the speed of climate change (Morgan et al., 2020; Visser, 2008). Survival in the face of an environmental modification at a lower time-scale can happen through acclimation and phenotypic plasticity (Somero, 2010). Adjustment of the physiological performances could improve immediate survival (Gerber et al., 2020; Mahanty et al., 2017; Narum et al., 2013), but not without consequences on life-traits determining individual fitness (Jonsson and Jonsson, 2019). Fitness and subsequent population sustainability are then ultimately depending on physiological limitations and tolerance (Koenigstein et al., 2016; Somero, 2010).

Physiological studies are of great importance to characterise the ability of species to cope with rapid environmental change (Bozinovic and Pörtner, 2015; Pörtner and Farrell, 2008; Somero, 2012). Ecophysiological approaches were identified as a priority for contemporary science on climate change, to study climate change-induced stress and to determine individual trade-offs that could set limits to the acclimation potential (Bozinovic and Pörtner, 2015; Jarrold et al., 2019). To comprehend and predict the consequences of climate change on organisms survival and physiology, different approaches and tools have been developed, each presenting benefits and limitations (See Boyd *et al.*, 2018). One approach is the design of controlled manipulative experiments, which allow to mechanistically describe the consequence of a driver on a response trait measured (Riebesell and Gattuso, 2015). Extensive research have

been carried out this last decades in this regard, mainly focusing on ocean warming or acidification effects on marine organisms. Yet, single driver experiments, while of high scientific value, have poor ecological relevance (Boyd et al., 2018), especially since warming and acidification are indivisible processes driven by CO_2 emissions. Marine organisms will face simultaneous multiple modifications of their environment, and their consequences are not always additive, requiring multiple-stressor experiments to increase predictions accuracy (Baumann, 2019; Todgham and Stillman, 2013), especially considering indivisible effects as warming and acidification. Warming will strongly affect ectotherms that have limited capacity to use metabolic heat to maintain their temperature and subsequent metabolic rate and physiological processes (Burraco et al., 2020; Kingsolver et al., 2013; Lefevre, 2016). Particularly fish are mainly poikilothermic, with a body temperature close to the ambient temperature in their surrounding environment, thus warming and acidification may lead to energetic trade-offs (Nilsson and Lefevre, 2016). Beyond their ecological importance, fish are also an important food source for humans. Climate change effects on the productivity of marine fish stock is then uncertain and need more investigations as in addition to the impact on ecosystems, it raises questions of food security (Koenigstein et al., 2016; Myers et al., 2017). While the entire life cycle of fish can be affected by global change, the larval stage is considered more vulnerable to environmental fluctuations, that can play a major role in species survival (Pankhurst et al., 2011; Petitgas et al., 2013).

A critical period of the development: the larval phase

Fish larvae, recruitment and fisheries

Fish stock fluctuations of commercial species have socio-economic impacts on human activities, and thus have been monitored and studies since decades (Hart and Reynolds, 2002).

Although the sustainability of exploited populations depends on various factors, the renew of the population is firstly linked with recruitment success (Ottersen et al., 2013). In fishery sciences, the recruitment is defined by new individuals entering the exploited population. Recruitment variability is mainly driven by mortality rates at early life stages (Levin and Stunz, 2005). Most marine teleost have a reproductive strategy with high production of offspring (eggs and larvae) without parental care (Juanes, 2007), leading to mortality rate before maturation close to 99.9% (Le Pape and Bonhommeau, 2015). Understanding and predicting recruitment dynamics is fundamental for fishery management (Bailey and Houde, 1989; Sharma et al., 2019). Yet mortality of early life stages is not stable over years and could generate important year to year fluctuations in recruitment (Houde, 2008; Szuwalski et al., 2015). Thus abundances data of eggs and larvae collected from oceanographic cruises, which reflect the spawning biomass, are generally poorly correlated with recruitment success of the cohort (Le Pape et al., 2020). The larval stage is defined as a period of huge mortality (Bailey and Houde, 1989; Houde, 2008), mainly due to predation and starvation (Cushing, 1990), representing a bottleneck period in fish life cycle (Levin and Stunz, 2005).

Fish larvae of most teleost spend their larval phase in the pelagic zone within the planktonic compartment (Juanes, 2007). They are part of the meroplankton as not all of their life cycle is planktonic. As any planktonic animal, they drift with the currents and depend directly on planktonic production at lower trophic level for their feeding (Checkley et al., 1988; Heath et al., 1989; Tiedemann et al., 2021). Numerous studies have been conducted to understand the main drivers of larval survival in the field, with Hjort's work being the first to highlight the crucial role of the larval phase in recruitment fluctuations (Hjort, 1926, 1914a). Hjort formulated the "critical period hypothesis" which states that the most critical period during the development of fish is the transition between endogenous and exogenous nutrition, at

which the mortality may be particularly high, especially when prey availability is suboptimal. This body of research has refined “critical period hypothesis” (Houde, 2008), discussing the fact that only one short period during the larval phase is critical for the survival. The different hypotheses developed after Hjort’s “critical period hypothesis” are not mutually exclusive and are even complementary. In line with Hjort idea that food limitation is the main factor leading to larval mortality, Cushing’s “Match-Mismatch hypothesis” (Cushing, 1973; Cushing, 1990) proposed that timing between primary production and zooplankton bloom, and fish spawning and larval production should be coordinated to support survival. Iles and Sinclair (1982) “stable retention” hypothesis, states that physical retention processes of early-life stages are more important than food availability in recruitment success, which is close to the “Aberrant drift” hypothesis of Hjort (Hjort, 1914a), arguing that mortality could also be strongly affected by larval dispersal in unfavorable currents. Other hypotheses combined nutritional and hydrodynamic processes to explain survival: Cury and Roy (1989) hypothesized that an “Environmental window” exists within which larval foraging success is linked to wind and micro-turbulence in an upwelling ecosystem; and Lasker (1978, 1981) suggested that a “Stable ocean” with vertical stratification could aggregate larvae and their preys, supporting high feeding and subsequent survival and recruitment success.

Decades of research have highlighted that biological and physical factor act in concert to influence larval nutrition and subsequent growth or mortality, revealing that environmental mechanisms linked with larval survival are multi-factorial (Somarakis et al., 2018). To better understand recruitment success fluctuations and improve fish-stock assessment, biotic and abiotic environmental information are progressively implemented in models to move toward more complete ecosystem-based fisheries management (Marshall et al., 2019). However, key drivers for a successful recruitment still remains poorly understood.

Larval development and condition

The larval phase of fishes is a period of important morphological and physiological changes to go from embryonic shape to a functioning juvenile/adult (Gisbert and Sarasquete, 2008). The fact they are not fully developed increase their vulnerability to environmental changes (Pimentel et al., 2019). Generally, four main developmental stages are described for fish (5 for flatfish), based on morphological criteria: yolk-sac presence, notochord's shape and fins development (Doyle, 1977; Ryland, 1966).

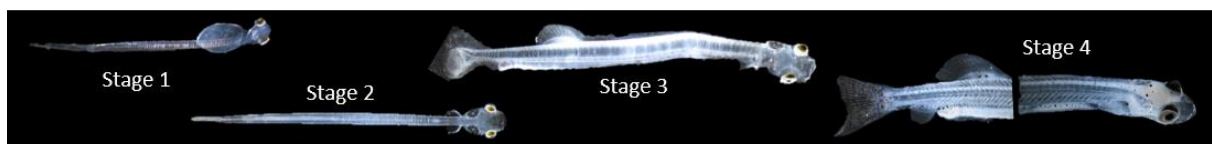

Figure 1 – The four developmental stages of herring larvae. (1) yolk-sac stage, (2) pre-flexion stage, (3) flexion stage and (4) post-flexion stage

The first stage corresponds to the yolk-stage (Figure 1), or vitelline stage. None of the fins are developed, the notochord is straight and the main criteria is the nutrition that is endogenous, i.e. the larvae feed on the yolk-sac. The second stage (pre-flexion stage) is marked by the depletion of the yolk-sac, when there is no more endogenous reserve and the nutrition is only exogenous, still with a straight notochord and only the pectoral fins are more developed. The transition between stage 1 and 2 is the critical period highlighted in Hjort's hypothesis (1914, 1926). The third stage (flexion stage) is distinguished by the flexion of the notochord where the caudal fin developed, and the development of the caudal and anal fins. The last stage (post-flexion stage) is reached when the pelvic fins start to develop. The end of the larval stage and the beginning of the juvenile stage is marked by the development of scales on the body and the completion of the fins. Considering that the stages are morphologically, anatomically,

and physiologically different, individuals at different stages do not have the same abilities to cope with environmental stress.

Survival through the larval stage, as previously stated, depends mainly on predation and starvation. While mortality by predation is complicated to assess in the field, mortality due to starvation, by lack of food or impairment of the digestive system can be assessed by physiological indices. Nutritional condition indices are mainly measured at three levels of organisation: whole-body, tissue, and biochemical levels (Ferron and Leggett, 1994). For tissue and cellular level, assessment of the nutritional condition, histological and biochemical index have been extensively used in the wild to determine year-to-year variations in condition or the potential of survival of a cohort (Chícharo and Chícharo, 2008; Giraldo *et al.*, 2013; Di Pane *et al.*, 2019). Condition indices reflect organ health, energy storage, and thus the ability to face a stressful situation, they serve as proxies of growth and fitness (Araújo et al., 2018). Global change could lead to energetic trade-offs in organisms, reorganising their energy allocation strategy (Koenigstein et al., 2016), impacting their nutritional condition and potential to go through the entire phase of the larval stage. Thus, understanding which mecanisms could be affected during ontogeny by biotic and abiotic environmental changes is necessary.

Evaluated the impact of global change on fish larvae

The larval stage is a period of high mortality depending mainly on hydro- and trophodynamic factors. The survival through this phase is determining for recruitment success and subsequent population dynamics within the ecosystems. Accordingly, investigating the effects of potential stressors on the development and survival of early life stages is a growing area of research.

Experimental research has focused on the effect of warming on eggs and fish larvae physiology, to determine thermal range tolerance and the effect of warming on life traits and survival (Madeira et al., 2016b; Moyano et al., 2017). Warming can induce thermal stress (Politis et al., 2017) and have negative impacts for some species in terms of survival, linked to cost of cellular protection (Madeira et al., 2016a). Growth, developmental rate and swimming speed are generally increased by non-lethal warming (Green and Fisher, 2004; Moyano et al., 2016). Research on ocean acidification effects on fish larvae showed negative impacts on behavior and olfactory system (Dixson et al., 2010; Lopes et al., 2016; Munday et al., 2009a; Rossi et al., 2018), as well as in condition and organ's integrity (Frommel et al., 2016, 2012a) or growth and survival (Baumann et al., 2011). Ocean acidification has also in other experiments promoted or had no effect on larval growth and development (Crespel et al., 2017; Rossi et al., 2015; Michael Sswat et al., 2018). However, as for warming, the response is not simple and similar for all individuals, suggesting species-specific effects and responses toward global change. However, the majority of the studies so far focused on single stressor effects. Yet, multiple environmental parameters are changing simultaneously which lead to additive, antagonistic, or synergistic effects. These effects are impossible to predict from the knowledge gained from single-stressor experiment (Baumann, 2019; Todgham and Stillman, 2013). Recent research has started to investigate the combined effects of ocean warming and acidification, revealing indeed that a stressor could exacerbate the effect of another (Flynn et al., 2015). Negative combined effects of warming and acidification were described for behavioral and physiological performance (Id et al., 2019; Laubenstein et al., 2019, 2018), and positive or lack of effects were also reported on the health, development and survival (Frommel et al., 2019; McMahon et al., 2020). In manipulative experiments focusing on abiotic parameters is easier that biotic one, yet studies combining one or two abiotic stressors

(warming, acidification) with food quantity could also change or exacerbate the effect of the abiotic stressors (Cominassi et al., 2020; Koenker et al., 2018). There is therefore an urgent need to assess the influence of global change on the larvae of commercial fish, such as herring, as survival at this life stage influences the size of the stock, and has an impact on human health and activities. The North Sea herring stock is of particular interest as it is already experiencing significant fluctuations.

The biological model: North Sea herring

Herring biology, ecology and fisheries

Herring appears to be a species with a wide range of temperature tolerance (Geffen, 2009), as well as flexible life-history strategy with production of eggs almost during the entire year. Different sub-populations or spawning components inside a stock are identified in functions of their time and spawning locations. In the North Sea (Figure 2), autumn-spawners contribute more than spring-summer spawner to the total stock (Daan et al., 1990). As the different components migrate differently for the reproduction but are mixed outside of the spawning season they are exploited together as one stock of herring (Payne, 2010).

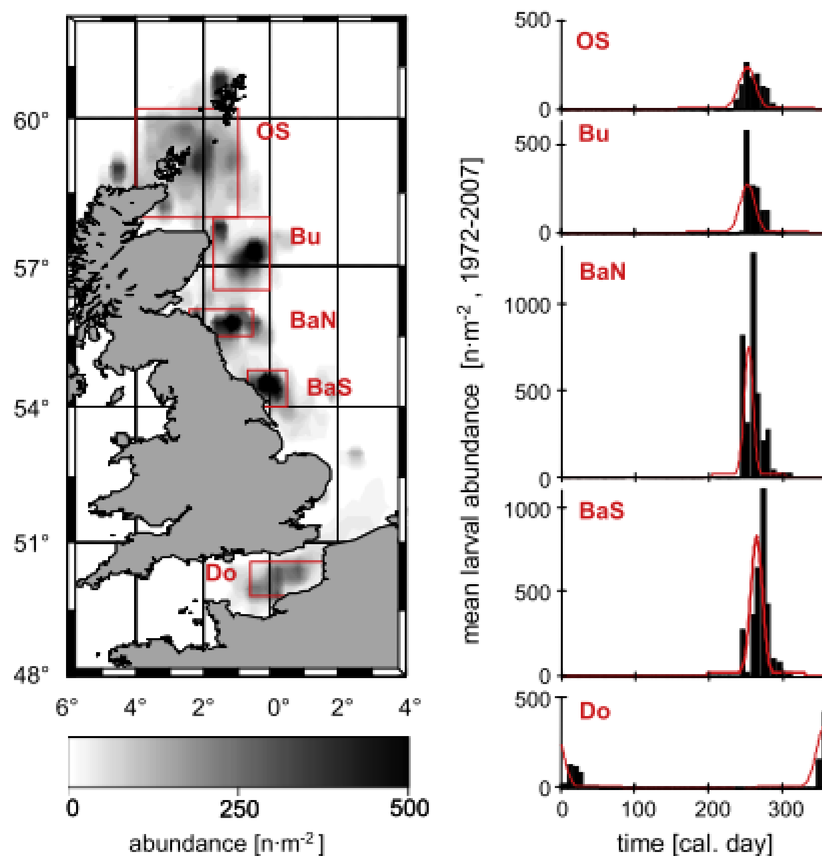

Figure 2 – Graphic from Hufnagl and co-authors (2015). Representation of the spawning location and time, with larval abundances of the different spawning component of the North Sea autumn spawning herring. The data are based on the international Herring larvae survey data, collecting larva < 10 mm, after hatching, between 1997 and 2006. Spawning components; OS: Orkney/Shetland; BU: Buchan; BaN: Banks north; BaS: Banks south; Do: Downs

Four main spawning components compose the North Sea autumn spawning herring (Figure 2), most of them spawn between August and October (Bank north and south components are commonly regrouped. The Downs component is distinguished by its spawning location, at the south of its distribution limit in the English Channel, associated with a period of spawning mainly in December. The larvae then develop in autumn and winter when the phytoplankton and zooplankton production is low (Hurst, 2007), which is the example of a mismatch between preys and larvae. Still this reproductive strategy might provide advantages such as lower feeding requirements with the lower temperature, lower rates of predation, or even less competition for resources as most larvae are developing in spring (Denis et al., 2016; Hufnagl

et al., 2015). Each spawning component is also characterised by different fecundity, size at hatch, growth rates, migrations paths toward nurseries and recruitment rates (Cushing and Bridger, 1966; Geffen, 2009). After hatching, the planktonic larvae from the different spawning sites are transported with the currents to nursery grounds in the eastern part of the North Sea, at the coastal part of the south German Bight and to the Skagerrak and Kattegat (Corten, 1986). Generally, after 2 years in the nurseries, the individuals join the adult population in offshore deeper waters (MacKenzie, 1985).

Past collapse and current concerns about the North Sea autumn spawning stock

This spawning stock has been exploited since middle ages and was the largest sea fishery in Europe at the beginning of the 20th century (Payne et al., 2009), having an important socio-economical place in European countries. Due to the commercial value of Herring, the fluctuations of the population in the North Sea has led to decades of research since the 18th century focusing on population dynamics, which formed the basis of fisheries research (Geffen, 2009) and are at the source of the first conceptual theories in fisheries ecology (Sinclair, 2009).

While North Sea autumn spawners herring biomass is naturally variable, the stock collapsed between the 1950s and the 1980s due to overfishing (Dickey-Collas et al., 2010). During this period the stock went from more than 5 million tons to only 50 000 tons (ICES, 2009, 1998; Payne, 2010), where the maximum age of fish caught was 5 instead of previously 14 years old (Dickey-Collas et al., 2010). The collapse was not homogenous inside the stock, the spawning component collapsed one after the other from south to north (Cushing, 1992). For the first time, a ban on fishery was adopted by different European countries by mid-1977, to stop

herring fisheries in the North Sea (Dickey-Collas et al., 2010). Around five years later, the ban was lifted and the stock is since then sustainably exploited (ICES, 2018), but the recovery was not homogeneous and immediate as the Downs component took around 25 years to recover (Dickey-Collas *et al.*, 2010). The dynamic of the contribution of each spawning component to the global stock is also variable, but for about ten years now the Downs component is the main contributor (Fässler et al., 2011; Payne, 2010).

Over the past 20 years, fluctuations in the recruitment have been recorded, with continuous years with low recruitment despite a sustainable exploited stock and a consequent spawning-stock biomass (Payne et al., 2009), pointing toward a higher mortality during the overwintering larval phase. Despite being one of the most studied fish stock dynamic, the drivers of recruitment, and potential cause for its recent decrease, are still poorly understood (Payne et al., 2013). Different hypotheses have been put forward, highlighting mainly warming (Fässler et al., 2011; Payne et al., 2009) or changes in the planktonic community (Alvarez-Fernandez et al., 2012; Payne et al., 2013; Weijerman et al., 2005) as potential causes for stronger larval mortality. Results from studies on the effects of global change on larvae of Atlantic (A) and Baltic (B) herring are summarized in Figure 3.

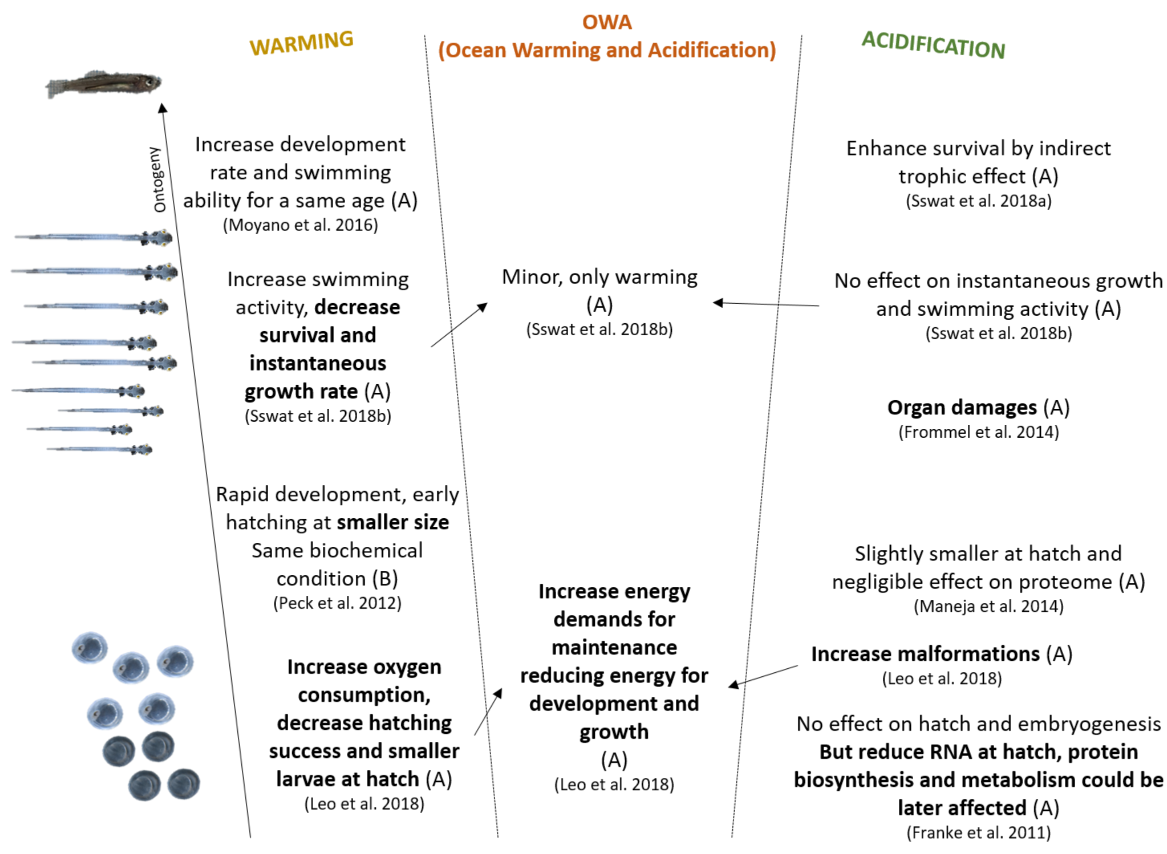

Figure 3 – Summary of the research investigating the impact of warming, acidification and both parameters combined on herring larvae from the Baltic (B) or the Atlantic (A) at different developmental stages. In bold are highlighted the deleterious effect of the parameter, increasing the vulnerability of the larvae.

Globally, warming increases the mortality, oxygen consumption, and swimming abilities, whereas it decreases the period of development and the size at hatching (Peck *et al.*, 2012; Moyano *et al.*, 2016; Leo *et al.*, 2018; Sswat *et al.*, 2018). The effect of acidification reported were either null (Maneja *et al.*, 2014; Sswat *et al.*, 2018), sub-lethal (Franke and Clemmesen, 2011; Frommel et al., 2014; Leo et al., 2018), or beneficial (Michael Sswat et al., 2018). In a mesocosm study manipulating acidification, Sswat and co-authors (2018) highlighted that acidification increased phytoplankton productivity, and that increased food availability might have compensated the negative effect of acidification. This reinforces the idea that for relevant and more accurate predictions, the potential indirect biotic effects of global change

need to be included in experiments. Understanding the mechanisms driving herring larval survival is now a priority in a changing world threaten by climate change. While the fate of herring larvae toward global change have started to be investigated, a lot of uncertainties remain as the effects of combined abiotic and biotic drivers, from the cells to the entire organism, have not been explored. The present manuscript will attempt to fill a part of this knowledge gap

Aim and structure of the thesis

As Atlantic herring is an ecologically and socio-economically important species of the North Sea, assessing the fate of the larvae in a context of global change and multiple–stressors is crucial. In this context, the general questions this thesis want to answer are: How may global change affect herring larvae survival and development?

To assess these questions two complementary axes are used in this manuscript. The objective of the first axis is to gain knowledge on the development of herring larvae under optimal condition. Indeed, success of nutrition depends on the one hand on food availability, and on the other hand on the maturation of the digestive system. Yet, information about the development of herring's digestive system are lacking in the literature. In the second axis, I investigated the potential effect of combined global change-stressors, namely warming, acidification, and changes in food quality, on herring larvae. I cultured herring larvae under different scenarios, and investigated how these scenarios influenced larval fitness. For this, I focused on different levels of biological organization, using traits directly related to fitness and describing underlying physiological mechanisms. More specifically, I investigated growth, development, as well as physiological differences along the larval stage. I complemented this work by focusing on the oldest larvae, which went through most of the larval stage, and compared their histological and lipid condition, their organ's integrity and biochemical composition.

<u>Axis 1:</u> Increase our knowledge on herring larval ontogeny with a special focus on the digestive system

- What is the timing of digestive organs development and digestive system maturation in herring larvae?

  To answer this question I reared herring larvae and used length and stage data to assess growth and development, as well as histological and enzymes analyses to describe the maturation of the digestive system during this development.

  Chapter 1 - Maturation of the digestive system of Downs herring larvae (*Clupea harengus*, Linnaeus, 1758): identification of critical periods through ontogeny
  Léa J. Joly, Christophe Loots, Cédric L. Meunier, Maarten Boersma, Sophie Collet, Valérie Lefebvre, José-Luis Zambonino-Infante and Carolina Giraldo
  <u>Published in Marine Biology</u> (submitted the 3rd of December 2020 and accepted in April 2021)

<u>Axis 2:</u> Assess the combined effects of warming, acidification, and food quality on growth, development, condition and physiology of herring larvae

For this axis, one experiment was conducted during which herring larvae were reared in a control treatment (11°C, pH8.0) and under the RCP 8.5 Ocean Warming and Acidification scenario (14°C, pH7.6), and subjected to two feeding conditions (prey of different nutrient and DHA content).

- How do warming, acidification, and food quality have influence growth rate, development rate, and metabolism of herring larvae?

  To answer this question samples were taken during the entire experiment to assess size and stage, and stage 3 larvae were sampled to conduct transcriptomics analyses and assess if the treatments led to different regulation at the transcriptional level of genes involved in metabolism (aerobic, lipid, glycogen) and stress response.

  Chapter 2 – Future environmental conditions affect herring larval development and physiology
  Léa J. Joly, Maarten Boersma, Carolina Giraldo, David Mazurais, Lauriane Madec, Sophie Collet, José-Luis Zambonino-Infante, Cédric L. Meunier
  Submitted in Biology Open the *13th* of January, 2022

- Question: How do warming, acidification, and food quality influence herring larval condition and biochemical composition?

  To answer this question larvae were sampled at the end of the experiment. Larval condition was assessed with histological observations and lipid index measurements. The biochemical composition and the effect of the treatments on lipid membranes and reserves were assessed using fatty acid analysis.

  Chapter 3 – Herring larval condition under different global change scenarios
  Léa J. Joly, José-Luis Zambonino-Infante, Maarten Boersma, Sophie Collet, Véronique Loizeau, Cédric L. Meunier, Éric Tavernier, Carolina Giraldo
  Draft format

- Question: Could warming and acidification modify fish biomarker of development?

  To answer this question, larvae in each treatments were sampled during the entire experiment and the morphogenesis of the otoliths were assessed

  Chapter 4 – Effect of climate change on the morphological development of sagittal otoliths in Atlantic herring (Clupea harengus) larvae
  Kélig Mahé, **Léa J. Joly,** Solène Telliez, Jose-Luis Zambonino-Infante, Cédric L. Meunier, Kirsteen MacKenzie, Carolina Giraldo
  Submitted in Journal of Experimental Marine Biology and Ecology the 14th of January, 2022

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AXIS 1 – Increasing our knowledge on herring larval ontogeny with a special focus on the digestive system

The aim of this axis is to describe the timing of the digestive organs development and digestive system maturation of herring larvae. To do so herring larvae were reared from hatching to the last developmental stage, histological and enzymes analyses were used to describe the ontogeny of the digestive system.

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CHAPTER 1 - Maturation of the digestive system of Downs herring larvae (*Clupea harengus*, Linnaeus, 1758): identification of critical periods through ontogeny

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ORIGINAL PAPER

Maturation of the digestive system of Downs herring larvae (*Clupea harengus*, Linnaeus, 1758): identification of critical periods through ontogeny

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1.1 Introduction

North Sea herring is a commercially and ecologically important stock of North Atlantic herring (*Clupea harengus*). This stock has experienced high biomass fluctuations, with an almost complete fisheries induced collapse in the past, resulting in the closure of the fishery in the 1980's (Dickey-Collas et al., 2010). Since then, the stock has recovered and, as a result of the very tight regulations, is currently sustainably exploited. Nevertheless, years of poor recruitment, i. e. quantity of juvenile fish entering the adult population, are regularly reported despite high adult biomass levels, suggesting that other factors are responsible for variability in larval survival , which are still poorly understood (Payne et al., 2009).

The economic implications of fish stock inter-annual variability have motivated research on population dynamics and fish biology for more than a century (Hart and Reynolds, 2002). Hjort (1914) was the first to point out larval survival, and not only spawning stock biomass, as a key factor determining annual recruitment and size of fish populations. He defined the transition from endogenous to exogenous nutrition as the critical period ("Critical period hypothesis"), i.e. the period when the larvae start feeding, and are most sensitive to starvation. First-feeding stage has been shown to be a bottleneck period for Western Baltic Sea herring, where young larvae reaching this critical period before the plankton bloom contribute little to the surviving year-class (Polte et al., 2014). Different recruitment hypotheses based on hydrodynamic and trophodynamic factors have been put forward since, and it is now well accepted that biological and physical processes act in concert to determine the survival of fish at early life stages (Houde, 2008), from fertilization to metamorphosis and beyond. For instance, feeding success and subsequent growth rate are advocated as two of the most important factors influencing

larval survival and recruitment (Anderson 1988; Bergenius et al. 2002; Jenkins and King 2006; Robert et al. 2007).

Thus far, condition and physiological state of field-caught larvae have been used as indicators of suitable environmental condition and potential recruitment success. These are typically measured by indices, including morphometric, histological and biochemical measurements (Ferron and Leggett, 1994). These indices are used to assess directly or indirectly the nutritional status of organisms, or to infer their ability to bear starvation (Ehrlich et al., 1976). Some of them are widely used in larval fish surveys, such as the relative content of RNA to DNA in tissue, or the size corrected weight of individuals. Recently, Denis et al. (2017) observed that Downs herring larvae, one of the most important spawning components of North Sea herring, undergo a change in their condition at around 13 mm. The authors observed a reduction in instantaneous growth rate (by RNA: DNA) along with an increase in ingestion rate and suggested this to be a possible critical period resulting from a change in the energy-allocation strategy (i.e., from growth to energy reserves). Because the response of RNA:DNA ratios differs with larval size (Foley et al., 2016) the former hypothesis of a second critical period needs to be validated with additional independent and well-controlled approaches. It is known that ontogeny and organogenesis of the digestive system can influence the physiological status of larvae (Suthers, 2000). Hence, knowledge of the development and maturation of the digestive tract, as well as the dynamics of energy storage by accessory glands, would be very helpful for a precise interpretation of field data. Furthermore, the study of ontogenic transitions could be of importance to determine critical periods during the development at which suboptimal feeding conditions could directly affect survival and recruitment.

Marine fish larvae are not fully developed at hatch and undergo simultaneous major ontogenic changes in morphology, anatomy and physiology. Growth and development are linked to the nutritional status of individuals which relies on internal storage, and after first feeding, on food quality, availability and functionality of digestive organs (Ronnestad et al., 2013). Maturation of the larval digestive tract is defined as the acquisition of adult digestive features; this process has been widely studied for digestive enzymes in teleosts, especially in the context of aquaculture (Zambonino Infante and Cahu, 2001). Most fish species lack a morphologically distinct and functional stomach (characterized by the appearance of gastric glands, (Zambonino Infante and Cahu, 2001)) before metamorphosis, and digestion processes in larvae mainly depend on other digestive organs, especially the pancreas (Ronnestad et al., 2013). Among pancreatic enzymes, trypsin activity has been related to food ingestion (Pedersen et al., 1987) and has been used to experimentally identify starving from well-fed herring larvae. Trypsin measurements reflect changes in food availability and nutritional condition at a shorter-term range than RNA/DNA ratios which has been estimated > 5 days (Foley et al., 2016; Ueberschär and Clemmesen, 1992). Intestine maturation is characterized by changes in enterocytes enzymatic activities, i.e. a decrease in cytosolic activities concomitant with a sharp increase in brush border membrane enzyme activities (Zambonino Infante et al., 2008). Consequently, digestive enzymes activities are used as indicators of larval development and potential of survival. Although these aspects are essential to understand larval recruitment, which is necessary to assess stock fluctuations, there is a striking lack of information on the ecologically and economically important North Sea *C. harengus*. Teleosts have similar developmental patterns, but large species-specific variations exist regarding organ differentiation timing and metabolism efficiency (Gisbert and Sarasquete, 2008). For a more complete assessment of potential recruitment success it is hence essential to acquire

in-depth knowledge on the processes during larval development, both structural changes as well as changes in physiology.

In the present study, we focused on North Sea herring (Downs component) and aimed to (1) describe the development and maturation of the digestive tract and associated organs (oesophagus, stomach, intestine, liver and pancreas), and (2) identify physiological changes that could potentially be linked to critical periods during ontogeny. To do so, herring larvae were reared in the laboratory from hatch to the last larval stage, and digestive enzyme activities (trypsin, alpha amylase, leucine-alanine peptidase, aminopeptidase N and alkaline phosphatase) as well as histological structure of digestive organs were assessed at different times through development. Hence, the present study provides important basic information on the structural and biochemical changes of herring's digestive system throughout the larval stages that can be useful for future work assessing the condition of field-caught larvae in relation to environmental conditions.

1.2 Materials and methods

1.2.1 Artificial fertilization

Mature herring from the Downs component were obtained in November 2018 from a local organization of fishermen (Coopérative Maritime Etaploise, CME) in Boulogne-Sur-Mer (France). Artificial fertilization was carried out using ready-to-spawn fish at the Ifremer Manche-Mer du Nord laboratory. Eggs of 27 females (mean standard length: 27.2 $\pm$ (SD) 1.92 cm; mean weight: 166.4 $\pm$ 32.54 g) were stripped-spawned in seawater and placed on four plastic plates (PVC) of 490 cm^2 each before being fertilized with the sperm of 9 males (mean standard length: 28.0 $\pm$ 3.84 cm; mean weight: 179.2 $\pm$ 32.0 g). The eggs and sperm were

incubated during 10 minutes at 13.5°C and 32.7 psu, before rinsing. To ensure genetic diversity, on each plate eggs from at least six females were spread and the mixed sperm of at least two males was added for the fertilization. Along with the four PVC plates a smaller slide of 21 cm^2 was used to monitor closely the fertilization and eggs development without having to take out of water the eggs intended for the experiment. Eggs were stripped-spawned on the slide and fertilized with the same protocol in order to give us an approximation on fertilization success. The fertilization success of the slide was of 42%. Around 200 eggs were laid on 21 cm^2, thus we can estimate that approximately 4600 eggs were present on each plate of the experiment.

The plates were transported to Ifremer-Centre de Bretagne laboratory in boxes with oxygenated and thermoregulated sea water (variation range: 13.5 to 15°C). Eggs were then incubated in a 200 L tank in the dark. For the entire experiment, natural seawater was pumped from the Bay of Brest (32.5 psu, pH 8.0), passed through a sand filter, heated to avoid natural temperature fluctuations, filtered (2 µm) and UV sterilized (PZ50, 75W, Ocene, France).

1.2.2 Larval rearing

Most of the eggs hatched on the 9th day of incubation (starting point of the experiment: 1 dph) and larvae were kept until 69 days post hatch (dph). Three days after hatching (3 dph) about 4500 larvae were counted and distributed equally in three 38 litres conical black tanks (1500 larvae by tank), constituting the replicates of the experiment. A continuous flow-through system of 20 L h^{-1} was used and the temperature was set to 13°C (13.3 ± 0.25°C). Oxygen saturation was measured daily with an oximeter (WTW Oxi 340, Bioblock scientific) and was always higher than 98%. In order to prevent any food limitation, the daily food quantity was

distributed four times during the day to maintain an *ad libitum* level, ensuring that there were always prey in the tank during the day. To be sure to allow proper feeding through time we used an increasing range of living prey sizes from phytoplankton to 24 h old nauplii, before weaning with feed granules.

Rhodomonas salina (Strain 2002) and *Oxyrrhis marina* (Strain 21.89) were obtained from the Culture Collection of algae (University of Göttingen, Germany). *R. salina* were grown in 10 L bottles under constant light in enriched Conway medium to provide quick growth (N and P concentrations doubled). *O. marina* were cultivated in a 5 L bottle under constant lighting and fed *R. salina*. Nauplii of the commercially available *Artemia salina* (VNBS, Viepearl) were hatched in 60 L tanks after 24 h at 28°C, and directly used to feed larvae early in the development. Thereafter, artemia nauplii were enriched 24 h (A1) in a mixture of fish oil (FO) and baker yeast (BY) (For 1 Million artemii = 4.3 g FO and 11.1 g BY).

The feeding protocol of Moyano et al. (2016) was followed for early feeding. Yolk sac larvae were fed the microalgae *R. salina* (mean: 30 000 cells mL^{-1}) and the heterotrophic protist *Oxyrrhis marina* (mean: 250 cells mL^{-1}). Two days after mouth opening, *Artemia salina* nauplii (A0) were supplied in the tanks (mean: 70 A0 $larvae^{-1}$ d^{-1}). From 22 to 39 dph a mix of *A. salina* nauplii (mean: 140 A0 $larvae^{-1}$ d^{-1}) and 24 h old enriched artemii (mean: 140 A1 $larvae^{-1}$ d^{-1}) were added. After that, A1 were supplied in decreasing quantities (mean: 200 A1 $larvae^{-1}$ d^{-1}) while introducing feed granules (Neo Supra, Le Gouessant, 58% proteins, 13% lipids) until 49 dph (from 0.2 to 0.5 g $tank^{-1}d^{-1}$). From 49 to 69 dph, larvae were fed only with the granules (1 g $tank^{-1}$ d^{-1}). The lighting sequence of the tanks was modeled on aquaculture protocols. The lighting period was of 24 h a day, progressively increased from 1 to 59 lux along the

development, in order to maximize larval feeding, in particular for young herring larvae which possess only cone cells prior to metamorphosis (Bell and Dick, 1993).

1.2.3 Larval sampling

A total of 761 larvae of *C. harengus* were sampled and measured (Total Length, TL in mm) throughout the experiment to estimate growth rates. A subsample of those individuals were used for enzyme analysis. Larvae were collected with a sieve in the morning, before feeding, and euthanized in ice water. As larvae are fragile organisms, homogeneous sampling through the tanks inevitably leads to a slight increase in mortality. Thus, for histological individual analysis we decided to limit the number of larvae sampled at the beginning of development and to increase it progressively according to the progress and success of the rearing. The samplings were adjusted to not go below a minimum, non-stressful, density of 25-30 larvae.L^{-1} per tank. Four vitelline larvae were directly sampled from the hatching tank at 3 dph. After that, from 2 to 13 larvae per tank were sampled at 12, 19, 21, 25, 28, 35, 49 and 69 dph. The larvae were preserved for 48 h in Bouin's solution, then rinsed and preserved in 70% ethanol in the dark until analysis. To describe the morphological development, larval stage and size were defined under binocular observations for 4 larvae (at 3 dph) + ((2-10 larvae) * 7 (sampling dates) * 3 (replicates)) + (13 larvae * 3 (replicates)) = 169 larvae. To have a reference value on the level of shrinkage of the conservation technique, larvae sampled at 69 dph were measure before and after conditioning (13 larvae per tank). A total of 35 larvae were used to histologically describe the digestive system development through time.

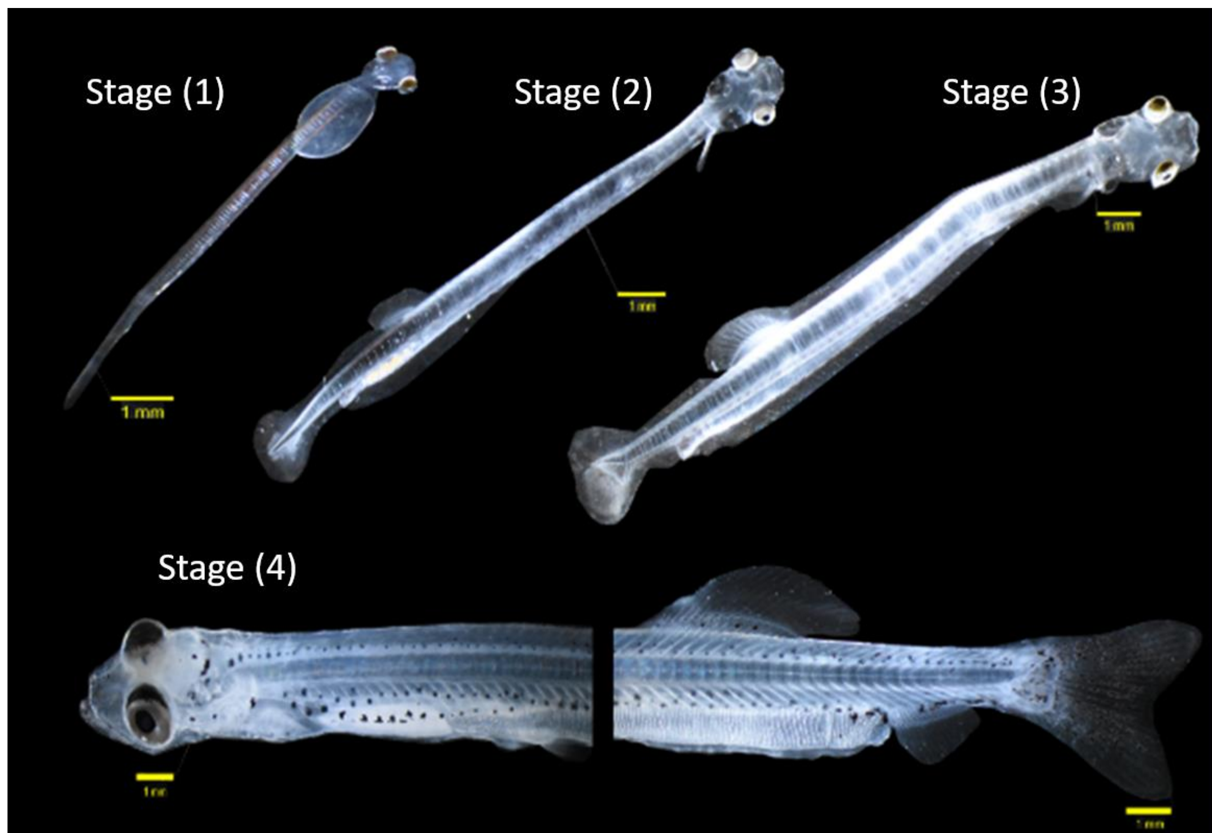

Figure 4 - Developmental stages of herring larvae: (1) yolk sac stage – 3 dph, 8-9 mm TL , (2) pre-flexion stage – 12 dph, 9-14 mm TL, (3) flexion stage – 28 dph, 12-18 mm TL and (4) post-flexion stage, the larvae is cut in two parts because it was unfortunately too long to fit entirely under the binocular – 69 dph, 15-30 mm TL, binocular observations

The developmental stage (Figure 4) for each larva was determined according to Doyle (1977): 1) yolk sac larval stage, 2) pre-flexion stage; yolk sac absent and straight notochord, 3) flexion of the notochord stage and 4) post flexion stage with visible pelvic fins.

Enzyme activities analyses by spectrophotometry require the pooling of individuals to work with a sufficient biomass, thus we started the sampling when larvae were 14 dph old. Between 30 and 50 larvae were sampled in each tank for enzymes analysis at 14, 21, 28, 35 and 49 dph. The larvae were put in vials and stored at -80°C, yielding a total of 5 (sampling days) * 3 (replicates) = 15 samples.

1.2.4 Organogenesis of the digestive system

Organogenesis of the digestive system was studied using histology. Individuals were dehydrated and embedded in paraffin blocks, as described by Di Pane et al. (2019), substituting the xylene by diasolv (non-Carcinogenic, Mutagenic or Reprotoxic substitute of xylene/toluene). Sagittal section of 7 µm were made with an automated-microtome (Leica, RM2255) and mounted on slides. Larval sections were dewaxed and rehydrated in successive baths of diasolv and alcohol progressively less concentrated (100 %, 95%, 70%) and tap water. Two histochemical colorations were used on different larvae. Half of them were stained using alcian blue (AB) pH 2.5 alone. The other half was double-stained with Periodic acid-Schiff (PAS) to stain glycogen and neutral mucosubstances, and alcian blue (AB) pH 2.5 for acid mucosubstances. Then all the slides were stained with Groat's hematoxylin and picro-indigo carmine for topographic coloration. Between 3 to 6 larvae per sampling date were observed under a microscope (Leica, DM6B) to describe the development of the larval digestive system. We focused on the histological organization of the digestive tract (oesophagus, stomach, intestine) and accessory digestive glands (liver and pancreas) (Table 1). Presence of zymogen, inactive enzymatic precursor, in the pancreas and glycogen, indicative of energy reserve, in the liver were examined. The liver condition and reserve amount were scored based on hepatocytes vacuoles characteristics following the protocol developed by Di Pane et al. (2019). Image acquisition was done with a software (LAS X) and a camera (Leica, DMC4500) connected to the microscope.

Table 1 - Synthesis of the histological structures observed for the digestive system development description (Zambonino Infante et al. 2008; Ronnestad et al. 2013; Di Pane et al. 2020)

Organs	Tissue	Features		
Oesophagus	Tube shape	short and narrow	elongated	folded
	Epithelium	simple	pseudo-stratified	stratified
	Epithelial cells shape	cubic		prismatic
	Goblet cells	present		absent
	Mucins	acid	neutral	both
Rudimentary Stomach	Epithelium	simple	pseudo-stratified	stratified
	Epithelial cells shape	cubic		prismatic
	Villi	present		absent
Differentiated Stomach	Cardiac stomach	simple epithelium	prismatic cells	prominent villi
	Fundic stomach	simple epithelium	prismatic cells	gastric glands
	Pyloric stomach	simple epithelium	prismatic cells	pylorus and pyloric caeca
Rudimentary Intestine	Epithelial cells shape	prismatic		

	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	present		absent
	Brush Border	present		absent
Differentiated intestine (anterior and posterior)	Epithelial cells shape	prismatic		
	Goblet cells	present		absent
	Mucins	acid	neutral	both
	Villi	moderate		prominent
	Brush Border	present		absent
	Vacuoles	present		absent
Exocrine Pancreas	Acini stain	dark		pale
	Tissue organisation	Indistinct acini, detachments		Distinct acini, contiguous
	Zymogen	present		absent
Endocrine Pancreas	Langerhans islets	present, number		absent
Liver	**Score**	**1**	**2**	**3**
	Hepatocytes size	small	medium	large
	Vacuoles	absent	small / medium	large
	Glycogen quantity	absent / small	medium	huge (everywhere)

	Tissue structure	some detachments	joined cells	joined cells

1.2.5 Enzymatic assays

Activity of five digestive enzymes was assessed during larval development: trypsin (EC 3.4.21.4), alpha amylase (EC 3.2.1.1), leucine-alanine peptidase (Leu-Ala), aminopeptidase N (aminP, EC 3.4.11.2) and alkaline phosphatase (AP).

Larvae from the first two sampling dates (14 and 21 dph) were entirely homogenized with a polytron (PT – MR2100, Polytron, NR: 324412). Older ones (28, 35, 49 dph) were dissected and measured on ice under a binocular microscope (Cahu and Zambonino Infante, 1994) in order to obtain pancreatic and intestinal segments. Samples were homogenized in five volumes (w/v) of ice-cold distilled water. Brush borders were extracted and purified from the intestinal segment (Crane et al., 1979). Homogenates of younger larvae and pancreatic segments from older larvae were used to determine activities of pancreatic enzymes, i.e. trypsin and alpha amylase, according to Holm et al. (1988) and Métais and Bieth (1968) respectively. Cytosolic leucine-alanine peptidase activity was assessed from intestinal homogenates (Nicholson and Kim, 1975). Assays of the brush borders enzymes were performed for alkaline phosphatase and aminopeptidase N following Bessey et al. (1946) and Maroux et al. (1973), respectively. All measurements were carried out using a spectrophotometer (Thermo Scientific, Evolution 201) at 37°C.

For each analysis, protein content was determined in the sample with Bradford's method (Bradford, 1976). All the activity measurements were done three times for each sample and averaged to give a value per tank and time. Enzymatic activities were expressed as specific

activities in mUnit mg protein^{-1} and segmental activities, i.e. the total activity of one enzyme per larva in the intestinal segment. The level of intestinal maturation was calculated as the ratio of brush borders enzymes related to cytosolic enzymes based on the segmental activities (Zambonino Infante et al., 1997).

1.2.6 Statistical analyses

Data analyses were performed under the R software (R core Team, 2019) with an alpha level of 0.05. Differences in growth rates among tanks were investigated with a linear mixed-effects model (LMEM) using the package "lme4" and the function *lmer* (Bates et al., 2015). "Length" was the outcome variable with "dph" used as a fixed effect and "tank" as a random effect. Following recommendation by (Barr et al., 2013) we fit a maximal random effects structure which allows for random slopes (for the different tanks) and random intercepts. The function *ranova()* from the "lmerTest" package (Kuznetsova et al., 2017) was then used to test the effect of the random effect on the model (likelihood ratio tests), an normality of residuals was checked. The random effect "tank" was not significant (P > 0.05) and so replicates of the different tanks were pooled for subsequent analysis. The data are expressed as mean ± SD. Enzymes data normality was checked with Shapiro's test and homoscedasticity with Bartlett's test. As normality and homoscedasticity were respected, specific activities and enzymatic ratio data were compared by one-way ANOVA, with time as a factor, followed by post hoc HSD Tukey tests when significant differences were found.

1.3 Results

1.3.1 Size distribution during developmental stages

LMEM fixed effect estimate of the variation of length over time was of 0.22 mm.d^{-1} (Table 2).

Table 2 - Estimated model fixed effects of the variation of length over time using "tank" as a random effect for 761 observations.

Effect	Estimate	Std. Error	df	t	P value
Intercept	8.87	0.47	2.18	18.59	0.001
Dph	0.22	0.01	2.15	18.10	0.002

All larvae presented a yolk-sac and hatched without a functional mouth but with fully pigmented eyes. The mouth opened at 3 dph (8-9 mm) and the yolk sac was fully resorbed at 8 dph (9-10 mm). Phytoplankton was observed in the intestine from 5 dph (8-10 mm), and artemia nauplii from day 7, before total resorption of the vitellus.

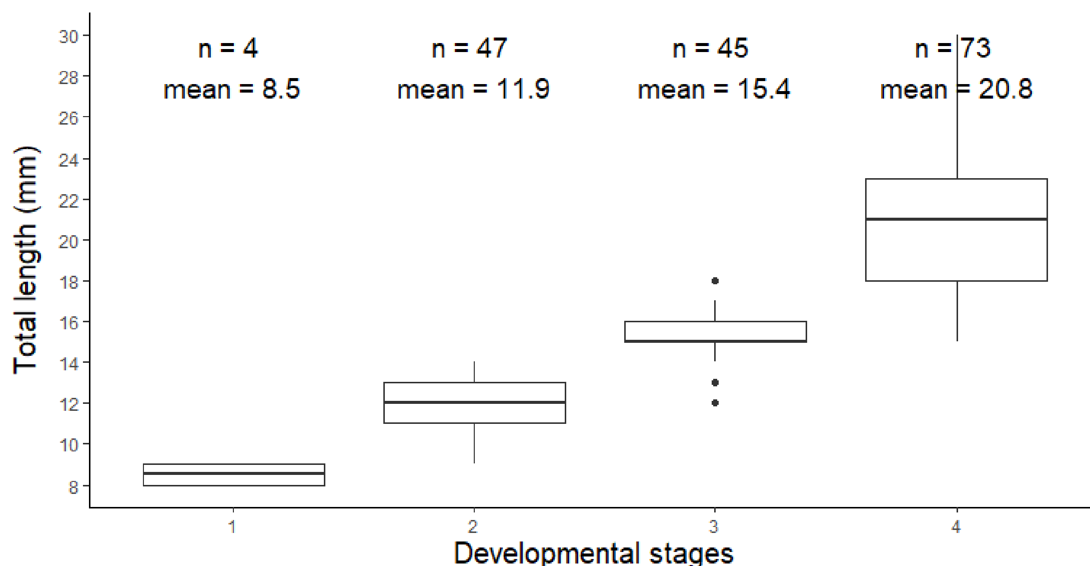

Figure 5 - Boxplot representation of the larval total length (mm) observed for each developmental stage

Additional to size measurements, the developmental stage was determined for 169 larvae sampled for histological analysis. Size ranges between each stage are well separated (Figure. 5). Stage 1 larvae were found at 3 dph, the larvae were thin and long with a straight notochord, had a visible intestine and vitelline reserves. In stage 2 larvae between 12 and 35 dph, the vitellus was totally resorbed and the caudal and dorsal fins started to differentiate. For stage

3 larvae between 21 and 35 dph, body height was higher, the intestine bigger and the caudal, dorsal and anal fins fully differentiated. Melanophores were also present along the superior part of the intestine and some above the head and on the caudal fin. For stage 4 larvae from 35 to 69 dph, all the body features were bigger, the whole body was thicker and the pelvic fins started to differentiate. Melanophores were present all over the body and concentrated above the intestine, on the dorsal part of the body, above the head, around the mouth and on the dorsal fin. The measurements came from individuals stored successively in Bouin's solution and 70% ethanol for histology. The conditioning method can result in shrinkage of the larvae. Individuals measured before and after conservation shrank by an average of 11.2 % (± 7.4 %, n = 39).

1.3.2 Digestive system development

Yolk-sac stage (stage 1)

At 3 dph, larvae were around 8 mm and the mouth was open with the yolk sac still present. The oesophagus was relatively short, narrow and folded, with an important amount of goblet cells (Figure. 6.C). Only acid mucosubstances (AB positive, Figure 7.A) were detected. The stomach was not yet morphologically differentiated and appeared as a simple cavity with short and mostly flattened epithelial cells (Figure 6.D). In the intestine, the enterocytes formed a straight thick layer with some goblet cells in between, producing acid mucosubstances (AB positive). Microvilli forming the brush border at the top of the cells were visible (Figure 6.E). Pancreas and liver had an elongated shape going from the back of the pectoral fins to the beginning of the intestine. Pancreatic cells were organized into weakly distinct clusters with a moderate amount of zymogen (Figure 6.G). Only exocrine pancreas was visible. The liver had

large cells with large vacuoles (Figure 6.F) and a pronounced pink (PAS-positive) tint highlighting an important quantity of glycogen in the cells.

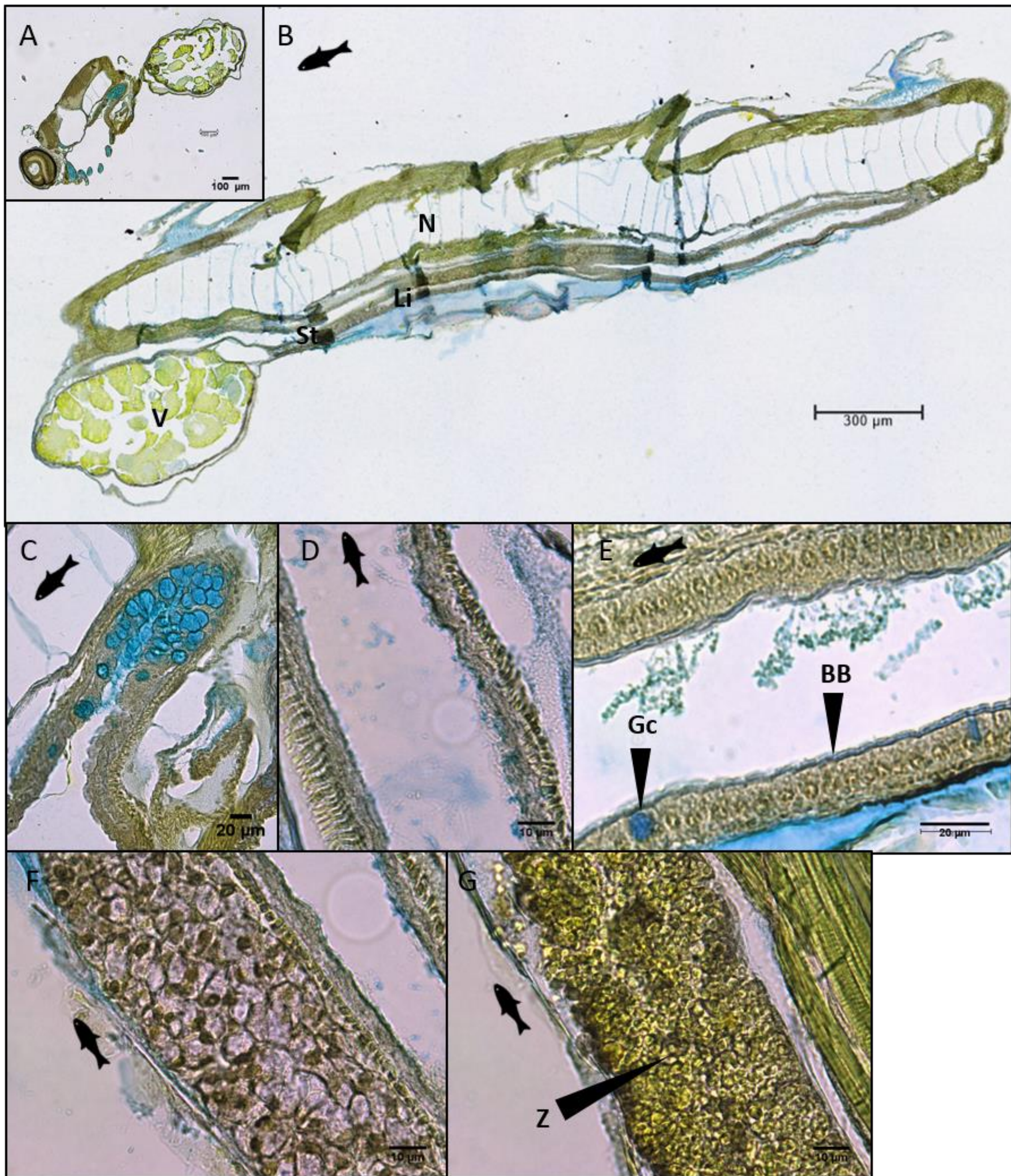

Figure 6 - Sagittal sections of Downs herring larvae (yolk sac stage, 8 mm, 3 days post hatch), colored with alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine. ***A*** *Section of the head and yolk sac, Magnification x200, scale bar = 100 µm.* ***B*** *General view of the larva, Scan x200, scale bar = 300 µm. Magnification x1000; oil immersion for C, D, E, F and G.* ***C*** *Details of the oesophagus with goblet cells, scale bar = 20 µm.* ***D*** *Section of the stomach cavity, scale bar = 10 µm.* ***E*** *Section of the intestine with goblet cells, scale bar = 20 µm.* ***F*** *Details of the liver with large vacuoles and glycogen (pink), scale bar = 10 µm.* ***G*** *Details of the pancreas with zymogen granules, scale bar = 10 µm. Abbreviations: BB Brush Borders, Gc Goblet cells, I Intestine, Li Liver, N Notochord, St stomach, V Vitellus, Z Zymogen granules within acinar cells, the black fish icon represents the orientation of the slide to help locate the head and the tail position*

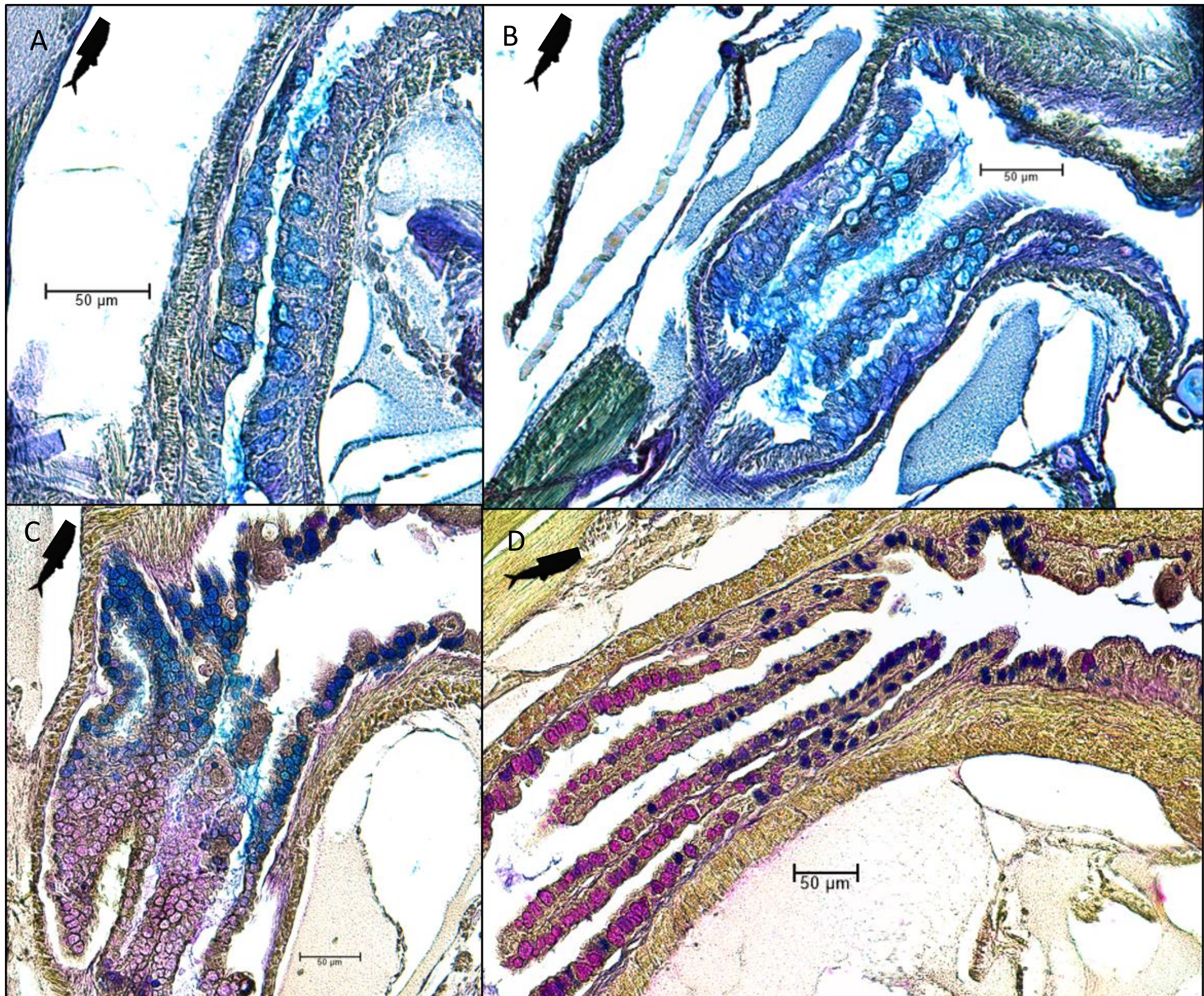

*Figure 7 - Oesophagus sagittal sections of Downs herring larvae at stage 1 (**A**), stage 2 (**B**), stage 3 (**C**) and stage 4 (**D**), colored with periodic acid-Schiff, alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine. Magnification x200, scale bar = 50 µm, the black fish icon represents the orientation of the slide to help locate the head and the tail position*

Pre-flexion stage (Stage 2)

At 12 dph, larvae were around 10 mm, the tissular organization was quite similar to the one described for stage 1 except for the yolk sac which was completely resorbed. The goblet cells in the upper part of the oesophagus were also tinted in blue, revealing acid mucosubstances production (Figure 7.B). Some stage 2 larvae showed few violet goblet cells in the lower part of the oesophagus, revealing both acid and neutral mucosubstances production (violet tint: AB and PAS positive). Anatomical changes began to appear at 19 and 21 dph when larvae were

around 11-12 mm and at the end of the stage 2. The distinction between the stomacal cavity and the intestine was clearly visible as a structured pylorus. In the intestine, two regions could be distinguished: the mid- and the hindgut, separated by a simple constriction of the organ. The epithelial structure was homogeneous for both parts, enterocytes began to form villi and still contained goblet cells. The endocrine pancreas was visible.

Flexion stage (Stage 3)

At 25 dph, larvae were around 14 mm and started transitioning into stage 3. The global morphology of the digestive tract remained the same but the organs became larger and thicker. The oesophagus interstice was wider and longer. A clear pattern appeared in the mucosubstances secretion by the goblet cells of the oesophagus: the proximal half produced acid mucus and the distal part neutral mucus (Figure 7.C). The oesophagus opened up to a stomacal cavity extended in the upper part. The epithelial cells height of the stomach increased and the villi of the intestine became larger. The endocrine portion of the pancreas took the form of one large cluster of cells called islet, with a central position. Before 28 dph, exocrine pancreatic cells seemed to be regrouped but without a clear pattern. At 28 dph, cells were organized into distinct and circular acini. The pancreas became more elongated, protruding beyond the beginning of the intestine. The liver underwent major changes at the beginning of stage 3, between 25 and 28 dph. Cells were smaller and the vacuoles were reduced or absent for some larvae. At 35 dph (end of the stage 3), the liver showed both large cells with huge vacuoles and small cells with smaller vacuoles. The stain remained pink through the stage highlighting the presence of glycogen reserves.

Post-flexion stage (Stage 4)

Stage 4 is the last stage before metamorphosis into a juvenile. Internal and external morphology of the larvae become increasingly closer to that of adults (Figure. 8.A). The oesophagus and its longitudinal folds were longer and the interstice wider than in stage 3 (Figure 8.B). As for the stage 3, two distinct parts were identified by the goblet cells mucus production, the coloration was stronger than before suggesting an increase in the quantity of acid and neutral mucosubstances produced respectively in the superior and inferior part of the organ (Figure 7.D). Epithelial cells of the stomach were higher at 49 dph (Figure 8.C). At 69 dph a coloration appeared in the stomach, the stomacal glands were not observed. Most of the larvae presented a strong blue coloration (AB positive: acid mucosubstances) for two third of the stomach length and a strong pink coloration (PAS positive: neutral mucosubstances) in the last part of the stomach directly connected to the pylorus. At 69 dph, the intestine was larger, the villi were prominent and the ceca pyloric were visible after the pylorus (Figure 8.E,D). At 49 dph the acini were still distinct but at 69 dph the structure of the exocrine pancreas was more diffuse. At the end of the stage 4, one large endocrine islet was still present in the middle of the pancreas and small islets were observed on either side of the larger one (Figure 8.F). The pancreas expanded further along the intestine. The liver of stage 4 larvae, like for stage 3, was highly variable with some individuals presenting numerous and large vacuoles (Figure 8.G, 9.A) while for others the vacuoles and amount of glycogen were reduced or absent (Figure 9.B,C).

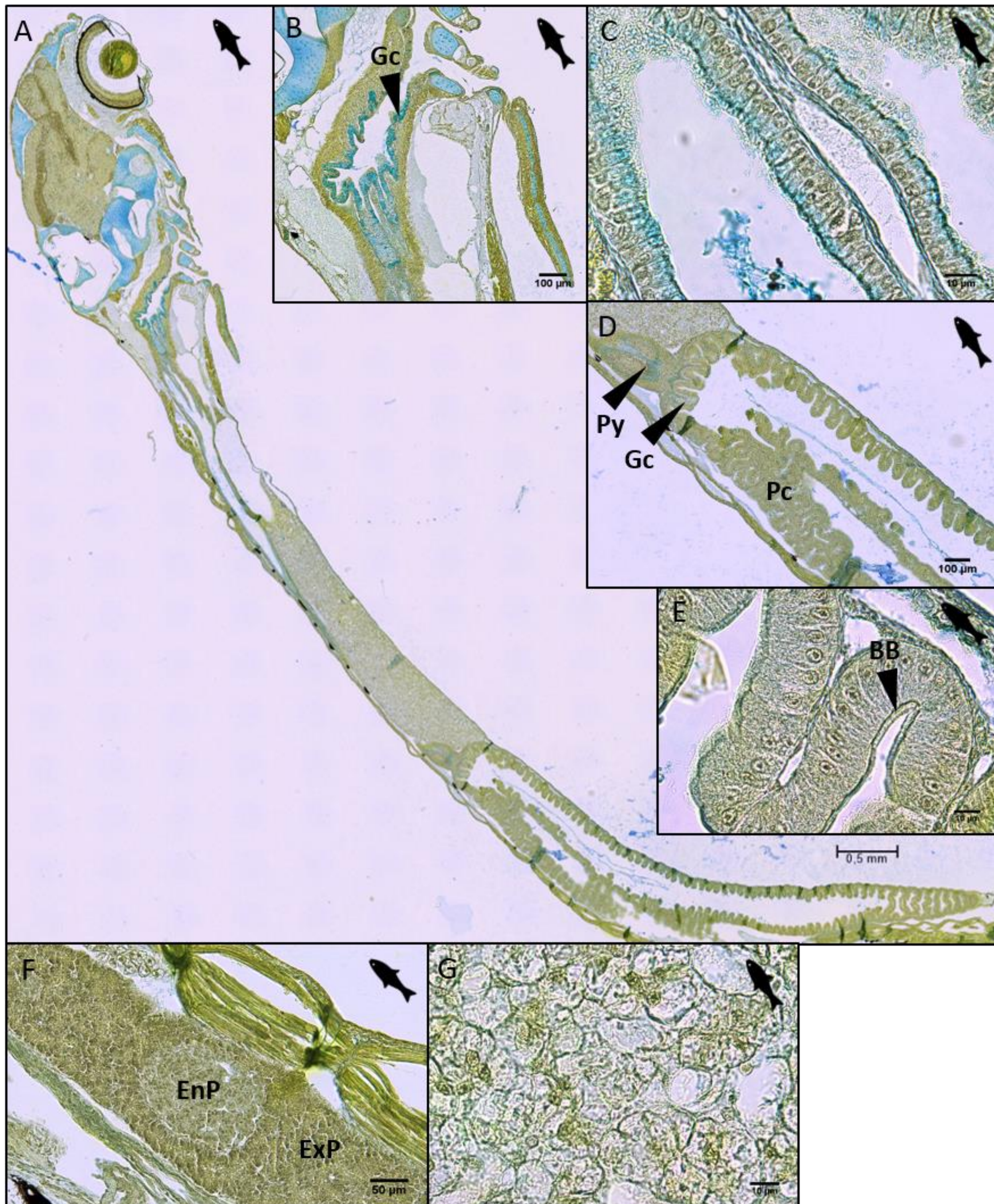

Figure 8 - *Sagittal sections of Downs herring larvae (stage 4, 27 mm, 69 days post hatch), colored with alcian blue pH2.5, Groat's hematoxylin and picro-indigo carmine.* ***A****. General view of the larva, Scan x200, scale bar = 0.5 mm.* ***B*** *Section of the oesophagus and upper part of the stomach, Magnification x200, scale bar = 100 µm.* ***C*** *Details of the stomach, Magnification x1000; oil immersion, scale bar = 10 µm.* ***D*** *. Section of the pylorus and start of the intestine with goblet cells, prominent villi and a small part of pyloric ceca, Magnification x200, scale bar = 100 µm.* ***E*** *Details of de villus structure of the intestine and the Brush Border, Magnification x1000; oil immersion, scale bar = 10 µm****F*** *Details of the pancreas organization with pancreatic islets and exocrine pancreas, Magnification x200, scale bar = 50 µm.* ***G*** *Details of the liver with large cells and vacuoles, and a small amount of glycogen (pink), Magnification x1000; oil immersion, scale bar = 10 µm. Abbreviations: BB Brush Borders, Gc Goblet cells, I Intestine, Li Liver, N Notochord, Pancreas with ExP Exocrine Pancreas and EnP Endocrine Pancreas, Oe Oesophagus, Pc Pyloric ceca, Py Pylorus, Sb Swim bladder, St stomach, V Vitellus Z Zymogen granules within acinar cells, the black fish icon represents the orientation of the slide to help locate the head and the tail position*

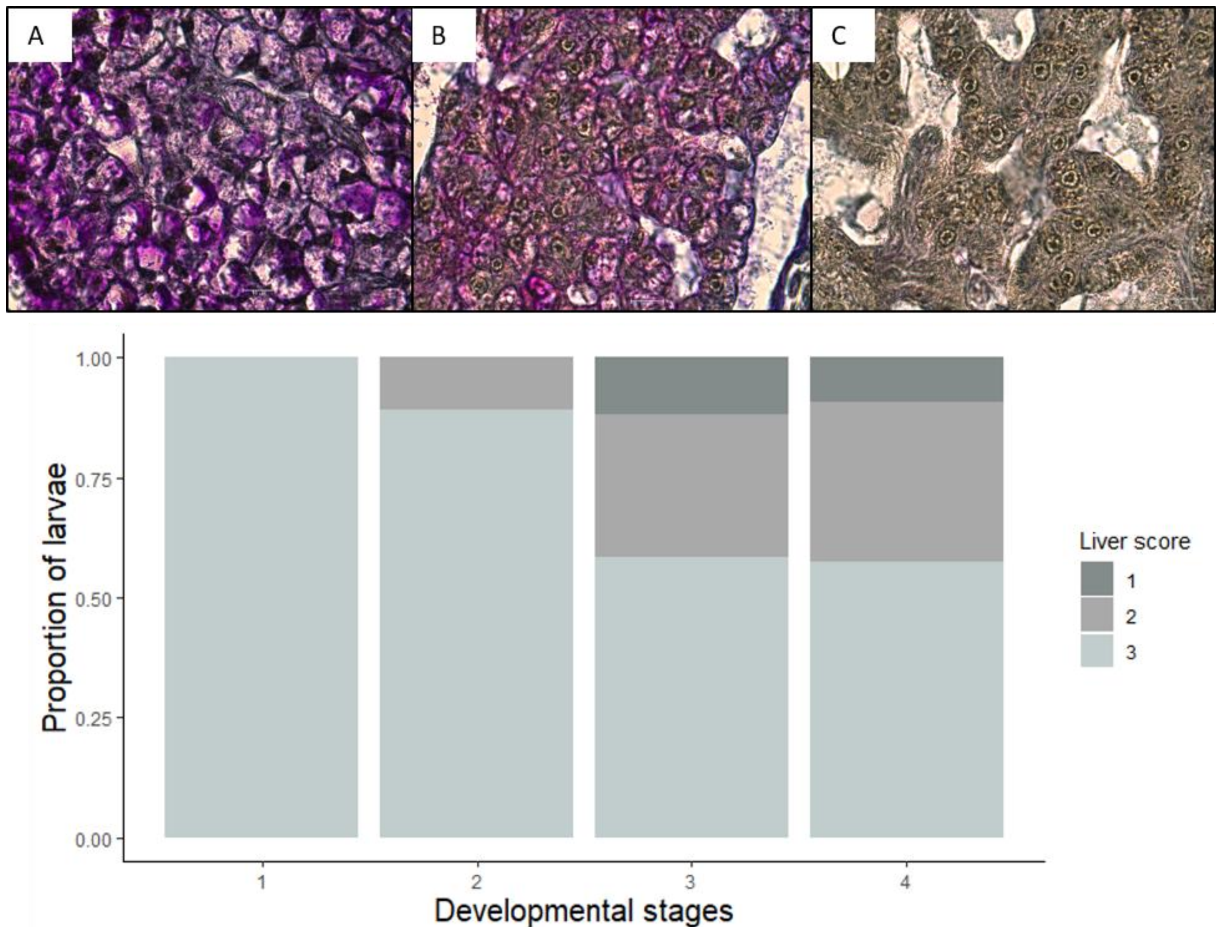

*Figure 9 - Sagittal sections of Downs herring liver at different scores and liver score proportion as a function of developmental stages. (**A**) Score 3: wide vacuoles, (**B**) Score 2: scattered vacuoles, (**C**) Score 1: no vacuoles, Magnification x1000; oil immersion, scale bar = 10 µm. (**D**) Larval proportion of liver score for the developmental stages 1 (n = 3), 2 (n = 8), 3 (n = 11) and 4 (n = 13)*

1.3.4 Activity of digestive enzymes

The level of maturation of the digestive system was determined based on the variations in the intestinal enzymes activities (Cahu and Zambonino Infante, 1995). Trypsin specific activity remained constant from 14 to 49 dph (31.0 ± 5.11 mU mg protein^{-1}; ANOVA, $F_{(4,10)} = 1.67$, $P = 0.233$) (Table 3). Amylase specific activity was different through time (ANOVA, $F_{(4,10)} = 12.14$, $P < 0.01$), a post hoc Tukey test showed that the activity was higher at 14 dph ($P < 0.05$) and decreased later from 5079 ± 470.6 to a mean of 818.1 ± 324.7 mU mg protein^{-1} at 49 dph. Intestinal enzymes specific activities were tested for larvae at 28, 35 and 49 dph. No statistical

significant differences were found for the intestinal enzymes specific activities Aminopeptidase N (ANOVA, $F (2, 6) = 2.79$, $P = 0.139$), Alkaline phosphatase (ANOVA, $F (2, 6) = 4.80$, $P = 0.057$) and Leucine-alanine peptidase (ANOVA, $F (2, 6) = 0.637$, $P = 0.561$), at the three different ages. The evolution of the ratio between segmental activities of the brush border and cytosolic enzymes differed through time (AP/Leu-Ala: ANOVA, $F (2,6) = 9.55$, $P = 0.014$; AminP/Leu-Ala: ANOVA, $F (2,6) = 8.37$, $P = 0.018$), a post hoc Tukey test highlighted a shift between 28 and 35-49 dph (Figure 10, $P < 0.05$).

Table 3 - *Summary of the specific activities (mU mg protein^{-1}) of pancreatic (trypsin and amylase) and intestinal (aminopeptidase N, alkaline phosphatase and leucine-alanine peptidase) enzymes during herring larval development. Values represent mean ± SD (n=3), * indicates a statistical difference over time (P < 0.05)*

	Enzymes		**Day Post Hatch**				
			14	**21**	**28**	**35**	**49**
	Total length (mm)		/	/	15.0 ± 2.2	16.9 ± 2.0	18.5 ± 2.4
Specific activity (mU mg protein^{-1})	Pancreatic	Trypsin	27.9 ± 6.5	34.9 ± 10.8	29.0 ± 1.1	37.7 ± 6.6	25.5 ± 5.6
		Amylase	5079 ± 470.6*	2215 ± 70.2	1724 ± 1047	1530 ± 1387	818.1 ± 324.7
	Intestinal	Aminopeptidase N	/	/	171.4 ± 17.8	257.8 ± 74.0	215.9 ± 15.3

Alkaline phosphatase	/	/	164.0 ± 54.5	436.5 ± 166.3	405.8 ± 107.5
Leucine-alanine peptidase	/	/	395.4 ± 137.2	450.7 ± 87.6	507.0 ± 132.4

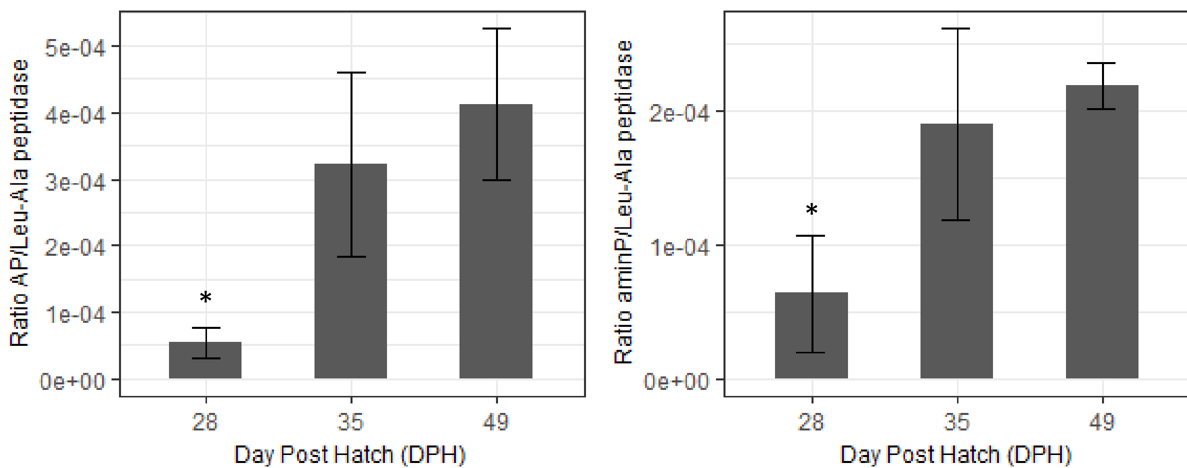

*Figure 10 - Intestinal maturation of the enterocytes during Clupea harengus larval ontogeny indicated by the change in ratio in segmental activities of brush border enzymes (AP: alkaline phosphatase, aminP: aminopeptidase N) / cytosolic enzymes (Leu-Ala: leucine-alanine peptidase). Results are presented in mean ± SD (n=3). * indicate significant differences between ratios (P < 0.05)*

1.4 Discussion

Here, we report for the first time a detailed description of the development and maturation of the digestive system of Down's herring larvae, an important component of the North Sea herring stock. The larval growth rate obtained during our experiment is similar to other experiments on Atlantic herring (Ehrlich et al., 1976; Moyano et al., 2016) and field data estimates on Downs (Denis et al., 2017). The combination of histological and enzymatic

approaches allowed us to identify and discuss developmental periods at which herring larvae may be more vulnerable to fluctuating environmental conditions and suboptimal feeding conditions.

1.4.1 Maturation and functionality of the digestive system

Anatomical and cellular changes of the oesophagus, stomach and intestine as well as accessory glands drive the digestion processes, the efficiency of nutrients transport and absorption. Functional timing of development is the major difference between fish species and appears to be genetically programmed (Zambonino Infante and Cahu, 2001).

Oesophagus

In teleosts, the alimentary canal generally develops from an undifferentiated straight tube at the yolk-sac stage to a segmented one where the oesophagus, the stomach and the intestine are differentiated. For Down's herring larvae, at 3 dph, all digestive organs, including the liver and pancreas were already distinguishable. With the opening of the mouth at around 3 dph, presence of microplankton in the gut was observed before yolk sac resorption in agreement with previous observations (Checkley, 1982; Munk, 1992). Busch (1996) showed that the first feeding, on prey larger than phytoplankton cells, is allowed by the decreasing of the yolk sac diameter. He argued that the oesophagus diameter increases by mechanical action in response to a decrease in the pressure exerted by the yolk sac when it is consumed. Consumption of larger preys is also facilitated by the presence of goblet cells that play different roles but are mostly involved in the lubrication of the digestive system (Zambonino Infante et al., 2008). Goblet cells can be present before the onset of exogenous feeding or appear later in the development depending of the species (Lazo et al., 2011). In addition, they also differ in their histochemical features. For example, in dover sole, senegal sole and

yellowtail flounder, oesophagus goblet cells only secrete acid mucosubstances (Zambonino Infante et al., 2008), whereas a mix of neutral and acid glycoconjugates were observed for the california halibut and white seabream. The nature of this secretion varies along the ontogeny of herring larvae, there is an initial production of acid mucus during stage 1 and 2 and an increased production of neutral mucosubstances for stages 3 and 4. The changes in histochemical properties of the glycoconjugates is species-specific, for the siberian sturgeon goblet cells in the larval oesophagus first started to produce neutral glycoproteins only, and later a mix of neutral and acid mucins (Gisbert et al., 1999). The mature pattern of stage 3 and stage 4 herring larvae in terms of histochemical properties is similar to the one of sea bream (Sarasquete et al., 2001), with a large amount of neutral mucosubstances secreted in the distal oesophageal zone and acid mucosubstances secreted in the proximal oesophageal zone.

Stomach

The presence of pyloric ceca and gastric glands in the stomach are commonly used to histologically define the transition from larvae to juvenile stage, and to an adult mode of digestion (Zambonino Infante et al., 2008). No gastric glands were observed or found during the experiment, suggesting that metamorphosis from a digestive point of view is not totally completed. However, the height of the stomacal cells starts to increase during the stage 3 and until the end of the stage 4 (~30 mm at 69 dph). The stomach was the latest organ to start its development, and by 69 dph it was not fully developed into the Y-shaped form typical of adult clupeids (Whitehead and Teugels, 1985), allowing for the ingestion of large preys.

Pancreas

In the exocrine pancreas, enzyme precursors are stored in the form of zymogen granules that were present throughout the development of herring larvae. The most studied pancreatic

enzymes are alpha amylase, involved in the hydrolysis of glycogen and starch (Lazo et al., 2011), and trypsin, considered as the most important proteolytic enzyme for fish early-life stages (Ronnestad et al., 2013). The expression pattern of these enzymes has been well described during larval development, with a lowering amylase activity, while there is an increase in trypsin activity (Zambonino Infante and Cahu, 2001). In the present study, we observed the decrease in amylase activity but trypsin activity remained unchanged during the two months of herring larval development. As trypsin intestinal activity is stimulated by food ingestion (Pedersen and Hjemeland, 1988), and given that we sampled in the morning before the first-feeding of the day, the stable value along the experiment might represent the basal trypsin content of larval herring. This suggests that herring larvae have an early functional exocrine pancreas, and that its maturation continues beyond 49 dph (18.5 $\pm$ 2.4 mm TL), as indicated by the decrease in amylase activity until 49 dph and the progressive change from a compact organ to a more elongate and diffuse one, like in herring adult.

Intestine

The intestine development generally corresponds to the maturation of enterocytes which is characterized by a sharp decrease in cytosolic enzymes activities in parallel to an increase in enzymes activities in brush border membranes (Zambonino Infante and Cahu 2001). These changes mark the beginning of the adult mode of digestion in fish larvae. In the present study, we assayed a set of intestinal enzymes belonging to these different enterocyte compartments. Leucine-alanine peptidase located in the cytosol, (Zambonino Infante et al., 2008) and aminopeptidase N located in the brush border (Govoni et al., 2015) ensure proteins digestion, while alkaline phosphatase is involved in the hydrolysis of phosphorylated proteins and may also be involved in amino-acids transport (Cara et al., 2003). In addition to playing a role in

nutrient transport and absorption, intestinal alkaline phosphatase is a key protective enzyme in fish involved in gastrointestinal health, limiting inflammation (Lallès, 2019). Ratios of brush border enzymes to cytosolic enzymes activities are largely used as indicators of intestinal maturation. In herring, the change from a larval to an adult mode of digestion in enterocytes occurred between 28 (15.0 $\pm$ 2.2 mm TL) and 35 dph (16.9 $\pm$ 2.0 mm TL), the size class and dph correspond to stage 3 larvae. The brush border structure was visible since the start of the development but was not mature until the end of the stage 3, at 35 dph when larvae were around 16 mm. In the meantime, the size of the intestine increased with more prominent villi. Stage 4 larvae were observed close to 35 dph, suggesting a synchronism between the physiological and the morphological developmental changes. As the larvae were pooled to assess enzyme activity our results reflect the general pattern of digestive maturation in herring larvae, and help into identifying the moment when most of the individuals have reached this particular developmental sequence. Further analysis using other techniques (e.g. radioimmunoassay or fluorescence technique) might be useful into assessing the degree of individual variability on the maturation of the digestive system linked to ontogeny.

1.4.2 Critical periods and implications for Downs herring recruitment

The critical period was defined by (Hjort 1914) as the major mortality event occurring during larval development if suitable environmental conditions are not met (Houde 2008) and was hypothesized to happen at transition from endogenous to exogenous feeding. In the present study, we observed that Downs herring larvae started to feed early and before the exhaustion of vitelline reserve. Moreover, liver of stages 1 and 2 larvae depicted large and numerous glycogen vacuoles showing that young herring larvae tend to promote energy storage when food is available. Glycogen plays an important role as cellular source of energy to be used later

on in the development (Furukawa et al., 2018) and to cope with environmental stress like changes in food availability (Gisbert and Sarasquete, 2008). Later on in the ontogeny, significant changes in liver reserves were observed. Heterogeneity in liver vacuoles size along with a decrease in glycogen was observed during stage 3 and 4, some larvae were found with small or no vacuoles. Glycogen was still present quite homogeneously but in lower quantity compared to smaller larvae and the general appearance of hepatocytes was less inflated.

Vacuoles depletion in the liver can be explained by the transition from stage 2 to stage 3 which entails a high energetic cost. Transition to stage 3 implies important morphological changes such as the development and growth of caudal and dorsal fins, as well as an increase in body height. Anatomically the organs of the digestive system also increased in size, all these changes likely being high energy demanding. These results are consistent with the decrease in growth rate observed for wild collected Downs herring larvae between 13 and 14 mm (Denis et al., 2017). Growth rate continues to decrease with the increase of larval length which may be indicative of a shift after 14 mm to a storage-oriented development strategy. Our results do not directly support this hypothesis since vacuoles in the liver were not more numerous in stage 4 larvae. These larvae are at the onset of metamorphosis, a challenging period where major physiological changes (e.g. increase in muscle development (Batty, 1984), muscle fibers recruitment and surface increased sharply after 25 mm in Clyde herring (Johnston et al., 1998)) also occur and require a huge amount of energy. While this could explain the depletion of liver vacuoles, change in the type of storage linked to a shift from a glycogen-based metabolism to a lipid-based one can be another hypothesis. Further analyses of larval lipid content and lipid class composition would be useful and complementary to disentangle and confirm energetic trade-offs between glycogen and lipids dynamics linked to larval ontogeny and nutritional condition.

For North Sea herring, the critical period in the wild was assessed to occur before 19 mm (Nash and Dickey-Collas, 2005) since abundance of larger larvae are used to estimate the recruitment index at one year and are well correlated with abundance of age 1 herring the year after (ICES, 2020). Then, rather that the transition from stage 1 to stage 2, the transition from stage 2 to 3 appears more as the critical period for North Sea herring. According to our results on the variation pattern of liver vacuoles and reserves during larval ontogeny, variability in the mortality rate experienced during this critical period may rely on the ability of larvae to make reserves before the transition, i.e. during stage 2. Stage 2 larvae in a favorable feeding environment could be able to store reserve while maintaining efficient growth, which would lead to a better potential of survival when passing to the next development stage. The use of liver reserve in stage 2 larvae as an indicator of potential of survival/mortality rate should then be investigated. Downs herring larvae, caught each year during the IBTS could be analysed using histology to estimate year to year differences in proportion of individuals with liver reserves. These results should be then confronted to annual variations in recruitment and environmental parameters. In a long term monitoring of larval herring condition, the use of the RNA/DNA ratio could be an easiest way, but since it does not directly inform on the reserve storage, and can differ from the histological index (Di Pane et al. 2019), it firstly required to be calibrated.

1.5 Conclusion

The combination of histological and histochemical techniques gave us significant insights on the ontogeny of the digestive system for Downs herring larvae. All the digestive organs were present at hatching, except for the stomach. Digestive accessory glands were even well

developed before mouth opening and containing an important amount of reserves (zymogen, glycogen). The use of biochemical measurements combined with histology was complementary and indicated not only the ontogeny of cell structures but also their functionality (i.e. brush borders of the enterocytes). The fluctuations in liver reserves quantities suggest that the transition from stage 2 to 3 and the end of the stage 4, at the onset of metamorphosis into juveniles, are highly demanding in energy. These results could serve as a reference for nutritional histological condition of herring larvae from field studies. These transitions can be perceived as critical or challenging periods for larvae that have to cope with a changing environment in the wild. Results suggest that only larvae that were able to store sufficient energy during the first feeding stage could pass these critical periods, develop and survive. These findings have a high level of implication to explain inter-annual fluctuations in Downs herring larvae survival and recruitment. Inter-annual variations in the level of energy storage (e.g. based on the evaluation of the amount of vacuoles in the liver) in first feeding stages should be examined and confronted to recruitment variability.

AXIS 2 – Assessing the combined effects of warming, acidification, and food quality on growth, development, condition and physiology of herring larvae

The aim of this axis is to investigate the potential impact of global change on herring larvae, their ability to cope with future predicted changes in the environment.

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CHAPTER 2 – Future environmental conditions affect herring larval development and physiology

The aim of this chapter is to assess the impact of warming, acidification and food quality on growth and development of herring larvae. As the stage 3 was identified as potentially critical in the Chapter 1, stage 3 larvae were sampled to conduct transcriptomics analyses and assess if the treatments led to different regulation at the transcriptional level of genes involved in metabolism (aerobic, lipid, glycogen) and stress response.

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2.1 Introduction

Anthropogenic activities have led to strong environmental modifications of the Earth system since the industrial revolution, leading to the name "The Anthropocene" for our era (Lewis and Maslin 2015), the era characterised by human-induced global changes. Among these environmental changes, warming is the driver expected to have the most significant impact on organisms (Barley et al., 2021; Huey and Kearney, 2020). In addition to warming, the continuous increase in CO_2 emissions have also led to ocean acidification (OA) (Doney et al. 2009). Independent of the emission reductions humanity manages to attain, these direct physico-chemical changes will continue. Additionally indirect effects could arise, such as changes in the quality or quantity of food sources for consumers. For example, increasing temperatures reduce the proportion of essential omega-3 polyunsaturated fatty acids (n-3 PUFA) in phytoplankton (Thompson et al. 1992; Guschina and Harwood 2009; Tocher 2010; Hixson and Arts 2016). Phytoplankton food quality is also directly influenced by dissolved nutrient availability, and dissolved phosphorus concentrations have steadily decreased in European coastal waters (Grizzetti et al. 2012), resulting in increasingly P-limited coastal systems (Sarker and Wiltshire 2017). Thus, both the quality as well as the quantity of planktonic food have changed, and will probably continue to change, with potential cascading effects through the food chain (Boersma et al. 2008; Jin et al. 2020).

As fisheries are an important part of the global food production (Béné et al. 2016), the potential decline in fish catch that could happen with global change (Cheung et al. 2011) raises socio-economical and food security concerns, especially for commercial species such as herring. While fish stock biomass fluctuations are affected by fishery intensity, environmental variations also play a major role as they modulate larval fish condition and associated

recruitment success (Rouyer et al. 2014; Polte et al. 2021). The larval stage of fish is typically characterized by high mortality, and represents a bottleneck period for many species (Houde, 2008). Hence, recruitment strongly depends on the survival and development of individuals during the larval period. Crucial morphological and physiological changes characterize the larval phase, such as organ morphogenesis and maturation of physiological functions (Zambonino-Infante et al. 2008). There is thus a strong need to understand how global change, and especially the interaction between warming and acidification on the one hand and prey quality on the other hand, affect larval fish survival and development.

Decades of experiments on fish larvae testing the influence of single stressors have contributed to the understanding of effects/responses toward environmental conditions. However, these are insufficient to reflect the complexity of simultaneously changing stressors in the natural environment, and the resulting organism's responses (Baumann 2019). This lack of integration is best represented by contrasting results which vary from null or beneficial (Crespel et al. 2017; Frommel et al. 2019; McMahon et al. 2020) to negative impacts of environmental conditions (acidification, warming, food supply) on growth and survival (Baumann et al. 2011; Frommel et al. 2016; Gobler et al. 2018). These depend on the species, the response variable, and the level of temperature and pH changes investigated. This becomes particularly clear when comparing the results of Frommel and co-authors (2014), who described strong acidification-induced organ damage, especially of the liver of larval herring, with those of Sswat and co-authors (2018), who reported a positive impact of acidification on survival of larval herring. The main difference between those studies was that Frommel and colleagues used unrealistically high pCO_2 levels in a single species set-up, whereas Sswat and co-authors investigated food web effects, using realistic scenarios in mesocosms.

North Sea Atlantic herring (*Clupea harengus*) is an ecologically and socio-economically important fish species, with highly variable recruitment success (Payne et al. 2013). Assessing how predicted co-exposure of warming, acidification and change in food quality could affect development and metabolism early in life, and subsequently the sustainability of fish population, is of crucial interest. Therefore, the first aim of the present study was to investigate the influence of global change drivers (temperature, pH, food quality) on growth in terms on total length in function of time, and development in terms of morphological changes (notochord flexion and fins development) of herring larvae. The focus was made on these two criteria as temperature alone differentially increased growth rate and development rate on herring larvae (Baltic herring, Moyano *et al.*, 2016), and combined effect of warming and acidification had no impact on these rates (Atlantic herring, Sswat *et al.*, 2018). Then the second aim was to assess the underlying mechanisms of herring larval response at the transcriptional level and identified potential coping mechanism or metabolism disruption. We investigated changes in the expression of genes linked to metabolism (aerobic, lipid and glycogen) and stress response, expecting differential regulation of genes involved in metabolism between treatments and higher level of heat shock proteins transcripts in the 2100 scenarios.

2.2 Materials and Methods

2.2.1 Strip-spawning and eggs incubation

North Sea herring comprises different spawning components, with different spawning times and locations (Geffen, 2009). In this study, we focused on the Downs winter component of the population, which spawns in the English Channel in November-December. Mature wild Downs

herring were collected from local fishermen (CME, Coopérative Maritime Etaploise, Boulogne-Sur-Mer, France, 22nd of November, 2019). Females (n= 324, mean length: 25.8 ± 2 cm; mean weight: 163.8 ± 44.7 g) and males (n= 221, mean length: 25.7 ± 2 cm; mean weight: 163.5 ± 43.4 g) were strip-spawned on PVC plates. To maximize genetic diversity, the eggs and the sperm of at least ten females and five males were mixed on one PVC plate and incubated for 10 minutes in natural filtered seawater (11°C, natural pH). A total of 30 PVC plates were prepared and incubated for 48 hours in 70 litres tanks (13°C, natural pH). The eggs were then transferred to the Ifremer Centre de Bretagne Laboratory (agreement number: B29-212-05), equipped for long-term experiment, and incubated in 200 litres tanks (10.6°C, natural pH). For the whole experiment, we used natural seawater, which was pumped from the Bay of Brest (France, 20m depth, 32.5 salinity, pH8.0, pCO_2 ~500 µatm in winter (Bozec et al. 2011)), filtered through sand, temperated, degassed, filtered through a membrane (mesh: 2 µm), and finally UV-sterilized (PZ50, 75W, Ocene, France). After 11 days of incubation, 21 000 larvae were randomly distributed over twelve 38 litres flow-through tanks. The experiment followed the French national regulations and was authorized by the Regional Ethics Committee (authorization number: 22555 – 2019102319251011).

2.2.2 Experimental design

The twelve experimental tanks were divided into four treatments with three replicates each. Two different physical environments were tested. Since global change affects both, temperature as well as pH, and the aim of this study was to provide an integrated prediction of future conditions, we chose to combine OA and temperature effects, following IPCC scenarios. The role of the first physical environment (Control) was to represent as best as possible the current physical conditions met by the larvae in the field during their hatching

period (November-December). Temperature data available from the International Council for the Exploration of the Sea (ICES), for these months and the geographic zone of interest (ICES, IVc and VIId), showed a mean temperature of 10.2 ± 2.3°C (standard deviation) over 95 years of data collected between 1893 and 2007 (ICES data portal, Dataset on Ocean HydroChemistry, Extracted November 5, 2021. ICES, Copenhagen). Hence, the control treatment had a constant temperature set at 11°C (due to infrastructure constraints) associated with natural seawater pH of 8.0. The second treatment (Ocean Warming and Acidification; OWA) corresponded to the RCP 8.5 (Representative Concentration Pathway, IPCC 2014) scenario (Δ+3°C, Δ-0.4 pH units) thus yielding a temperature of 14°C and a pH of 7.6. These different environmental treatments were applied to 6 tanks each. To reach the targeted temperature and pH of the OWA environment, the seawater temperature was progressively increased from 11 to 14°C over 48 hours, and each tank was supplied with water by a header tank (200 litres) where CO_2 was bubbled through the water column via a gas diffuser. CO_2 diffusion was manually controlled by a flow meter and bubble counter to adjust the pH at 7.6. Temperature and pH were manually controlled two to three times a day (WTW 330i, Xylem Analytics Germany). Total Alkalinity (TA) was measured once a week according to the protocol of Anderson and Robinson (1946), and Strickland and Parsons (1972). The excel macro CO2sys (Lewis and Wallace 1998) was used to calculate pCO_2 from water chemistry parameters (Mehrbach et al. (1973) refit by Dickson and co-authors (2007) constant). Oxygen saturation (WTW Oxi 340, Xylem Analytics Germany) and salinity (WTW LF325, Xylem Analytics Germany) were measured once a week. Oxygen saturation was above 88% during the whole experiment. The mean values of abiotic conditions in the experimental tanks are shown in Table 4. The larvae were reared under a 10:14h Light:Dark cycle, with the light dimmed at dusk and dawn.

Table 4 - Environmental parameters (mean ⎕ SD) of temperature (T, °C), pH, Salinity, Total Alkalinity (TA, µmol/kgSW) and pCO2 (µatm). "+" notify treatments enriched in phosphorus and docosahexaenoic acid.

Treatments	**T (°C)**	**pH**	**Salinity**	**TA (µmol/kgSW)**	**pCO_2 (µatm)**
Control (11°C, pH 8.0)	11.6 ± 0.4	8.03 ± 0.01	32.7 ± 0.4	2277.0 ± 117.7	562.9 ± 53.2
Control+ (11°C, pH 8.0)	11.3 ± 0.4	8.03 ± 0.05	32.7 ± 0.4	2273.3 ± 161.5	555.3 ± 59.7
OWA (14°C, pH 7.6)	14.3 ± 0.5	7.63 ± 0.11	32.7 ± 0.4	2271.8 ± 279.3	1549.3 ± 279.3
OWA+ (14°C, pH 7.6)	14.1 ± 0.4	7.59 ± 0.10	32.7 ± 0.4	2288.9 ± 185.9	1773.8 ± 264.6

Given the decrease in P-loads into the North Sea, and the predicted changes in the availability of essential fatty acids, both as a result of warming as well as caused by the changing availability in phosphorus, the quality of the available food for larval fish is likely to strongly change in future. To test the consequences of these changes in a current and future physical environment, the environmental treatments (Control vs OWA) were crossed with two different integrated feeding treatments (Figure 11) to assess if food quality could compensate or exacerbate abiotic effects. As temperature is a driver of metabolic rate and can influence growth and development rate, we planned the feeding sequences and sampling using the Cumulative Degree Days (CDD) approach (Chezik et al. 2014), representing the cumulative sum of average temperature encountered per day by the larvae since hatching (0 CDD). CDD represent the thermal opportunity met by individuals. Despite this, CDD as a measure is rarely used in fish and fisheries studies (Neuheimer and Taggart 2007). This method, however, provides a physiologically relevant temperature metric (Neuheimer and Grønkjær 2012) to compare individuals reared at different temperatures (Trudgill et al. 2005). In our experiment, 0 CDD corresponds to the hatching and 35 CDD is the mean sampling point before the

distribution of larvae in the different conditions (2 and 3 dph), the starting point of the experiment.

Based on the validated feeding sequence for rearing Downs herring larvae with increasing prey

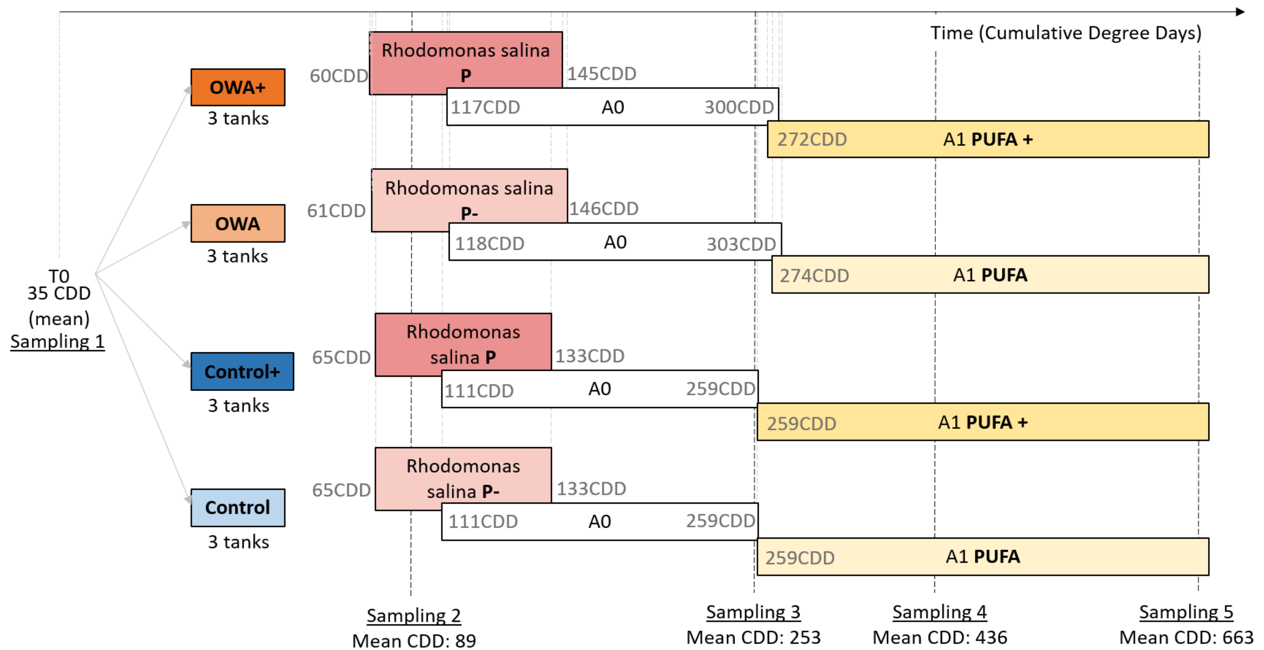

Figure 11 - Feeding sequences and sampling points for herring larvae in four treatments in Cumulative Degree Days (CDD). OWA-OWA+ (Ocean Warming and Acidification), an environmental treatment at 14°C and pH7.6 and Control-Control+ an environment at 11°C and pH8.0. Three type of prey were supplied to the larvae: phytoplankton (R. salina) cultured under different phosphorus quantities (P / P-), freshly hatched Artemia nauplii (A0), and 24 hours-old Artemia nauplii (A1) enriched or not in polyunsaturated fatty acids DHA (Docosahexaenoic Acid, PUFA / PUFA+).

size (Joly et al. 2021), all larvae were fed ad libitum with a succession of Rhodomonas salina, freshly hatched Artemia nauplii and 24-hours old enriched Artemia (Figure 11), supply in the tanks between 8 am and 5 pm. Rhodomonas salina (Strain 2002, University of Göttingen, Germany) were grown under constant light in 10-litre bottles in enriched Conway*2 medium (double quantities of N and P) and maintained at a concentration of 1 Million cells.mL^{-1}. For the nutrient-poor treatment (Rhodomonas salina P-, Fig 1), a quarter of the cells grown in the previous medium were isolated and grown 48 hours in a modified enriched Conway*2 medium without phosphorus, enabling the manipulation of algal stoichiometry within a

realistic range observed in natural systems (Meunier et al. 2016). The phytoplankton was supplied into the rearing tanks between 60 CDD, when the mouth was opened but the yolk-sac still present, and 146 CDD. Freshly hatched Artemia nauplii A0 (VNBS, Viepearl) were incubated 24 hours at 28°C, in 60-litre tanks, and supplied to the larvae between 110 and 303 CDD. Artemia nauplii A1 were either enriched 24 hours with a diet (Larviva Multigrain, Biomar) containing a high content of DHA, a polyunsaturated fatty acid (22:6n-3, Docosahexaenoic Acid; +2 %) for the PUFA+ treatment, or in a mixture of fish oil and baker yeast for the PUFA treatment. A1 Artemia were supplied to the tanks from 259 CDD to the end of the experiment. Larvae were reared from hatching to the beginning of the stage 4 (post-flexion stage), corresponding to 47 dph at 14°C and 60 dph at 11°C (around 660 CDD). Tanks were cleaned every two days and mortality was regularly checked, the number of dead larvae was only quantified at high mortality events.

2.2.3 Sampling

Total length (mm) and developmental stages were measured and observed at 5 points during the experiment. The developmental stage characterizations were based on Doyle's classification (1977) and modified for rapid identification under binocular (see Figure 12).

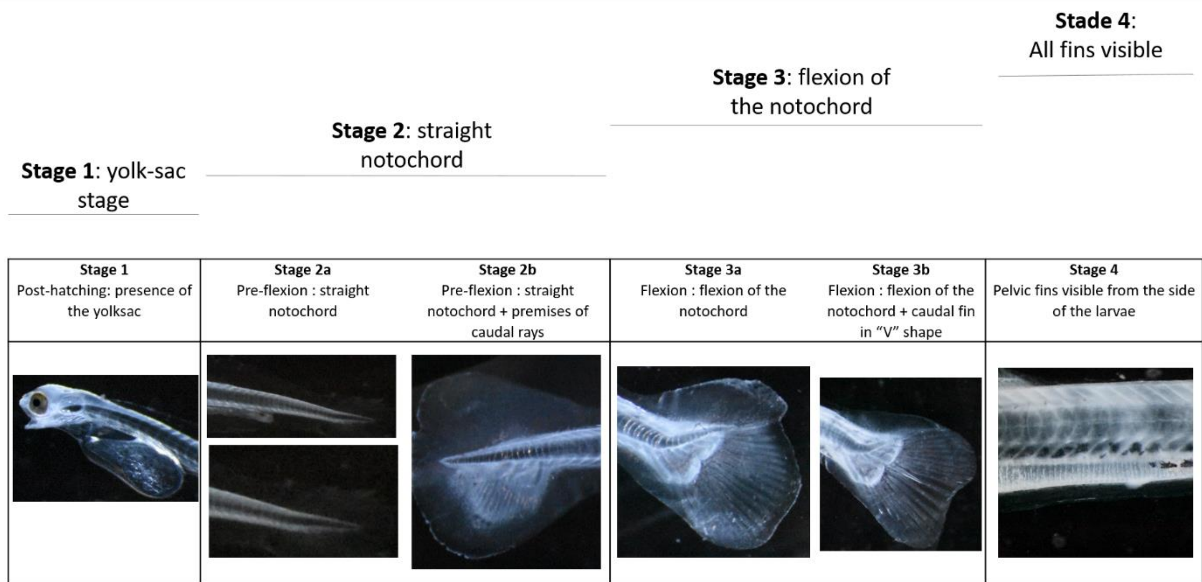

Figure 12 - Illustration of herring developmental stages and sub-stages.

The five sampling points were defined as follows to sample key stages, with a different number of larvae sampled and euthanized in ice-water (between 21 and 96 larvae for each tank) depending on biomass requirements for further analyses. At sampling 1 (T0) 35 larvae were collected between 2-3 dph to obtain the mean size before tank transferring. Sampling 2 was made to target stage 1 and 2A at 89 CDD (~20 larvae by tank). Sampling 3 was performed to sample mostly stage 2B at 253 CDD (~60 larvae by tank), Sampling 4 for stage 3A at 436 CDD (~20 larvae by tank) and Sampling 5 for stage 3B and 4 at 663 CDD (~90 larvae by tank).

The beginning of the stage 3 was previously identified as a potential critical period for Downs herring larvae (Joly et al. 2021), with depletion of energetic reserve even with *ad libitum* feeding. Thus, we focused on this stage at the 4th sampling point (~436 CDD) to investigate metabolism disruption or compensatory mechanisms to cope with global changes at the molecular level. Seven larvae were sampled by tank, stored in RNA later and kept at -20°C to preserve RNAs.

2.2.4 Gene expression analysis

The aim was to have an overview of the regulation that might occur in herring larvae facing changes in temperature, pH and food quality, focusing on pathways linked with energy metabolism and focusing on genes for which cDNA sequences were available in NCBI database. The gene regulation of CS, Idh1 and Idh2, key enzymes in the Krebs cycle, can reflect modification in aerobic potential, a decrease in these gene expressions could be linked with acid-base regulatory imbalance (Pimentel et al. 2019). Global change and stress response in marine organism could have two different consequences on the energy reserve dynamics. On the one hand an increase in energy expenditure (Araújo et al. 2018; Wang et al. 2020) that could be revealed by a decrease in expression of genes involved in lipid anabolism (Dgat2, Fasn) as well as glycogen synthesis (Gys2), representing energy storage, along with an increase in lipid catabolism (Lipe, Pnpla2). On the other hand, an increase in Fasn could reveal lipid accumulation to cope with stress (Strader et al. 2020) or disruption of the lipid metabolism and abnormal lipid accumulation as found in several acidification and pollution experiments on fish larvae (Frommel et al. 2012a, 2014; Sun et al. 2019). Growth Hormones (Igf1-x1, IgfII-x1, IgfII-x2) expression were also investigated to be compared to larval growth rates and assess if an increased in growth rate generally associated with higher temperature can be observed at the transcriptional level (Madeira et al. 2016). Finally heat shock protein expression (SerpinH1-like1, SerpinH1-x1) were assessed to determine if the treatments were sources of stress for the larvae. Under stress, Heat Shock Proteins (HSPs) are synthesized and function as molecular chaperones, involved in the maintenance of protein homeostasis (Wickner et al. 1999), they prevent protein aggregation and apoptosis (Roberts et al. 2010). Among sampled larvae, between 2 to 3 Individuals only in stage 3A or 3B were pooled by treatment, replicate and by stage, to obtain a minimum wet weight of 30 mg to facilitate RNA

extraction. Between 2-5 pools where available for each treatment. Total RNA was extracted from pooled samples using NucleoSpin RNA® kit (Macherey-Nagel, Germany). RNA concentration and purity were assessed by spectrophotometry using NanoDrop 2000 (ThermoScientific, USA) and RNA quality with Bioanalyseur 2100 (Agilent Technologie Inc, USA). Relative levels of mRNA expressions were quantified by RT-qPCR analysis. The RNA extracted by treatment were reversed-transcribed in cDNA using iScript® cDNA Synthesis kit (Bio-Rad Laboratories, USA). QPCR was used to quantify the relative levels of 16 transcripts involved in different metabolic pathways (Table 5). The specific primers for each gene were designed with Primer 3 plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) from sequences in the NCBI database. Each biological pooled sample was analyzed in technical triplicates using iQ SYBR® Green Supermix (Bio-Rad Laboratories, USA). The relative quantities of transcripts in larvae were normalized with the ΔΔCt method using the CFX-manager software associated to the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories Inc.). Initially EF1a, Rpl13 and Actin were selected as control gene for normalization of gene expression, but only Rpl13 and Actin expression were used as reference genes since their expression profiles were stable among samples (coefficient of variation and expression stability M values lower than 25% and 0.5, respectively.)

Table 5 - Presentation of the selected genes for investigation, the biological process their associated with and primer specification for real-time qPCR

Abbreviation	Full name	Function / biological process	Reference (NCBI database)	Primer sequence 5’-3’
EF1a	Elongation factor 1-alpha	Elongation factor Protein biosynthesis	XM_012840422.2	F: AGTACCCTCCACTGGGTCG R: GTGGAGTTGGGTGACCTCTG
CS	Citrate synthase	Krebs cycle	XM_012824897.2	F: *TTGCGCCGAAGATCCTGAAT* R: *TGCCACCATACACCATGTCG*
Idh1	Isocitrate dehydrogenase 1	Krebs cycle	XM_012819824.2	F: TCCACTAACCCCATTGCCTC R: CCCCTTGATACAAGCTGCCA
Idh2	Isocitrate dehydrogenase 2	Krebs cycle	XM_012833616.2	F: CACTGTCTTCCGTGAGCCAA R: CTGTTGCTTTGTACTGGTCGC
Dgat2	Diacylglycerol O-acyltransferase 2	Triacylglycerol biosynthesis	XM_012827712	F: TACTTCCCCATCCGGCTCAT R: ATCCGGAAATTCCCAGCCAG
Fasn	Fatty acid synthase	Fatty acid biosynthesis	XM_012814622.2	F: ATCATCACTGGTGGCCTTGG R: TAGCTTGGTATCCGTTGCGG
Lipe	Hormone-sensitive lipase	Triacylglycerol degradation	XM_012839365.2	F: CACCAGTCTGGCATAGGAGT R: AGCTCAGGGTCTATGGCGTA
Pnpla2	Patatin-like phospholipase domain containing 2	Triacylglycerol degradation	XM_012840949.2	F: TCTGATCCAGTCGCTAGGCA R: CCACATGGTACGAGAAACGTG
Gys2	Glycogen synthase 2	Glycogen biosynthesis	XM_012824488.2	F: CTGCACAGGAACCCAGATGT R: GGACAAACTCCTGGATGCGA
Igf1-x1	Insulin-like growth factor 1, transcript variant x1	Growth factor	XM_012830244.2	F: CCTGCGCAATGGAACAAAGT R: GACAGCACATGGTACACTTGA
IgfII-x1	Insulin-like growth factor II, transcript variant x1	Growth factor	XM_031564361.1	F: GCTGAAATCAGAGTGATGTCCT R: GCCGGTCGGTCTACTGAAG

IgfII-x2	Insulin-like growth factor II, transcript variant x2	Growth factor	XM_012832374.2	F: CGCAGCACAAACAAGGCTAC R: TGCCGGTCGGTCTACTGAAG
SerpinH1-like1	Heat Shock Protein 47 - Serpin1a	Chaperone Stress response	XM_012832341.2	F: *AGCATAGTGCGGTGAACTCC* R: *GAAAGCCATAGATGCCAGTGC*
SerpinH1-x1	Heat Shock Protein 47 - Serpin1b	Chaperone Stress response	XM_031573303.1	F: *ATTGTTCTCGAGACATCCGC* R: *TTGCCACGTTGTGGTACAGG*
Rpl13	Ribosomal protein L13	House-keeping gene	XM_012817263.2	F: CATGGCCCCCAGTAGGAATG R: CGAGCCTTATGTCTGCGTTG
Actin	Actin cytoplasmic 1	House-keeping gene	XM_012839274.2	F: TCAGCGCTCCTAATCCCAAA R: CCACCATCACACCCTGATGTC

2.2.5 Statistical analysis

Growth rates of larvae in each treatment were estimated by linear regression between T0 and the following sampling points. A comparison of slopes was used to determine differences in larval growth rate between treatments using day post hatch as covariable. Due to a mistake in the feeding treatment for one tank in the OWA treatment, all the larvae from this tank were excluded from the study. Then, Linear Mixed Effect Models (LMEM) were used to test the influence of temperature and pH (Environment; Enviro in the formula), levels of nutrient availability (Food) and their interaction on (1) Total Length (TL) over time in function of the Cumulative Degree Days, (2) Total Length at each sampling point, and (3) levels of Gene expressions (normalised relative quantities). Enviro and Food were used as fixed factor with two levels corresponding to the crossed treatments. Stage and tank effects were included as random effects.

Effect on total length over time in CDD:

(1) TL ~ CDD + Enviro + Food + Enviro:Food + CDD:Enviro + *(CDD|tank)*

Effect on total length at each sampling date:

(2) TL_{M_DD} ~ Enviro + Food + Enviro:Food + *(1|tank)*

Effect on gene relative differential expressions:

(3) Gene(X) ~ Enviro + Food + Enviro:Food + *(1|tank) + (1|stage)*

Homoscedasticity of the residuals was graphically verified for all models. Statistical analyses were performed in R using the package 'lmerTest' (Kuznetsova et al. 2017).

2.3 Results

2.3.1 Larval survival, growth and development

We observed a mortality event in all the tanks at 44 dph for the OWA and OWA+ treatments and at 57 dph for the Control and Control+. It is important to note that these events occurred roughly at the same developmental-temperature corrected time (610-630 CDD), with respectively 201, 236, 139 and 254 dead individuals for OWA, OWA+, Control and Control+. For all treatments a sub-sample of these dead larvae was used to characterise their developmental, which revealed that between 94 and 99% were in stage 3B.

The slopes used to compute growth rates were similar in all treatments (ANCOVA, dfd = 2341, dfn = 4, F = 2.07, p = 0.083) with an average growth around 0.22 $mm.d^{-1}$ (general linear equation: 8.32 + 0.22*dph, R^2 adjusted = 0.76, n = 2351).

Table 6 - *Results of a mixed-effects model testing for the effect of environmental treatment (Enviro, 11°C*pH8.0 / 14°C*pH*7.6), food quality (enriched in phosphorus and docosahexaenoic acid / not enriched), time in Cumulative Degree Days (CDD), and their interactions on herring larvae size.*

Aim	**Response variable**	**Explanatory variables**	χ^2	**df**	**p-value**
Fish growth over time in cumulative degree days	TL (mm)	CDD	1435.92	1	<0.001*
		Enviro	4.16	1	0.041*
		Food	1.65	1	0.198
		Enviro*Food	0.12	1	0.727
		CDD*Enviro	4.17	1	0.042*

*Table 7 - Results of mixed-effects models testing for the effect of environmental treatment (Enviro, 11°C*pH8.0 / 14°C*pH*7.6), food quality (enriched in phosphorus and docosahexaenoic acid / not enriched) and their interactions on herring larvae size at four sampling points*

Aim	Response variable	Explanatory variables	X^2	df	p-value
Size at sampling point (mean cumulative degree days)	TL_{89_DD}	Enviro	2.10	1	0.147
		Food	0.08	1	0.773
		Enviro:Food	0.03	1	0.861
	TL_{253_DD}	Enviro	0.01	1	0.905
		Food	3.38	1	0.066
		Enviro:Food	0.06	1	0.802
	TL_{436_DD}	Enviro	21.71	1	<0.001*
		Food	0.12	1	0.727
		Enviro:Food	0.52	1	0.471
	TL_{663_DD}	Enviro	14.19	1	<0.001*
		Food	0.80	1	0.369
		Enviro:Food	0.79	1	0.373

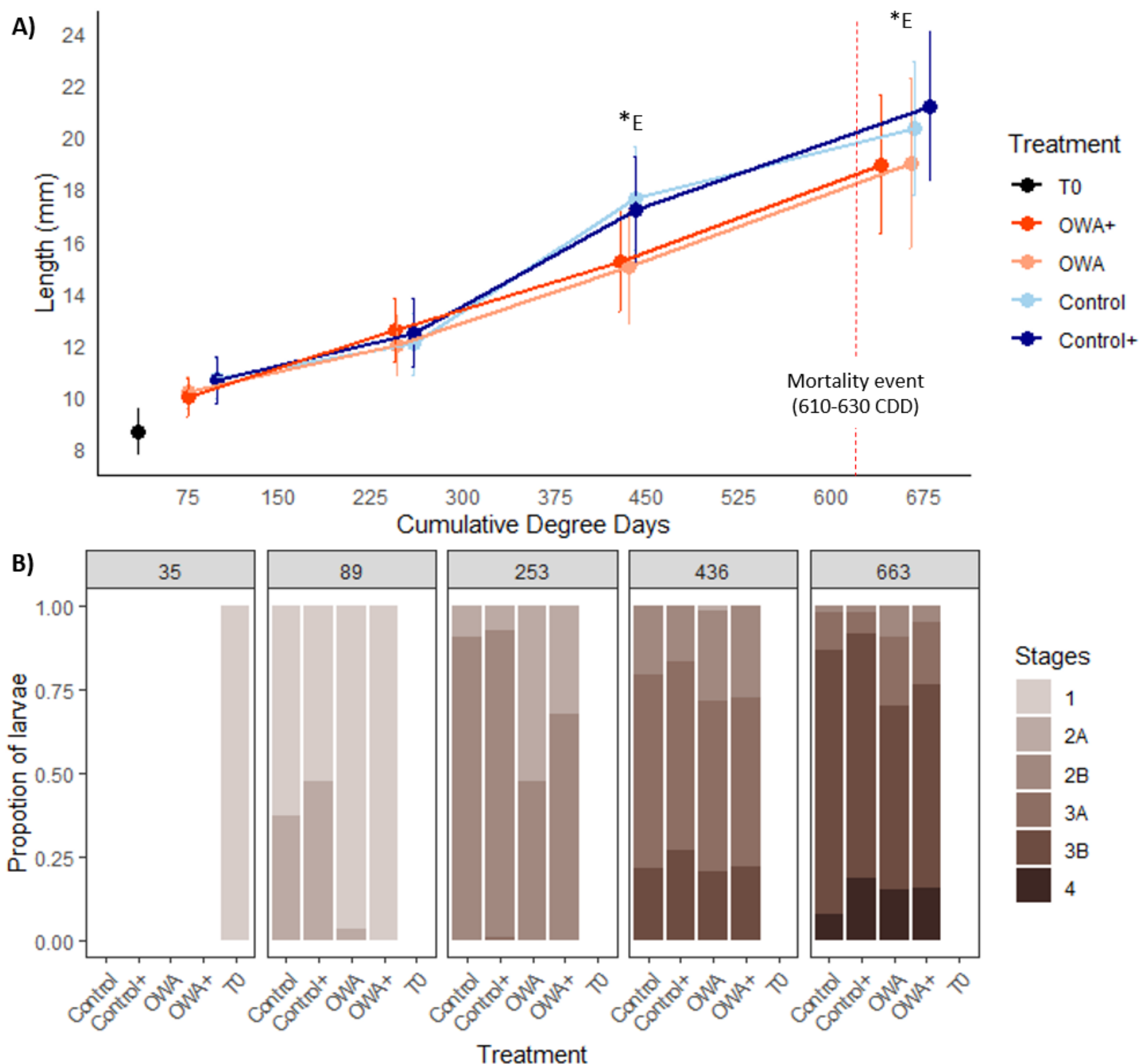

Figure 13 - Size variation (A) and stage proportions (B) of herring larvae sampled at five mean sampling points (in Degree days), in four experimental treatments. In blue are represented the larvae reared at 11°C and pH 8.0 (Control), in orange the ones reared at 14°C and pH 7.6 (OWA). The "+" represents an enriched diet in phosphorus and docosahexaenoic acid. The (*) followed by the letter "E" denotes significant differences based on a LMEM model due to the environmental treatment). T0 is the sampling point before transferring the larvae in the different treatments. The red dotted line notifies a mortality event common to all treatments.

When comparing size (total length) as a function of the cumulative degree days (CDD), or thermal time, the environment was responsible for a significant difference in size in herring larvae (Table 6). The difference in size was not significant early in the experiment (Table 7), at 89 and 253 CDD. For 436 and 663 CDD herring larvae were smaller in warming and acidification treatment (Table 7, Figure. 13.A) for the same thermal time. At 89 and 253 CDD more larvae in Control-Control+ treatments were in an advanced developmental stage than in OWA-OWA+

(Figure 13.B). At 89 CDD between 38.2% to 45.6% of larvae were in stage 2A in the Control-Control+, treatments whereas only 0% to 4.5% of larvae were in this same stage for OWA-OWA+. Similarly, at 253 CDD most of the larvae were in stage 2B for Control-Control+ (89.4%-90.4%) against 49.2%-68.2% for OWA-OWA+. At 436 and 663 CDD the proportion of stage were similar between treatments with mainly stage 3A at 436 CDD (between 50-57 % for OWA and Control) and stage 3B at 663 CDD (between 55-79% for OWA and Control). Larvae reached a mean size of 15.1 ± 2.02 mm at 436 CDD and 19.1 ± 3.0 mm at 663 CDD in OWA environment versus 17.4 ± 2.02 mm and 20.9 ± 2.77 respectively at the same CDD in Control environment. Larvae started to reach the post-flexion stage (enter in stage 4) at 22 mm in Control and 21 mm in control+ whereas it was at 18 mm for OWA and OWA+

2.3.2 Gene expression profiles

*Table 8 - Results of mixed-effects models testing for the effect of environmental treatment (Enviro, 11°C*pH8.0 / 14°C*pH*7.6), food quality (enriched in phosphorus and docosahexaenoic acid / not enriched) and their interactions on the level of gene expressions of herring larvae size at 436 cumulative degree days*

Aim	Response variable	Explanatory variables	χ^2	df	p-value
Gene expression	EF1a	Enviro	6.29	1	0.012*
		Food	0.02	1	0.886
		Enviro:Food	0.03	1	0.853
	CS	Enviro	1.27	1	0.260
		Food	0.63	1	0.427
		Enviro:Food	1.12	1	0.289
	Idh1	Enviro	8.76	1	0.003*
		Food	1.92	1	0.166
		Enviro:Food	0.06	1	0.809
	Idh2	Enviro	1.65	1	0.003*
		Food	4.76	1	0.030*
		Enviro:Food	0.03	1	0.869
	Dgat2	Enviro	0.40	1	0.527
		Food	0.01	1	0.997
		Enviro:Food	0.10	1	0.746
	Fasn	Enviro	1.23	1	0.267
		Food	0.11	1	0.742
		Enviro:Food	0.12	1	0.730

Lipe	Enviro	1.17	1	0.278
	Food	0.18	1	0.671
	Enviro:Food	0.34	1	0.560
Pnpla2	Enviro	1.81	1	0.178
	Food	3.83	1	0.050
	Enviro:Food	0.08	1	0.774
Gys2	Enviro	1.52	1	0.218
	Food	0.64	1	0.422
	Enviro:Food	0.01	1	0.903
Igf1-x1	Enviro	0.59	1	0.443
	Food	1.60	1	0.206
	Enviro:Food	0.77	1	0.381
IgfII-x1	Enviro	0.63	1	0.426
	Food	6.72	1	0.009*
	Enviro:Food	0.01	1	0.981
IgfII-x2	Enviro	5.90	1	0.015*
	Food	1.00	1	0.317
	Enviro:Food	0.29	1	0.597
SerpinH1-like1	Enviro	7.76	1	0.005*
	Food	0.68	1	0.409
	Enviro:Food	0.42	1	0.515
SerpinH1-x1	Enviro	457.10	1	<0.001*
	Food	2.44	1	0.118
	Enviro:Food	3.23	1	0.072

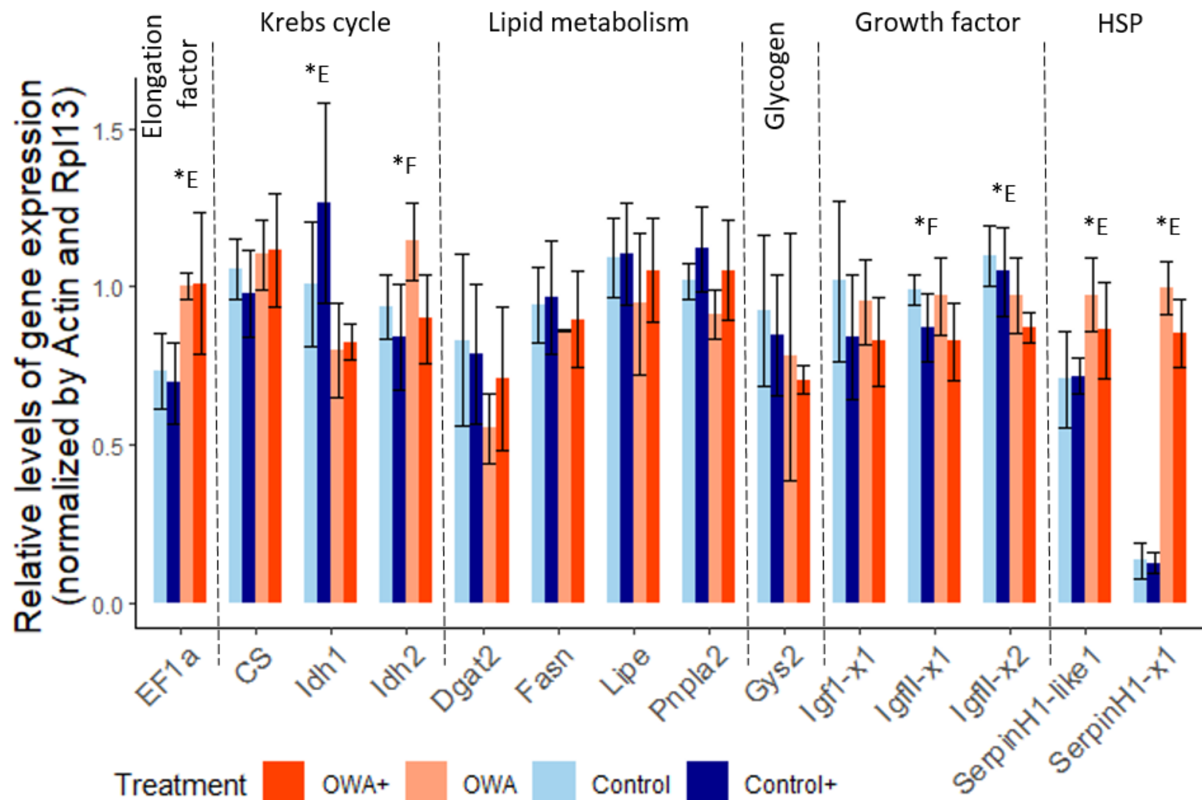

Figure 14 - Gene expression profiles in four experimental treatments. In blue are represented the larvae reared at 11°C and pH 8.0 (Control-Control+), in orange the ones reared at 14°C and pH 7.6 (OWA-OWA+). The "+" represents an enriched diet phosphorus and docosahexaenoic acid. The (*) marks a difference due to "E" Environment treatment and "F" Food treatment

Mixed-effects models revealed that the expression of 7 genes out of 14 were differentially regulated between treatments (Table 8). The regulation was low for six of them in comparison to the control environment (Figure 14), with a difference inferior at 0.5 relative level of expression. The elongation factor EF1a, initially selected to be a reference gene had a higher expression in OWA and OWA+ treatments (Figure 14). Among the genes playing a role in the Krebs cycle, idh1 was higher in Control environment and idh2 in environment with the non-enriched diet. Within lipid and glycogen metabolism, none of the expression of the genes tested were modified. The expression of growth factors IgfII-x1 and IgfII-x2 were respectively higher in non-enriched diet treatment and higher in Control environment. The expression of SerpinH1-like1 was higher in OWA environment. The main difference was for the SerpinH1-x1

(heat shock protein 47, serpin1b) which displayed almost a 7-fold increase in the OWA environment in comparison to the Control one.

2.4 Discussion

In the present study, we found no direct lethal effects of combined warming and acidification on Downs herring larvae, independent of the nutritional quality of the food supply. While the food quality treatments we used did not influence larval fish fitness, we identified physiological disturbances through induction of stress-response indicators and smaller larval size-at-stage caused by warming and acidification, which occurred at the beginning of stage 3 which is a potential critical period for Downs herring larvae (Joly et al. 2021).

2.4.1 Warming and acidification: a stressful environment for herring larvae

The majority of the level expression of genes involved in the energetic metabolism were either low or not differentially regulated for the selected stage 3 larvae. Growth hormones expression and growth rate were not stimulated by the increase in temperature. Genes involved in Krebs cycle were largely unaffected by the treatments, suggesting no transcriptional modification of the aerobic metabolism of the larvae. Lipid and glycogen metabolism were unaffected by warming, acidification and food quality thus meaning that the energetic metabolism was not adjusted at the transcriptional level to cope with the stressors, in contrast for what has been shown for other fish larvae (Frommel et al. 2012a, 2014; Sun et al. 2019; Strader et al. 2020). For example gene expressions of citrate synthase, glycogen synthase 2, fatty acid synthase, and growth hormone were regulated at the transcriptomic level for Atlantic cod under acidification treatment more severe than the one we used (Frommel et al. 2020), the lipid metabolism disruption highlighted in the study was associated with organ damages (Frommel et al. 2012). The up-regulation of the elongation factor EF1a

during our experiment was an unexpected result as it is commonly used and recommended as reference gene for fish (Urbatzka et al. 2013; Cordero et al. 2016; Raposo de Magalhães et al. 2021), and have been already used for Pacific herring (Incardona et al. 2015, 2021). This elongation factor was also found to be upregulated in bivalves exposed to copper (Zapata et al. 2009), hypoxia (David et al. 2005) or low salinity (Jones et al. 2019). The relation with environmental stressor is unclear but might be associated with its role in protein regulation and biosynthesis. This reveals that the EF1a gene should be taken with caution when used as a reference gene in studies investigating transcriptomics regulation due to warming and acidification in fish larvae. Indeed, we did not find metabolism disruption for herring larvae and the major result was the strong adjustment of heat shock protein 47 (Hsp47, serpin1b), with higher gene expression in OWA environment. The gene expression profiles gave an assessment of the long-term (e.g. chronic, > 4 weeks) response to the OWA treatment. It represented the physiological adjustment made by the larvae after the initial stress response, with potential fitness consequences (Oomen and Hutchings 2017). Hsp47 belongs to the family of low molecular weight heat shock proteins (Basu et al. 2002), which are mainly induced during stress (Ciocca et al. 1993). HSPs are not only activated for heat tolerance and thermal acclimation (Mahanty et al. 2017) but also as protection towards other stressors, such as acidification (Mittermayer et al. 2019), or pollutants (Mitra et al. 2018). Increase in hsp47 transcript was found to play a key role in survival of a species of minnow encountering natural long-term exposure to a warmer environment (Mahanty et al. 2017). Coping with stress requires the synthesis of Hsp proteins, which represents an energetic cost (Harianto et al. 2018) that adds to the overall cost necessary for the development and survival of the species, possibly inducing energetic trade-offs (Somero, 2012).

2.4.2 Reaching stage 4 at smaller size: a trade-off in energy allocation?

The effect of temperature on body size and larval phase duration is a well-known process and has been demonstrated for herring (Johnston et al. 2001; Moyano et al. 2016). Temperature impact on growth as size as a function of time, and of development rate, influencing the larval stage duration (i.e time to go through the complete morphological and anatomical changes), are decoupled. Development generally has a stronger temperature dependence (Forster et al. 2011; Kamiński et al. 2013), reducing the larval phase duration and the final larval size at a same developmental age (Green and Fisher 2004). Here, even if warming and acidification did not impact growth rate, this treatment induced a developmental acceleration as larvae reached the post-flexion stage at different time, at 47 dph at 14°C*pH7.6 against 60 dph at 11°C*pH8.0, resulting in smaller larvae in OWA environment. Separately, warming has been shown to increase larval growth rate for Baltic herring (Moyano et al. 2016), and acidification to reduce size-at-stage in Atlantic herring (Frommel et al. 2014). Sswat and co-authors (2018) showed no effect of combined warming and acidification on growth and development rate of Atlantic herring. Reduction in body size with increasing temperature is a global phenomenon observed for different species from endotherms to ectotherms (Gardner et al. 2011), and especially important for ectotherm aquatic organisms (Audzijonyte et al. 2018). Oxygen limitation has been put forward as a key mechanism to explain a reduction of fish size in warming waters (Pauly 1981; Pörtner et al. 2017) but this theory is widely debated (Lefevre et al. 2017; Jutfelt et al. 2018), and the underlying mechanisms not known. Here, we pose that the decrease in size-at-stage was caused by a differential increase in development rate compared to growth rate (Verberk et al. 2021). As temperature directly affects the metabolic rates of ectotherms (Gillooly et al. 2001), warming likely increases metabolic energy demands. Fish larvae mainly allocate their energy resources towards physiological maintenance and

growth; however, if energy acquisition does not match the increased demand for both, the energy allocated towards growth could be reduced (Sheridan and Bickford 2011). The energy budget that determines growth of organisms is shaped by resource availability like food and oxygen (Verberk et al. 2021), which were not limiting factors in our experiment. Energy intake and assimilation are limited in organisms, and energy acquisition, conversion or allocation can be perturbed by stressors (Sokolova et al. 2012). We then hypothesized that a re-allocation of energy expenditure toward protection mechanism could be the main cause for the reduction in size-at-stage observed in this experiment. Although resource availability and quality are important drivers of larval fish condition, especially when combined with abiotic stressors (Cominassi et al. 2020), we did not observe any significant effect of food quality. However, unlike the natural environment, our feeding conditions were not limiting, which may have mitigated the effects of the low nutrient levels we chose for this study. Overall, the coping mechanism to tolerate the combined pressure of warming and acidification by inducing cellular protection, via heat shock proteins, likely increased the energetic demand and reduced the amount of energy directed toward growth.

2.4.3 Ecological implications for the population

Linking the results of growth and transcriptomics allows us to hypothesize that herring larvae are robust and able to cope with predicted levels of global warming and acidification. Indeed, Hsps production without further metabolism disruption can indicate a strong acclimation response, that can enhance survival in the face of a stress (Narum et al. 2013; Mahanty et al. 2017). Still, this phenotypic plasticity induced to compensate the environmental changes has an energetic cost which affected growth, and could be detrimental at the population level as growth is a key functions to organism’s fitness. In natural environment the sublethal effects

we observed could be exacerbated as access to food may be limited, potentially reducing the size at stage 3 and 4 even further. This is particularly important as we observed the size reduction effect at the beginning of the stage 3. This stage was previously identified as a potential critical period for Downs herring larvae (Joly et al. 2021) with depletion of energetic reserve even with *ad libitum* feeding thus being a period of high energetic cost during ontogeny. The larval phase is a period of high mortality (Hjort 1914) mainly caused by starvation and predation (Houde 2008), whereby mortality is selectively affecting smaller larvae (Meekan et al. 2006). Larval mortality might then increase because of an increase in predation pressure. Such consequence could lead to a decrease in population recruitment, especially as lower growth rate was already associated with decrease recruitment in North Sea herring (Payne et al. 2013). Further research should focus on longer-term experiments to investigate if reductions in size-at-stage can be compensated later in development. If this is not the case subsequent smaller juveniles would yield smaller adults at maturity and would negatively impact the reproductive success and fitness of individuals (Kingsolver and Huey 2008). Also, to better estimate the potential of herring larvae to survive on the longer-term in the face of global change, the condition of larvae could be investigated to detect carry-over effect, that could lead to death later in the development (Pechenik 2006).

CHAPTER 3 – Herring larval condition under different global change scenarios

The aim of this chapter is to assess the condition of the larvae at the end of the global change experiment, for those larvae that survived for most of the larval stage. In the previous chapter, the OWA and OWA+ environments were highlighted as stressful for the larvae, in the next chapter, the use of histological and lipid indices could determine whether this stress response is also identifiable at a higher level of biological organisation. The biochemical composition and effect of the treatments on membranes and lipid stores were assessed by fatty acid analysis.

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3.1 Introduction

Global warming of the Earth due to anthropic activities is leading to ocean warming and acidification (OWA), the “business as usual” scenario established by the Intergovernmental Panel on Climate Change predicts an increase in temperature around +3°C and a decrease of 0.4 pH units by the end of the century (RCP 8.5, IPCC, 2014). Those direct abiotic changes of the environment can induce species-specific modifications at the physiological level (Chown et al., 2010; Harvey et al., 2014). Temperature particularly affects biochemical reactions and subsequent metabolic rates, from aerobic metabolism (Schulte, 2015) to growth and maturation (Van Der Have and De Jong, 1996). To counteract the effect of temperature on the fluidity of membranes, poikilotherms modify their ratio of saturated and unsaturated fatty acids (Guschina and Harwood, 2006), which is called the homeoviscous adaptation (Sinensky, 1974). Upper consumer like fish have a limited *de novo* synthesis capacity of omega-3 and omega-6 polyunsaturated fatty acids (PUFA), most of PUFA included in marine organism’s membranes or involved in essential physiological processes are supplied at the higher trophic level by photosynthetic organisms (Dalsgaard et al., 2003). Among them, eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3), two major fatty acids incorporated in phospholipids (Dalsgaard et al., 2003) are considered as essential fatty acid (EFA) in fish, i.e. substances that must be obtained through the diet (Litzow et al., 2006). DHA in particular plays a role in homeoviscous adaptation (Colombo et al., 2020) and its biosynthesis by phytoplankton is predicted to decrease by 2100 in response to an increase in sea temperature (Hixson and Arts, 2016), reducing the overall availability for the entire ecosystem. This could have severe repercussions on all organisms through bottom-up processes (Yasuda et al., 2021). High dietary PUFA level generally enhance development and provide rapid growth in fish larvae and juveniles, promoting their survival (Tocher, 2003).

The combined effects of reduction in food quality in the ocean along with warming and acidification can thus significantly modify the conditions necessary for the good development of fish larvae, which could have repercussions for the recruitment of the fish populations and ultimately the survival of certain species. The North Sea herring stock is an important socio-economic stock of northern Europe that has been facing a decrease in recruitment since several years (Payne et al., 2013, 2009), despite a sustainable exploitation of the stock. In addition of being an important species of humans interest, Atlantic herring is also an important ecological species exerting a wasp-waist control on the North Sea ecosystem (Fauchald et al., 2011), being at the center of the trophic chain, both prey and predator. The larvae of the winter Downs spawning component of the North Sea stock, in particular, play an important role in the trophic system, as they are highly abundant in the planktonic compartment. Fish larvae are a key developmental stage and their survival through the larval phase largely determines recruitment success for commercial species (Somarakis et al., 2018; Stiasny et al., 2016). Larval mortality is mainly due to predation and starvation (Houde, 2008). Poor larval nutritional condition can be the result of low preys availability/quality but also the consequence of a poor ontogenic development of certain organs or physiological functions, leading for example to digestive system impairment (Ronnestad et al., 2013; Zambonino Infante et al., 2008). Insufficient nutrition is commonly investigated for fish larvae in the field using condition index, the histological index being the only one to directly assessed starvation (Chícharo and Chícharo, 2008; Ferron and Leggett, 1994; Suthers, 1992), to determine the potential of survival of individuals. Food intake, and particularly lipids consumed from prey are directly used for physiological processes and fatty acids derived from these lipids are incorporated into membrane structure (Tocher, 2003). When lipid intake exceeds the organism’s needs, the excess is mainly stored in the form of triglycerides (TAG), which

constitutes the main energy reserve of animals (Lehninger, 1975). The amount of energy reserves reflects larval nutritional status and conditions the potential of fish larvae to cope with a stress (Ferron and Leggett, 1994). Energy reserve can be assessed using biochemical lipid index TAG/CHOL, where the amount of TAG (reserve) in an individual is normalized by cholesterol (CHOL) quantities, a membrane constituent, increasing with somatic growth and development (Fraser, 1989). Abnormal lipid deposits and organ damages were observed in several fish larvae species exposed to ocean acidification (Frommel *et al.*, 2012, 2014, 2016). In the case of the Atlantic cod, the abnormal lipid vacuoles observed in different organs (Frommel et al., 2012b) were associated with lipid metabolism disruption at the transcriptional level (Frommel et al., 2020).

Under future global change scenarios, herring larvae will most likely face ocean warming and acidification in their habitats coupled with a reduction of the food quality that could potentially affect their physiology, condition and potential of survival. To test this hypothesis, we have implemented an experimental approach by testing four environmental treatments on Downs herring larvae. Although herring larvae seemed to be able to cope with such environmental and dietary changes (Chapter 2), we found that it would be interesting to examine potential carry-over effects and the compensatory mechanisms and/or trade-off that could happen at the structural and energy allocation level. To shed light on the long-term consequences of the combined stressors on herring condition we investigated the larval individual condition and status at the end of the experiment, around 660-degree days (thermal time), implementing histological observations and assessing lipid reserves using the TAG/CHOL index. To complete the information on the general health of herring larvae, fatty acid composition of lipid membrane and reserve were compared, as little is known about the combined effect of food quality and abiotic change on fatty acid profiles.

3.2 Materials and Methods

3.2.1 Experimental design and sampling

The complete experimental design was described in Chapter 2.

Two differently enriched 24-hours old artemia nauplii (A1) were supplied from 259 CDD to the end of the experiment, representing the major and longer change in food quality of the experiment. For the less-enriched treatment, the A1 were fed 24 hours with baker yeast and fish oil (labelled A1), as in Joly and al. (2021). For the enriched diet the focus was made on DocosaHexaenoic Acid level (DHA, 22:6n3), A1+ were fed 24 hours in a solid diet (Larviva Multigrain, Biomar). The DHA concentration in A1 was at 0.10 ± 0.01 mg.g Artemia Fresh Weight^{-1} (AFW), and of 0.41 ± 0.14 mg.gAFW^{-1} for A1+, which was significantly different (T-test, df=4, $p<0.01$).The relative proportion of the main fatty acids are represented in Table 9.

Table 9 - *Fatty acid composition of Artemia nauplii A0 (newly hatched), A1 (fed 24 hours in a mix of fish oil and baker yeast) and A1+ (fed 24 hours in a diet (Larviva Multigrain, Biomar) containing high level of DHA (DocosaHexaenoic Acid, 22:6n3). Values are reported as Fatty Acid (FA) composition (% of total fatty acids identified ± Standard Deviation)*

	A0	**A1**	**A1+**
14:0	2.1 ± 0.4	1.7 ± 0.2	1.9 ± 0.2
16:0	14.5 ± 1.7	12.8 ± 0.8	14.0 ± 1.1
18:0	4.6 ± 1.7	5.2 ± 0.2	5.0 ± 0.2
Σ SFA	21.5 ± 1.9	20.4 ± 0.9	21.4 ± 1.5
Σ MUFA	48.9 ± 2.3	50.9 ± 1.2	47.8 ± 0.6
ARA	4.4 ± 0.1	4.1 ± 0.2	4.3 ± 0.1
ALA	2.6 ± 1.2	2.4 ± 1.1	2.4 ± 1.2
EPA	13.8 ± 0.7	13.4 ± 0.9	13.0 ± 0.5
DHA	***0.34 ± ±0.2***	***0.93 ±0.1***	***3.0 ±0.6***
Σ PUFA	29.6 ± 1.4	28.8 ± 2.0	30.9 ± 1.2

The experimental design was then composed of four different treatments: Control+ (11.3 ± 0.4°C; 8.03 ± 0.05 pH; expressed as mean ± SD; diet enriched in DHA), Control (11.6 ± 0.4°C; 8.03 ± 0.01 pH), OWA+ (14.1 ± 0.4°C; 7.59 ± 0.10 pH; diet enriched in DHA) and OWA (14.3 ± 0.5°C; 7.63 ± 0.11 pH). The sampling around 660 CDD marked the last day of the experiment, were the larvae were mainly in stage 3B (Chapter 2), i.e. last part of the flexion stage, corresponding to 47 days post hatch at 14°C and 60 days post hatch at 11°C. Five larvae by tank were sampled and stored 48 hours in Bouin's solution, then rinsed and stored in 70% ethanol for histological analysis. Seven larvae by tank were sampled and stored at -80°C fir lipid class analysis. Larvae for histological and lipid class analysis were individually processed and stored. For fatty acid composition, between 17 and 30 larvae were sampled and pooled by tank, and stored at -80°C. Each larva sampled was measured (mm). Due to a manipulation error in the feeding treatment, one tank from OWA treatment was excluded from the analysis.

To determine the condition (histology and lipid class) the results of the control treatment that we considered optimal, Control+, were compared to those of the OWA and OWA+ treatments. For fatty acid profiles, all treatments were considered to compare the effect of diet quality on herring larvae in the same environment and to determine possible differences in a warming and acidification environment.

3.2.2 Analyses

3.2.2.1 Organ's integrity (histology)

Organ's health was assessed using histopathology. Larvae were dehydrated in alcohol bath progressively more concentrated (70%, 95% and 100% ethanol), rinsed in diasolv bath and embedded in paraffin blocks. Sagittal sections of 7 µm (Automated-microtome, Leica, RM2255) were placed on slides, dewaxed and rehydrated in alcohol baths (100%, 95% and

70% ethanol) and tap water. Larval sections were stained for topographic coloration with Groat's hematoxylin and picro-indgo carmine, and with Periodic acid-Schiff (PAS) and alcian blue (AB) pH 2.5 to stained respectively neutral and acid mucosubstances. PAS was also used to highlight glycogen presence in the liver. The slides were observed under microscope (Leica, DM6B).

Organ state was assessed with a particular focus on eyes, oesophagus, stomach, pancreas, liver, kidneys and intestine. Three level of state were attributed between 3 and 1, based on fish larvae damage previously described in literature (Di Pane et al., 2019; Frommel et al., 2019, 2014, 2013, 2012b). **Score 3** was the healthiest one, an overall good integrity of the cells within the organ are observed. Organs are well defined, without separation or basal detachment. For the organs of the digestive system the healthy appearance corresponds to the pattern described in stage 3 and 4 larvae in the first chapter. **Score 2** was an intermediate score, few detachments are observed within the organ. Size of the cells or structure are reduced, particularly for the hepatocytes. **Score 1** was the unhealthy score, organ integrity was poor with separation inside the organ and basal detachment, reduced cells and vacuoles. Herring glycogen presence in the liver was scored based on hepatocytes structure using descriptions made by Joly and co-authors (2021), for herring larvae, as reference. **Score 3** characterized an important amount of reserve with inflate hepatocytes vacuoles, **score 2** scattered vacuoles and less reserved, and **score 1** a total absence of reserve with no vacuoles.

To assign scores objectively, the origin of each larva was hidden from the observer. Initially five larvae by tanks, resulting in 15 larvae by treatments were sampled, but due to coloration problems, 15 larvae were observed in Control+, 11 in OWA+ and 10 in OWA.

3.2.2.2 Lipid class composition

Total lipid content of each larvae was extracted from freeze-dried (-20°C) and weighted individuals (DW: Dry weight) following a modified Folch method (Folch, 1957). Lipids were extracted in a mix of chloroform/methanol (2:1) containing 0.01% butylated hydroxytoluene and 0.88% NaCl, with final proportion of 8:4:3 (chloroform/methanol/water). Total lipids (TLi) were stored at -80°C before quantification using a TLC-FID (thin layer chromatography – flame ionization detector, Iatroscan MK-6s, Analyser Iatron Laboratories, Tokyo, Japan). TL were solubilized in chloroform (30µg/mL) and 2 µL deposited on chromarods (Type S5) with an automatic spotter (NTS 3000), analyses were done in triplicate. Lipids classes (polar lipids (PL) and neutral lipids: triglycerids (TAG), free fatty acids (FFA) and cholesterol (Chol)) were separated using a double development procedure with the following solvent systems: n-hexane:benzene:formic acid 80:20:1 (v/v/v) during 26 minutes followed by n-hexane:diethyl ether:formic acid 97:3:1.5 (v/v/v) for 25 min. The FID was calibrated for each compound class using commercial standards. TAG/CHOL ratio were calculated as condition index. Total Lipid (TL) content of each larvae was calculated by adding the quantity of neutral lipids and polar lipids.

3.2.2.3 Fatty acid composition

To determine the fatty acid composition of phospholipids and neutral lipids, the total lipid content of the larval pools was extracted as previously described with a modified Folch method. Then the total lipid contents were fractioned into neutral and polar lipid on gel micro-column. The neutral fraction was eluted with a mix of chloroform:methanol (98:2, v/v) and the polar lipid one with methanol. Each lipid fraction was transesterified with methanolic potash (0.5 mL KOH-MeOH) to obtain FA methyl esters (FAME) fractions. FAMEs were then

analysed with a gas chromatograph (CLARUS 500, Perkin-Elmer) coupled with a Flame Ionization Detector (FID). FA peaks were identified using a standard (37 component FAME mix, Sigma).

Thirty-one Fatty acids were identified and their relative quantity was expressed in % of FAME identified. 7-hexadecenoic (16:1n9) and palmitoleic acid (16:1n7) co-eluted and were grouped under the name 16:1n in the manuscript. To assess the selective incorporation, or need, of a fatty acid in the larva in regards to each feeding treatment we used the ratio between the relative proportion of this fatty acid in the fish (polar or neutral lipids) and the diet, also called fish to diet ratio (Mejri et al., 2021). The fish to diet ratio was calculated as follow:

$$(1) \qquad \frac{\%\ \text{FA (Fish polar or neutral lipids)}}{\%\ \text{FA in the Artemias}}$$

The ratio was calculated for a fatty acid in larvae in Control+ and OWA+ against relative proportion of the same fatty acid in preys A1+, and for larvae in Control and OWA against preys A1. A ratio above 1 would mean that the FA is not supply in sufficient quantity in the diet to meet the fish requirement, and is thus selectively incorporated or synthesized by the fish. The fish to diet ratio was calculated for saturated fatty acids (SFA) including myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0). It was also done for most abundant mono-unsaturated fatty acids (MUFA), which includes the pool 16:1n, vaccenic acid (18:1n7) and oleic acid (18:1n9). Then for abundant and important polyunsaturated fatty acids (PUFA), including linoleic acid (18:2n6), arachidonic acid (ARA, 20:4n6), and essential fatty acid (EFA) with α-linoleic acid (ALA, 18:3n3), docosahexaenoic acid (DHA, 22:6n3), docosapentaenoic acid (DPA, 22:5n3) and eicosapentaenoic acid (EPA, 20:5n3).

3.2.2.4 Data analyses

The analyses of the fatty acid profiles highlighted the fact that the Control+ may not be the optimal control environment as first thought (see Discussion), further analyses will be needed to properly investigate the differences in condition of the herring larvae. Thus, for the time being, no statistical analysis has been done for total lipid content, lipid class and histology, the general patterns of the results will be discussed. Normality and homoscedasticity were checked for proportions of SFA, MUFA and PUFA, DHA/EPA and EPA/ARA ratios, and differences tested with one-way ANOVA.

To visualize differences in fatty acid profiles between treatments and to distinguish similar groups between samples, for polar and neutral lipids, a Correspondence Analysis (CA) and a hierarchical clustering on the results of the correspondence analysis were used (R: FactoMineR package). To simplify graphical interpretation only the 13 FA with the highest contribution values to the axes were presented.

3.3 Results

3.3.1 Histopathology

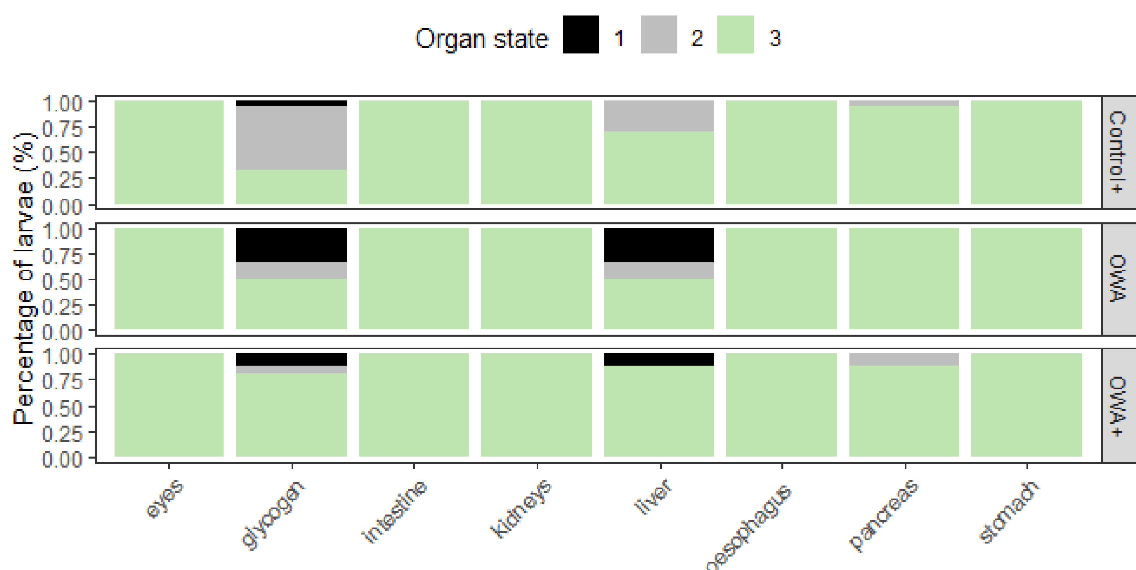

*Figure 15 - Distribution of each score (1: unhealthy with strong damages, 2: some disruption in the tissue, 3: healthy organ without damage) for each organ in function of the treatment. Treatments: Control+ (11°C*pH8.0, Enriched in DHA), OWA+ (14°C*pH7.6, Enriched in DHA), and OWA (14°C*pH7.6) treatments*

In general, little damages were observed in the larvae, regardless of the experimental treatment. All the damages or disruptions were linked to glycogen reserve in the liver, and liver and pancreas aspects (Figure 15). More disruption were found in Control +, but the ones found in OWA and OWA+ were more severe. Indeed, the liver was devoid of glycogen reserve in more than 25% of the larvae in OWA and in less than 25% of the larvae in OWA+.

3.3.2 Total lipid content and lipid class composition

Table 10 - *Size, weight and lipid composition of herring larvae reared in Control+ (11°C*pH8.0, Enriched in DHA), OWA+ (14°C*pH7.6, Enriched in DHA), and OWA (14°C*pH7.6) treatments. TAG = Triglycerids, CHOL= Cholesterol. Values are reported as means± Standard Deviation. Lipid classes are presented as proportion (%) of larval total lipid content.*

	Control+	**OWA+**	**OWA**
n	21	19	14
Standard Length (mm)	22.1 ± 2.51	19.31 ± 2.98	20.1 ± 3.47
Dry weight (mg)	3.89 ± 1.70	2.61 ± 1.50	3.09 ± 2.10
Lipid proportion (%.DW^{-1})	13.13 ± 3.05	13.03 ± 2.73	12.63 ± 3.11
Lipid class (% total lipid)			
Polar lipids	71.0 ± 6.25	73.5 ± 6.85	70.9 ± 6.71
TAG	20.47 ± 4.83	18.29 ± 6.52	19.92 ± 6.32
CHOL	7.26 ± 1.44	8.18 ± 1.50	9.20 ± 3.14
TAG/CHOL	2.92 ± 0.90	2.28 ± 0.78	2.34 ± 0.86

Polar lipids were the dominant lipid class and represented 70-74% of the total lipid content (Table 10) followed by TAG (18-20%) and Chol (7-9%). The low percentage of FFA (< 3.5 %) in the total lipid content for all samples indicated that lipids were not degraded.

3.3.3 Fatty acids profiles

3.3.3.1 Polar lipids

Among the 31 FA identified, 13 had values > 1% and accounted for 95-100% of total FA (Table 3).

*Table 11 - Fatty Acid (FA) composition (% of total fatty acids identified ± Standard Deviation) of the polar lipid fraction of pooled larvae reared in different treatments, only FA > 1% are shown in the table. Control+ (11°C*pH8.0, Enriched in DHA), Control (11°C*pH8.0), OWA+ (14°C*pH7.6, Enriched in DHA), and OWA (14°C*pH7.6). ∑ represents the sum of Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA) and Polyunsaturated Fatty Acid (PUFA). Ratios of total omega 3 and omega 6 and essential fatty acids are shown. EPA: Eicosapentanoic acid (20:5n3), DHA: Docosahexaenoic acid (22:6n3), as well as important ARA: Arachidonic acid (20:4n6). Sums and ratios are calculated on the 31 FA identified*

	Control+	**Control**	**OWA+**	**OWA**
n	3	3	3	2
14:0	1.02 ± 0.06	0.41 ± 0.01	1.09 ± 0.13	0.53. ± 0.01
16:0	11.39 ± 0.24	10.15 ± 0.27	11.90 ± 0.06	14.95 ± 0.27
18:0	8.45 ± 0.47	10.49 ± 0.34	8.58 ± 0.37	6.46 ± 0.26
∑ SFA	***21.08 ±0.37***	***21.89 ±0.89***	***22.35 ±0.39***	***22.55 ±0.48***
16:1n	6.63 ± 0.48	2.91 ± 0.13	7.81 ± 0.54	4.23 ± 0.06
18:1n9	12.61 ± 0.96	10.18 ± 0.14	14.68 ± 1.33	12.18 ± 0.22
18:1n7	13.55 ± 0.29	13.25 ± 0.21	13.45 ± 0.45	11.6 ± 0.01
∑ MUFA	***36.58 ±1.29***	***28.26 ±0.62***	***38.55 ±2.48***	***29.97 ±0.28***
18:2n6	3.97 ± 0.11	3.34 ± 0.04	3.90 ± 0.16	3.73 ± 0.01
20:2n6	1.01 ± 0.06	1.27 ± 0.03	0.99 ± 0.03	1.27 ± 0.07
18:3n3	2.62 ± 0.03	1.11 ± 0.04	1.37 ± 0.03	0.79 ± 0.02
20:4n6	8.08 ± 0.53	10.7 ± 0.09	7.38 ± 0.48	9.24 ± 0.08
20:5n3	14.98 ± 0.87	19.52 ± 0.37	14.98 ± 0.67	19.76 ± 1.55
22:5n3	1.53 ± 0.17	2.76 ± 0.03	1.25 ± 0.17	1.67 ± 0.13
22:6n3	9.84 ± 0.32	10.04 ± 0.35	7.59 ± 1.10	9.91 ± 1.67
∑ PUFA	***42.35 ±1.66***	***49.85 ±0.27***	***39.10 ±2.10***	***47.48 ±0.20***
∑n3/∑n6	2.17 ± 0.02	2.17 ± 0.02	2.03 ± 0.08	2.23 ± 0.01
DHA/EPA	0.66 ± 0.03	0.51 ± 0.03	0.50 ± 0.06	0.51 ± 0.12
EPA/ARA	1.86 ± 0.04	1.82 ± 0.02	2.03 ± 0.06	2.14 ± 0.15

In all treatments PUFA dominated the fatty acids profiles (39-50%), mainly with EPA (20:5n3, 15-20%) and DHA (22:6n3, 8-10%). Saturated acids were dominated by palmitic (16:0) and stearic (18:0) acids. Monoenes acids were dominated by oleic (18:1n9) and vaccenic (18:1n7) acids (Table 11).

Proportions of SFA were similar between treatments (anova, (Df = 3, 7), F = 1.39, p = 0.32). Proportions of MUFA (anova, (Df = 3, 7), F = 5.04, p = 0.04) and PUFA (anova, (Df = 3, 7), F =

6.69, p = 0.02) were different between treatments. Treatments with food enriched in DHA (Control+ and OWA+) had globally higher proportions of MUFA and lower proportions of PUFA (Table 3) than less-enriched treatments (Control and OWA). Among MUFA, the difference was mainly due to 16:1n, which had higher proportions in enriched treatments (Control+ and OWA+). Among PUFA, proportions of DHA were similar between all treatments whereas EPA proportions were lower in enriched treatments. DHA/EPA ratios were similar in all conditions (anova, (Df = 3, 7), F = 2.29, p = 1.17) and EPA/ARA ratios were different (anova, (Df = 3, 7), F = 6.05, p = 0.02), slightly higher in OWA and OWA+ treatments.

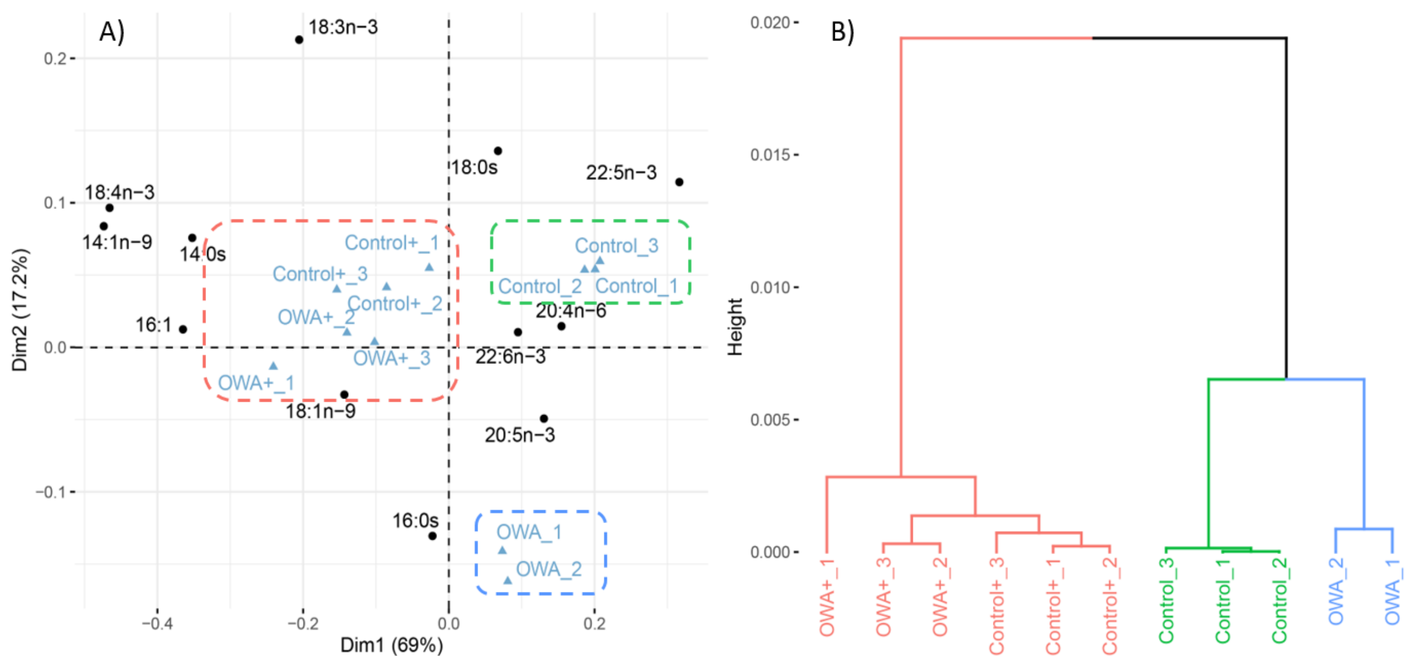

*Figure 16 - A) Biplot of Correspondence analysis (CA) of the polar lipids fatty acids (FA) profiles of pooled herring larvae reared in different treatments. Black points represent fatty acid descriptors and blue triangles represent treatments and their replicates. Red, green and blue rectangle represent groups identified by hierarchical clustering on the correspondence analysis. B) Hierarchical Clustering on results of Correspondence analysis of the fatty acids profiles of pooled herring larvae reared in different treatments. Main FA explaining the division between groups from results of CA are written. Control+_1, Control+_2, Control+_3: (11°C*pH8.0, Enriched in DHA); Control_1, Control_2, Control_3 (11°C*pH8.0); OWA+_1, OWA+_2, OWA+_3 (14°C*pH7.6, Enriched in DHA); OWA_1, OWA_2 (14°C*pH7.6).*

Correspondence Analysis (CA) was also used to explore differences on FA profiles among treatments (Figure 16.A). The combination of the first two factorial axes explained 86.2 % of the total variance. The first axis (69%) was mainly described by 16:1n (32.4%), 20:5n3 (12.8%) and 18:1n9 (11.27%). The second one (17.2%) by 16:0 (37.8%), 18:0 (28.4%) and 18:3n3 (11.2%) acids.

A hierarchical clustering on principle components (Figure 16.B) was then used to investigate groups discrimination according to their fatty acid profiles. The first division was made between positive and negative dimensions of the first axis, separating food treatments (enriched in PUFA vs non-enriched). The first axis was driven by 16:1n (32.4%), 20:5n3 (12.8%), 18:1n9 (11.3%), 20:4n6 (9.3%) and 22:5n3 (8%). The second clear division appeared only between Control and OWA treatment, whereas no strong distinction was made between Control+ and OWA+ treatments. Control and OWA were separated between the positive and the negative dimensions of the second factorial axis, separating environmental treatments and driven mainly by 16:0 (35.8%) and 18:0 (28.4%).

3.3.3.2 Neutral lipids

Among the 31 FA identified, 13 had values > 1% and accounted for 95-99% of total FA (Table 12).

Table 12 - *Fatty Acid (FA) composition (% of total fatty acids identified ± Standard Deviation) of the triglyceride fraction of pooled larvae reared in different treatments, only FA > 1% are shown in the table. Control+ (11°C*pH8.0, Enriched in DHA), Control (11°C*pH8.0), OWA+ (14°C*pH7.6, Enriched in DHA), and OWA (14°C*pH7.6). ∑ represents the sum of Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA) and Polyunsaturated Fatty Acid (PUFA). Ratios of total omega 3 and omega 6 and essential fatty acids are shown. EPA: Eicosapentanoic acid (20:5n3), DHA: Docosahexaenoic acid (22:6n3), ARA: Arachidonic acid (20:4n6). Sums and ratios are calculated on the 31 FA identified.*

	Control+	**Control**	**OWA+**	**OWA**
n	3	3	3	2
14:0s	1.05 ± 0.15	1.83 ± 0.16	1.29 ± 0.07	1.66 ± 0.07
16:0s	11.58 ± 0.14	12.32 ± 0.88	11.78 ± 0.23	11.87 ± 0.13

18:0s	6.95 ± 1.07	5.86 ± 0.28	7.14 ± 0.26	6.32 ± 0.13
∑ SFA	***20.78 ±1.22***	***21.08 ±1.37***	***21.43 ±0.09***	***20.94 ±0.02***
14:1n9	0.67 ± 0.12	1.18 ± 0.11	0.75 ± 0.04	0.98 ± 0.01
16:1n	9.30 ± 1.07	13.57 ± 1.29	9.92 ± 0.45	11.26 ± 0.04
18:1n9	26.83 ± 0.66	23.93 ± 0.78	27.38 ± 0.51	23.48 ± 0.42
18:1n7	18.55 ± 0.25	15.89 ± 0.77	18.02 ± 0.30	15.98 ± 0.13
20:1n9	0.97 ± 0.13	1.44 ± 0.07	1.12 ± 0.17	1.17 ± 0.19
∑ MUFA	***58.49 ±0.23***	***57.52 ±1.33***	***59.12 ±0.64***	***54.95 ±0.85***
18:2n6	4.91 ± 0.39	5.31 ± 0.33	4.46 ± 0.19	4.23 ± 0.32
18:3n3	1.90 ± 0.35	3.16 ± 0.24	1.05 ± 0.07	1.49 ± 0.02
20:4n6	3.54 ± 0.06	3.49 ± 0.17	3.29 ± 0.11	4.05 ± 1.15
20:5n3	6.83 ± 0.54	10.78 ± 0.53	7.25 ± 0.47	10.54 ± 0.03
22:6n3	1.60 ± 0.35	0.45 ± 0.03	1.27 ± 0.11	1.10 ± 0.34
∑ PUFA	***20.73 ±1.07***	***26.32 ±1.44***	***19.45 ±0.73***	***24.11 ±0.83***
∑n3/∑n6	1.15 ± 0.03	1.53 ± 0.01	1.13 ± 0.07	1.46 ± 0.02
DHA/EPA	0.24 ± 0.08	0.05 ± 0.005	0.18 ± 0.02	0.10 ± 0.03
EPA/ARA	1.93 ± 0.16	3.09 ± 0.01	2.20 ± 0.15	2.61 ± 0.09

In all treatments MUFA dominated the fatty acids profiles (54-60%), mainly with 18:1n9 (22-28 %) and 18:1n7 (14-19%). Oleic and vaccenic acid were in higher proportions in enriched treatments. As for polar lipids, proportions of SFA were not significantly different (anova, Df = 3, F = 0.14, p = 0.93) whereas differences were noted for MUFA (anova, Df = 3, F = 5.84, p = 0.03) and PUFA (anova, Df = 3, F = 14.1, p < 0.01). Saturated acids were dominated by palmitic (16:0s) and stearic (18:0s) acids. PUFA were dominated by EPA (6-11 %) and linoleic acid (18:2n6; 4-6 %). The highest difference between treatments was the proportions of PUFA especially due to a difference in EPA. EPA proportions were lower in enriched treatments. DHA proportion was strongly lower in Control (Table 4) but this result is to be taken with caution, because we cannot totally exclude an analysis problem specifically for this group. EPA/ARA (anova, Df= 3, F = 6.58, p= 0.02) ratios and DHA/EPA (anova, Df= 3, F = 26.68, p < 0.01) ratios were different, EPA/ARA were higher in OWA and Control whereas their DHA/EPA were lower.

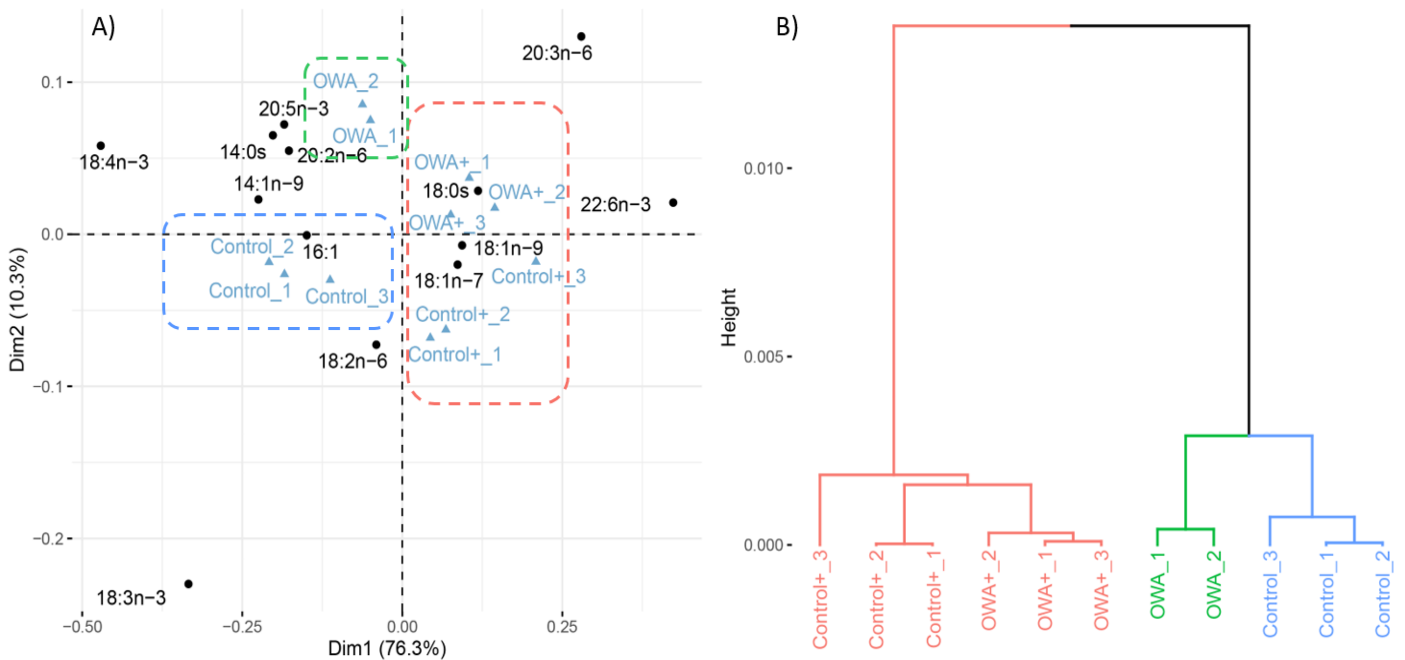

*Figure 17- A) Biplot of Correspondence analysis (CA) of the triglycerides fatty acids (FA) profiles of pooled herring larvae reared in different treatments. Black points represent fatty acid descriptors and blue triangles represent treatments and their replicates. Red, green and blue rectangle represent groups identified by hierarchical clustering on the correspondence analysis. B) Hierarchical Clustering on results of Correspondence analysis of the fatty acids profiles of pooled herring larvae reared in different treatments. Main FA explaining the division between groups from results of CA are written. Control+_1, Control+_2, Control+_3: (11°C*pH8.0, Enriched in DHA); Control_1, Control_2, Control_3 (11°C*pH8.0); OW+p_1, OWA+_2, OWA+_3 (14°C*pH7.6, Enriched in DHA); OWA_1, OWA_2 (14°C*pH7.6).*

Correspondence Analysis (CA) was also used to explore differences on FA profiles among treatments (Figure 17.A). The combination of the first two factorial axes explained 86.7 % of the total variance. The first axis (76.3%) was mainly described by 20:5n3 (17.2%), 16:1n (14.2%), 18:1n9 (12.9%), 18:3n3 (12.5%) and 22:6n3 (11.5%). The second one (10.3%) by 18:3n3 (43.9%), 20:5n3 (6.4%) and 18:2n6 (10.8%) acids. A hierarchical clustering on principle components (Figure 17.B) was then used to investigate groups discrimination according to their fatty acid profiles. As for polar lipids, the first division separated food treatments (enriched in PUFA vs non-enriched) explained by 20:5n3 (17.2%), 16:1n (14.2%), 18:1n9 (12.9%), 18:3n3 (12.5%), 22:6n3 (11.5%), 18:1n7 (7.48%) and 18:4n3 (6.7%). The second

division appeared between Control and OWA treatment, separating environmental treatments with 18:3n3 (43.9%) and 20:5n3 (19.5%).

3.3.3.4 Selective incorporation

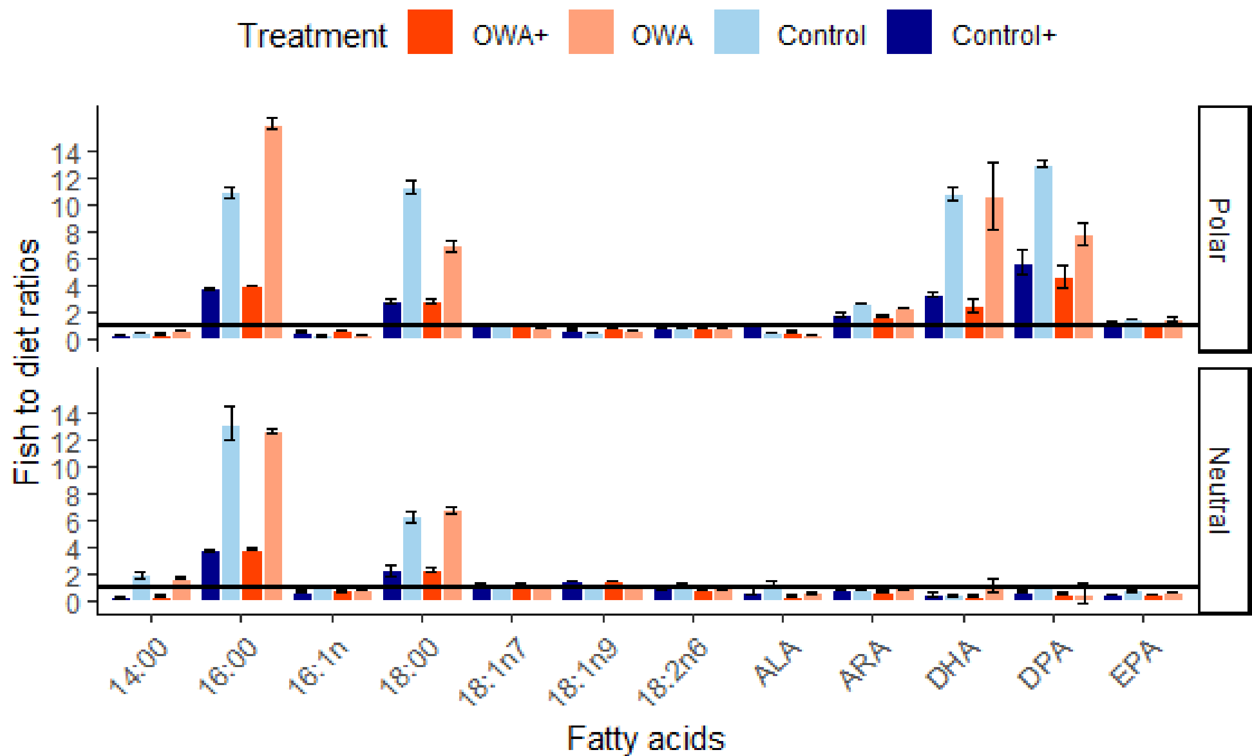

*Figure 18 - Fish to diet ratios of herring larvae in polar and neutral lipid fractions. Control+: 11°C*pH8.0, Enriched in DHA; Control: 11°C*pH8.0; OWA+: 14°C*pH7.6, Enriched in DHA; OWA: 14°C*pH7.6.*

Fish to diet ratios (Figure 18) showed that selective incorporation happened especially in the polar lipids and for few fatty acids. The major results in the membranes are for 16:0, 18:0, ARA, DHA and DPA. ARA and DHA with higher exhibited higher retention rate for the non-enriched diet. Conversely, the selective incorporation of DPA was higher for non-enriched diet but different between OWA and Control with ratios at 7.8 ± 0.9 and 13.0 ± 0.2, respectively. It appears that 16:0 and 18:0 are strongly and selectively retained in the lipids of the membranes of larvae receiving unenriched diets; conversely, a very low retention is noted

when the larvae receive enriched food. The same strongly expressed pattern is observed in the reserve lipids for these two former fatty acids as well as for 14:0, but in a lesser extent.

3.4 Discussion

The present study examines the effect of combined warming (current temperature +3°C), acidification (current pH -0.4) and diet quality (content in DHA) on late herring condition status and investigates the extent to which this condition depends on dietary fatty acid profiles. At this stage of the study, all the data (histology and lipid index) concerning the condition could not yet be analyzed and we will only discuss the general patterns.

The larvae were exposed 660 degree days to ocean warming and acidification, corresponding to 47 dph in the OWA+ and OWA, and 60 dph in Control+ and Control, and a total of 400 degree days to different dietary DHA level. The histopathology and lipid class results are still partial, but they do not seem to indicate strong differences between the different experimental groups. It is very likely that our choice of ad-libitum feeding may have erased differences that would have appeared in the natural environment, since food restriction or fasting is one of the main factors responsible for organ damage and depletion of energy reserves (Gisbert and Sarasquete, 2008). However, previous studies investigating the effect of global change or diet composition on early life stage of fish, using feeding sequence not limiting and providing satiation, found different possible consequences on different species. Responses such as disruption of the lipid metabolism and organ damages caused by acidification (Frommel et al., 2020, 2016, 2012b), impairment of the digestive capacity caused by warming and acidification (Pimentel et al., 2015) or by inadequate food supply (Li et al., 2017) have been described. Yet, even though deleterious effect on organ state have been previously described for herring larvae (Frommel et al., 2014), it was not the case in our study. The main difference observed was concerning the liver, based on the amount of reserve rather than tissue degradation. The

proportion of larvae with lower reserve was not treatment specific, but the severity of the depletion might by higher in OWA and OWA+ and need further investigations. Variability in liver reserve has previously been described in Downs herring larvae at the same developmental stage (Joly et al., 2021). Close to the end of the larval period, it could be related to a change in energy storage from glycogen to lipid or to a period of high energy consumption, i.e. the glycogen stored in the liver can be quickly mobilized to support the physiological processes linked to metamorphosis (Stirling, 1976). The lipid reserve under the form of TAG was important and similar in all treatments, representing 20% of the total lipid content. The fish to diet ratio makes it possible to identify the fatty acids particularly necessary for the metabolism of the larva, which are preferentially retained or synthesized to meet requirements. In all treatments, fish to diet ratio evidenced high proportions of 16:0 and 18:0 and are the only ones with a ration above 2 in the neutral lipid compartment. These fatty acids are commonly synthesized in all organisms (Sargent et al., 2003), to constitute a potential reserve for use in oxidative phosphorylation in order to produce ATP, which fulfills cellular energy demands (Robin and Skalli, 2007) of the larvae, which is consistent with the large amounts of reserve measured in the larvae.

The fish to diet ratio must be interpreted considering the fact that the diets differ only in the DHA content, which is higher in the so-called "enriched" treatments. DHA and DPA (docosapentaenoic acid; 22:5n3) levels in polar lipids were largely higher than the intake levels in all dietary treatments, but in higher proportion in non-enriched dietary regimes. Generally PUFA composition of fish is largely determined by the composition of their diet (Arts et al., 2009), but can also be affected by internal bioconversion, especially to synthetize DHA from precursors such linolenic acid (ALA), EPA and DPA (Guo et al., 2021). Given that relative similar proportions of DHA were found in polar lipids in all treatments, the higher Fish to Diet Ratios for DPA and DHA could likely reveal that herring larvae have a DHA synthesis capacity, as shown for salmonids (Betancor et al., 2015b). As internal conversion is energy costing, converting from a closer HUFA in the biosynthesis pathway would be more efficient, but

depending on its dietary availability (Guo et al., 2021). DHA thus seemed to be of great importance for herring larval development and physiological requirements. DHA is involved especially in brain and vision development (Pilecky et al., 2021; Tocher, 2015), it has been directly showed for herring larvae (Bell et al., 1995), and also for metamorphosis of fish larvae (Shields et al., 1999). Along with the proportion of SFA, DHA is the most important PUFA involved in maintaining membrane fluidity (Niebylski and Salem, 1994). Supply in DHA is then determining for fish larval development, and loss in PUFA at the basis of the trophic level was hypothesized to threaten fish recruitment by bottom-up processes (Litzow et al., 2006). For Baltic herring in the field, low DHA level were associated with low growth and lower level of DHA within fish (Paulsen et al., 2014). Herring might then play an important buffering role in trophic ecology, by adjusting its DHA level to go through the larval phase, and transfer energy to higher trophic level as a small pelagic (Lindegren et al., 2011).

It should be pointed out that the additional DHA supply by the enriched treatment did not lead to a better development or growth rate (see Chapter 2), despite the essential role of this fatty acid in growth and development. This finding is intriguing and leads us to analyze the effect of this DHA intake in a more global way, by focusing on EPA and ARA. Firstly, according to the PCA analysis, EPA seems to have a structuring and central role that differentiates the two dietary regimes. With both dietary regimes, EPA levels in fish are approximately equal to intake levels, indicating that the nutritional need for this fatty acid is globally met. However, the proportion of EPA is 30% less, in the membranes of fish fed the enriched dietary regimes, and it is likely that EPA molecules are used for another function in the metabolism. EPA is known to possess anti-inflammatory and immunomodulatory properties, and could be converted into series of downstream metabolites such as eicosanoids, leukotrienes and resolvins (Leuti et al., 2019; Wall et al., 2010). This response may indicate that enriched dietary

regimes in DHA are not necessarily beneficial for herring larvae. It was already reported that high dietary PUFA, particularly supplied as triglycerides, were not well handled from a metabolic point of view by cod and seabass larvae, and could induce malformations in bones structures and metabolic disorders in the liver (Gisbert et al., 2005; Wold et al., 2009, 2007). Excessive dietary DHA particularly increased oxidative stress due to its high degree of unsaturation (Betancor et al., 2015a, 2013). As no negative effect were observed at the tissue level, larvae seemed to be able to compensate the potential deleterious effect. Secondly, EPA/ARA ratios discriminate the two abiotic treatments, ratios being higher in OWA and OWA+ treatments compared to Control and Control+ due to less ARA incorporated. It has been shown that this fatty acid could be transformed by beta-oxidation into eicosanoids products, which are molecules involved in the inflammatory response (Regulska et al., 2021). Research in mammal cells has linked ARA release from membranes to stress response, via the induction of heat shock proteins (Gosslau and Rensing, 2000; Miwa et al., 2000). In the previous study on these herring larvae, the main metabolic difference at the transcriptional level between larvae reared in the control or in the ocean warming and acidification treatments, was precisely the production of heat shock protein transcripts (Chapter 2).

In conclusion, herring larvae might have the potential to adjust their level of DHA when their requirement are not met, consequently they do not seem vulnerable to the predicted decrease in DHA in the ecosystems for 2100, in response to global change. This can be positive for herring and the ecosystem as herrings, throughout their life cycle, are the preys of many species and play a central role in the trophic web. Our result did not highlight a strong difference in SFA or DHA proportion that could indicate a process of homeoviscous adaptation due to temperature effect. Considering that Atlantic herring is an eurytherm species, with an important phenotypic plasticity (Geffen, 2009), this suggests that 11°C and 14°C could both be

within its optimum thermal range. The lack of specific mortality in treatments (see Chapter 2) or organ damage or depletion in energy reserve, suggested that herring larvae is able to cope with combined effect of warming, acidification and dietary regime quality, as long as the food (prey) availability is sufficient: in such a case, herring might be one of the “winner” species face to global change. However, prey availability is random in the natural environment, which could dramatically alter the ability of larvae to cope with global change; future studies will need to implement experimental approaches that include limitations on prey abundance.

CHAPTER 4 – Effect of climate change on the morphological development of sagittal otoliths in Atlantic herring (*Clupea harengus*) larvae

The aim of this chapter was to evaluate the effect of combined warming and acidification on otolith morphogenesis in herring larvae. The feeding treatment were not taken into account as previous study have shown that dietary fatty acid supply does not affect otolith development.

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4.1 Introduction

Otoliths are calcified biomineralised structures overlying the sensory epithelia in the inner ear of fish. They are formed by calcium carbonate crystals embedded in a non-collagenous organic matrix composed of acidic proteins and polysaccharides (Degens et al., 1969; Popper et al., 2005). Otoliths are formed during embryonic development. They are metabolically inert and do not resorb in periods of stress, but grow throughout an individual's life, in correlation with its ontogenic growth (Casselman, 1987). Because of the positive correlation between otolith growth and organismal fitness, otoliths have been widely used in fisheries science as a proxy to evaluate age, growth, and stock segregations. In Atlantic herring (*Clupea harengus*), otolith microstructure has been used to study environmental effects (temperature, salinity or feeding activity) on the growth of the larvae (Berg et al., 2017; Denis et al., 2017; Folkvord et al., 1997; Johannessen et al., 2011; Tonheim et al., 2020), and otolith shape used as a tool for stock identification (Burke et al., 2008; Libungan et al., 2015; Turan, 2000). Atlantic herring are of high commercial importance, with around 1,640,000 tons caught in 2016 (FAO FishStat data), but are vulnerable to the effects of climate change, particularly at the larval stage (Hufnagl and Peck, 2011).

Otolith growth, and ultimately global otolith shape are well-known to result from the combination of genetic heterogeneity, ontogeny (physiological processes) and the influence of environmental (biotic and abiotic) factors (Hüssy et al., 2020; Mahé, 2019; Vignon and Morat, 2010). Global climate change is projected to cause warming of the ocean surface by 3 to 5°C by 2100, and acidification of waters with a drop of 0.4 pH units in the worst-case scenario (IPCC 2019, "Changing ocean, marine ecosystems and dependent communities"). The speed at which global change is occurring will not allow some species to adapt; in addition,

early life history stages which do not yet have fully functional physiological homeostatic mechanisms could be particularly affected (Melzner et al., 2009). Some ontogenic processes, such as otolith morphogenesis, could thus be particularly impacted by the alteration of environmental conditions.

A previous study on herring larvae testing four temperature conditions (4°C, 12°C and two others shifted twice with 4/8/4°C and 12/8/12°C) showed that the size and the width of the otolith daily increment were both temperature-dependent (Folkvord et al., 2004). Other studies on the effects of ocean acidification on fish otoliths have been mostly conducted on larval developmental stages. Several studies have shown that elevated levels of CO_2 caused an increase in otolith size or shape for multiple species (Bignami et al., 2013; Coll-Lladó et al., 2021; Maneja et al., 2013; Munday et al., 2011; Réveillac et al., 2015). Consequently, acidification can lead to decoupling of otolith and body growth (Reveillac *et al.* 2015). Furthermore, the effect of acidification is not observable on the otoliths of some other species (Franke and Clemmesen, 2011; Frommel et al., 2013; Perry et al., 2015). Changes in extracellular concentrations of carbonate and bicarbonate caused by acid-base regulation in a high CO_2 environment could increase the precipitation of $CaCO_3$ in the otolith (Payan et al., 1998). The intensity of these modifications are linked to the life stage but could be a species-dependent process.

Here, we aimed to investigate in Atlantic herring how otolith morphological variability and size within individuals, i.e. directional bilateral asymmetry, and between individuals during the first early life stages is affected by the warming and acidification conditions projected by 2100 under the worst-case IPCC scenario We then implemented an experiment where we monitored the larvae from growing degree days (GDD) 32°C.day to 662°C.day. Designated

by a number of expressions, including “thermal time”, “heat units”, “thermal units”, or “growth units”, thermal opportunity for growth is usually measured in “growing degree-days” (Bonhomme, 2000; Neuheimer and Taggart, 2007; Trudgill et al., 2005; Wang, 1960). Otoliths are important biomarkers that are widely used in fisheries management, particularly in the case of commercially and ecologically important species such as herring. Otolith size and shape allows us to evaluate growth in a species and to know the distribution of stocks. It is therefore crucial to reveal the consequences of climate change on otolith characteristics in order to continue to use them without introducing bias.

4.2 Materials and Methods

The rearing method was fully described in Chapter 2.

Herring larvae were reared until they reached the last larval development stage respectively from hatching to 47 days post-hatching in the OWA treatment (14°C, 1660 µatm) and 60 days post-hatching in the Amb treatment (11°C, 560 µatm). To follow the otolith growth and shape variation through time, 351 Atlantic herring from 32 to ~662°C.day were sampled five times over the course of the experiment. At 32°C.day, 15 individuals were sampled, and then 42 fish for each experimental condition were sampled at each of the next four samplings. To assess the potential effect of temperature on the growth and shape of otoliths, we used the “heat units approach” (e.g., Réaumur, 1735). GDD were calculated as follows:

$$GDD = \sum_{i=1}^{n} (Ti - Tth).\Delta d$$

where Ti represents the average temperature at day i, Tth is the threshold temperature below which growth is expected to cease and Δd is time (in days). In our study, Tth was set at 0°C to minimize the bias introduced by this factor. This threshold temperature Tth used to compute GDDs was tested by varying the value of this parameter with 5°C and 10°C and the significance level of tested effects was the same. Moreover, it is recommended to use standard Tth values (0, 5, 10, 15 °C) (Chezik et al., 2013). The GDD approach quantifies the thermal opportunity for growth by aggregating temperatures relevant to growth (McMaster and Wilhelm, 1997), and is thus more precise than the calendar time approach when describing growth (K. Mahé et al., 2019; Neuheimer and Taggart, 2007). The first sampling occurred at three days post-hatching (GDD=32°C.day). The four other samplings were then carried out after different time periods depending on the rearing temperature, the sampling date are the same as in Chapter 2.

4.2.1 Otolith shape analysis

After measuring the total length (TL± 0.1 cm) of fish, their sagittal otoliths (left and right) were extracted from the cranial cavity and cleaned. In order to minimize distortion errors in the normalization process during image analysis, each otolith was placed on a microscope slide with the sulcus facing downward and the rostrum pointing left. The outline of each otolith was digitized using an image analysis system consisting of a high-resolution camera (Hamamatsu Orca 3G) connected to an automated microscope using reflected light (Zeiss Axio Imager Z2). Each digitized image was analyzed using the image-analysis software TNPC (Digital processing for calcified structures, version 7). To compare the shapes of the left and right otoliths, mirror images of the right otoliths were used. Otolith shape was assessed by analyzing first the otolith

area (Oarea, µm²) and Elliptic Fourier Descriptors (EFDs) (Lestrel, 2008). Oarea seems to be a better univariate descriptor than longest length because the otolith shape is close to a circle without being a circle. As a result, the longest length of the otolith is too variable from one individual to another, without always being measured on the same axis. EFDs were obtained by using TNPC 7 software to perform Fourier analysis on binarized images. For each otolith, the first 99 elliptical Fourier harmonics (H) were extracted and normalized with respect to the first harmonic so as to be invariant to otolith size, rotation and starting point of contour description (Kuhl and Giardina, 1982). To determine the number of harmonics required to reconstruct the otolith outline, the cumulated Fourier power (F) was calculated for each otolith k as a measure of the precision of contour reconstruction obtained with n_k harmonics (i.e., the proportion of variance in contour coordinates accounted for by the n_k harmonics):

$$F_{(n_k)} = \sum_{i=1}^{n_k} \frac{{A_i}^2 + {B_i}^2 + {C_i}^2 + {D_i}^2}{2}$$

where A_i, B_i, C_i and D_i are the coefficients of the ith harmonic. n_k was chosen for each otolith k to ensure that it was reconstructed with a precision of $F_{(n_k)} = 99.99\%$ (Lestrel, 2008). The maximum number of harmonics $n = max(n_k)$ across all otoliths was then used to reconstruct each individual otolith. Only the first 6 harmonics were necessary to ensure reconstruction of each otolith shape with a precision of 99.99% and were thus used for further analyses.

The resulting matrix containing EFDs (as columns) for each otolith (as rows) was subjected to Principal Components Analysis (PCA) (Rohlf and Archie, 1984). and the 3 first principal components (PCs) were selected as otolith shape descriptors or shape matrix (S) according to the broken stick model (Legendre and Legendre, 1998), which, in this case, corresponded to a threshold of 3.4% of the total variance explained (Borcard et al., 2011). In total, these 3 PCs

explained 82.4% of the total variance in the EFDs. This procedure allowed us to decrease the number of variables used to describe otolith shape variability through EFDs while ensuring that the main sources of shape variation were kept, and to avoid co-linearity between shape descriptors (Rohlf & Archie, 1984).

4.2.2 Statistical analyses

Each environmental condition was the combination of temperature and pCO_2 concentration values. Otolith area and larvae size differences were analysed using a post-hoc Tukey-HSD test among several values of GDD. The relationship between larval total length (TL) and otolith area (Oarea) in response to the environmental conditions was tested using Analysis of Covariance after verifying the normality of residuals. To analyse the potential anatomical differences described by the directional bilateral asymmetry (DA) between left and right otolith shape, partial redundancy analysis (pRDA) was modelled on the selected principal components (PCs) matrix. In this pRDA, otolith side (left/right) was used as the potentially influential variable and the individual as the conditioned variable. To test the potential effect of climate change, a second pRDA was applied where the explained matrix was combined with permutation tests on the selected PC matrix and the explanatory matrix consisted of the tested effects (Temperature and pCO2 concentration) and the interactions between them. In all pRDAs, total length was used to correct for fish size. To visualise shape differences between right and left otoliths, or between 2 environmental conditions (temperature/pCO_2 concentration), average shapes were rebuilt based on EFDs averaged for each group of individuals. Directional asymmetry and environmental effect amplitude were then computed as the percentage of non-overlapping surface between the reconstructed otolith average shapes relative to the total area they covered after superposition.

Statistical analyses were performed using the following packages in the statistical environment R: 'Vegan' (Oksanen et al. 2013), 'sp' and 'rgeos' (Bivand et al., 2013).

4.3 Results

4.3.1 Otolith growth

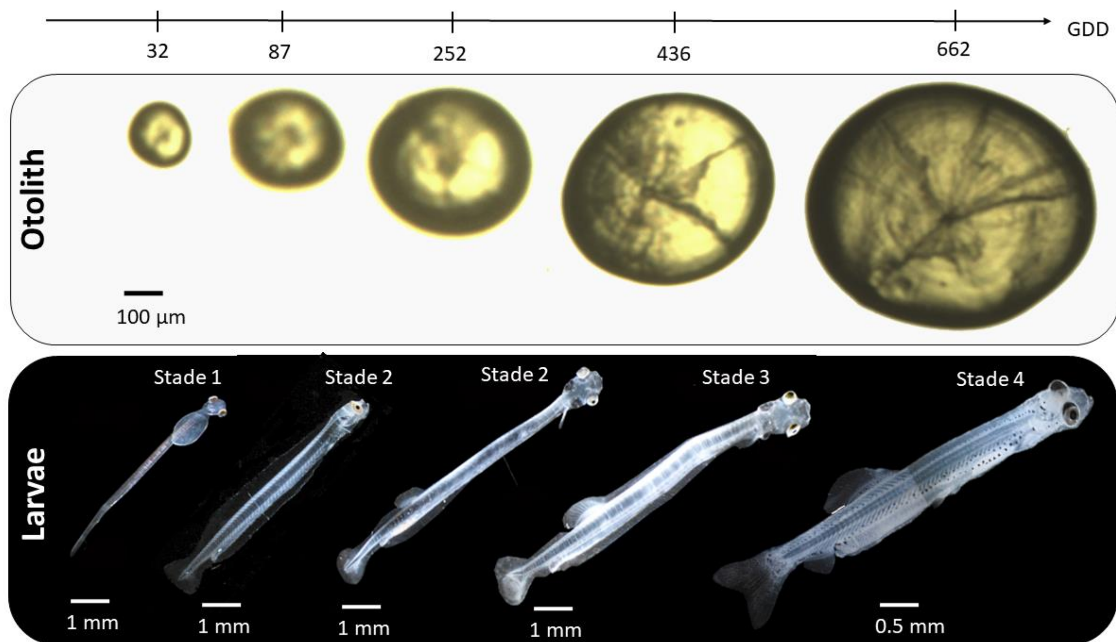


Figure 19 - Growth of otolith shape and larval fish by GDD value (°C.day).

Otolith and larvae sizes increased stepwise from 32 to 670°C.day (Figure 19). Over the course of the experiment, the ratio between the dorso-ventral and antero-posterior axis increased, leading to the loss of the initial circular shape of the otolith. Otolith morphogenesis was positively correlated with growing degree days. Herring larvae were long and thin at hatching, then progressively developed dorsal and caudal fins. Body height increased around 436°C.day, and the pelvic fins were the last fins to differentiate at around 662°C.day.

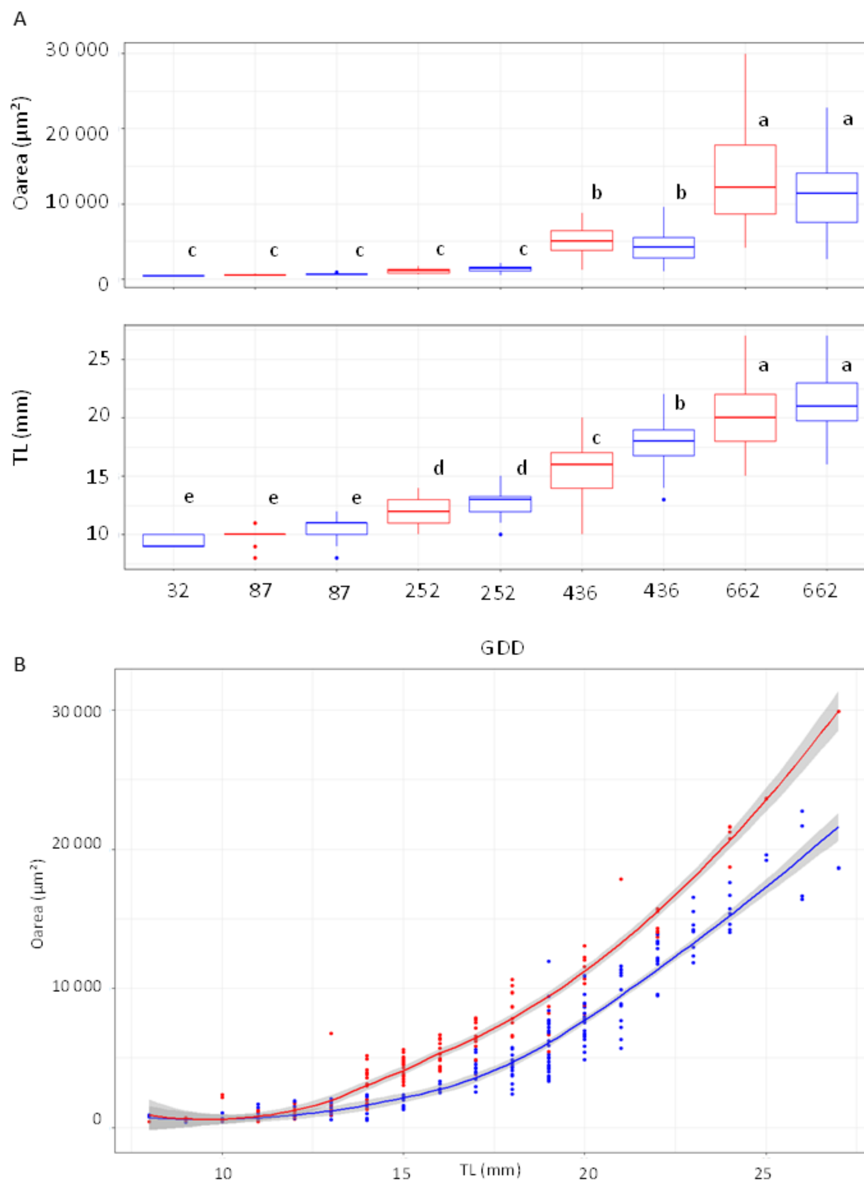


Figure 20 - (A) Box-plot of otolith area (Oarea µm²) and total length of fish (TL cm) values by GDD value (°C.day) and environmental conditions (red for 14°C and 1660 µatm and blue for 11°C and 560 µatm). A different letter within each sampling time denotes a significant difference between the two groups. (B) Relationship between otolith average area (Oarea) and larval body length (TL) in response to the environmental conditions (red for 14°C and 1700 µatm and blue for 11°C and 560 µatm).

Otolith area and larvae size differences were analysed among several values of GDD. Larvae size changed faster than otolith area (Figure 20.A). Larvae size increased significantly between 32°C.day and 87°C.day, while the first changes in otolith area appeared only at 436°C.day. However, after this GDD value, otolith area increased faster than larval size. Neither larvae

size nor otolith area were significantly different between the two environmental conditions (temperature/pCO2) at any value of GDD, with the exception of larvae size at 436°C.day ($P<0.05$). The relationship between otolith area (Oarea) and larval body length (TL) was always significant ($P<0.05$), but the slope was significantly higher in the OWA than in the Amb treatment (slopes difference; $P<0.05$; Figure 20.B). For the same fish length, the area of otolith was bigger for the 2100 scenario (14°C/pCO2 1660 µatm) than under present day conditions (11°C/pCO2 560 µatm) (Figure 20.B); this difference increased with fish size.

4.3.2 Otolith shape

Using the Fourier harmonics to describe the otolith shape, pRDA were used to test the explanatory variables of interest (i.e., side, combination of temperature and pCO2 concentration, GDD value). No significant difference in shape was observed between left and right otoliths (Redundancy analyses, $p > 0.05$). The average percentage of non-overlapping surface between the two sides never exceeded 1% (Figure 21). Similarly, the results showed no significant environmental effect on otolith shape in herring larvae, with the average percentage of non-overlapping surface ranging from 0.73% to 1.48% (Figure 21).

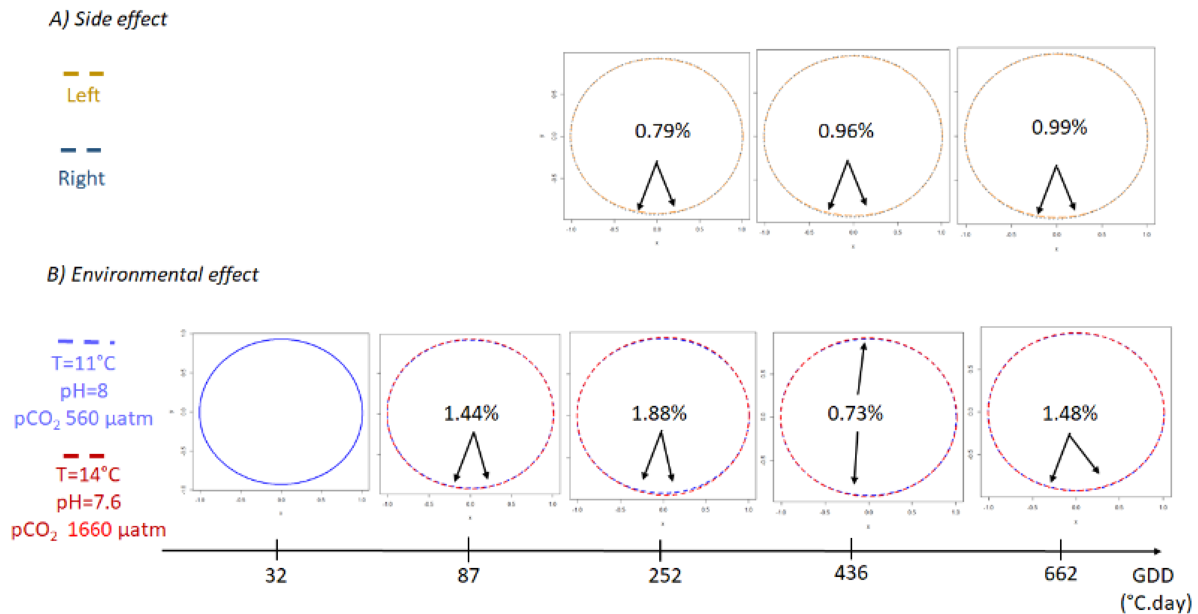


Figure 21 - Percentage of non-overlapping surface between left and right otolith shape and for two environmental conditions by GDD value (°C.day) (arrows identify the main areas of difference between both otoliths).

4.4 Discussion

Fish larvae otoliths are commonly used to estimate growth using the microstructure and the positive relationship between otolith and fish sizes. This method has been used for several species including herring (Folkvord et al. 1997; Johannessen et al. 2000; Berg et al. 2017; Denis et al. 2017; Tonheim et al. 2020). Our experimental study validated the results of these *in situ* studies and showed that this significant correlation between otolith and fish growth was still observed in present day environmental conditions as well as for the 2100 scenario. A previous study on herring larvae testing four temperature conditions (4°C, 12°C and two others shifted twice with 4/8/4°C and 12/8/12°C) showed that the size and the width of the otolith daily increment were both temperature-dependent (Folkvord et al., 2004). The otolith area per fish size in our study increased with higher temperature and pCO_2 (i.e. lower pH). It should be noted that the feeding protocol chosen for this study provides *ad-libitum* feed, in order to avoid under-feeding situations that could have interfered with the otolith study. Nevertheless,

it cannot be excluded that larvae under the 2100 scenario ingest more food than control larvae, which could also accelerate the increase of the otolith area in the 2100 scenario group. While growth mechanisms of otoliths and their morphogenesis during the early life stages of fish are poorly understood, shifts in otolith shape are linked to physiological modifications due to environmental disturbance (Geffen, 1987; Vignon, 2018). Early life stages are more vulnerable to environmental challenges because they have higher surface area to volume ratios and have not yet fully developed the homeostatic regulatory mechanisms which are found in adult fish (Hurst et al., 2013). For Downs herring, a shift affecting larval condition has previously been observed *in situ* for individuals at 13 mm, and was driven by environmental conditions (temperature and prey concentration) (Dennis et al. 2017). Our experimental study on Downs herring covered this length range but the otoliths did not show any ontogenetic differences due to environmental conditions during this larval life period.

To follow otolith morphogenesis, we used a univariate descriptor (i.e. otolith area) and EFDs. Only the first 6 harmonics were necessary to ensure reconstruction of each otolith shape with a precision of 99.99%. This low harmonics number indicates that the otolith shape of herring larvae is quite basic compared to other species such as Atlantic cod (*Gadus morhua*), in which a complexification of the otolith form occurs at the larval stage (Irgens et al., 2018). The otolith shape is regulated by a complex combination of endogenous and exogenous factors, including both abiotic environmental parameters (such as temperature and pCO_2), and biotic parameters (such as food availability). The influence of these factors is dependent on the ontogenetic stage (i.e. the development stage of the individual). In addition, the otoliths can be different between right and left inner ears as a consequence of potential developmental lateralization (e.g. side effect). Environmental factors, especially temperature, have a greater influence than genetic differences for Atlantic cod (Cardinale et al., 2011; Hüssy, 2008; Irgens

et al., 2018). For seabass (*Dicentrarchus labrax*), increased temperature speeds up otolith morphogenesis and modifies the developmental pattern of the otolith shape (Mahé et al. 2019). Ocean acidification can also alter otolith shape (Holmberg et al., 2019). For several species, individuals exposed to high pCO_2 had a larger otolith area and maximum length compared with controls; the increases were larger than could be explained by an increase in $CaCO_3$ precipitation in the otoliths driven by the modification of the pH regulation in the endolymph (Checkley et al. 2009; Munday et al. 2011; Réveillac et al. 2015; Coll-Llado et al. 2018). In studies on other species including herring, high pCO_2 had no effect on the larval sagittal otolith (Franke and Clemmesen, 2011; Munday et al. 2011; Frommel et al. 2013; Perry et al. 2015). Our study found that the relationship between otolith area and fish length changed with environmental conditions, although this result was not observed when using GDD values. Here, we hypothesize that the increase in otolith area by fish size could mainly result from water acidification (Checkley et al. 2009; Munday et al. 2011; Réveillac et al. 2015; Coll-Llado et al. 2018), as elevated seawater pCO_2 has been shown to cause such directional asymmetry (Holmberg et al. 2019). For herring, the environmental factors we studied (temperature/pCO_2) did not affect the otolith shape between sides at the observed larval stage. Consequently, it is likely that the level of response to pCO_2 increase might be a species-specific phenomenon. This capacity to maintain otolith shape in response to environmental changes is probably due to efficient intracellular ionic-regulation mechanisms in this species (Ishimatsu et al., 2008a; Melzner et al., 2009). This result may suggest that herring larvae are well equipped to cope with the environmental changes projected for 2100, as long as their energy and nutritional needs are well covered, which would not necessarily be the case in the natural environment. The faster growth of the larvae under the 2100 scenario may indeed lead to a greater prey requirement, and it cannot be ruled out that the increase in the area of

the otoliths may also have affected their hearing ability and behaviour. Nevertheless, it should be noted that this species certainly has a good intracellular ionic regulation because it is confronted with different environments. Indeed, herring larvae typically hatch in littoral regions where pH and temperature can fluctuate because of freshwater inflow, so they may often experience such fluctuations and therefore be quite resistant to them. For more extreme values, however, the homeostatic mechanisms might not be sufficient to compensate for environmental stress and this could induce changes in otolith morphological development (Coll-Llado et al. 2018).

Ontogeny and environment are the factors that control most of the otolith development during the early stages of life. The otolith shape evolves from the basic round form to elongated shape due to faster growth along the anterior-posterior axis than along the dorsal-ventral axis (Bounket et al., 2019; Galley et al., 2006; K. Mahé et al., 2019; Mille et al., 2015). Our study showed that the otolith shape of herring grew in this way during the larval period.

At the adult stage, significant directional asymmetry has been observed in herring (Bird et al., 1986), while no lateralization has been observed at the larval stage. This bilateral effect increases over the life of the fish when considering a different trajectory of otolith morphogenesis between left and right sides. Although otolith shape is influenced by both abiotic and biotic environmental parameters and depends on individuals' genotype, environmental effects can also be perceived more with ontogeny, consequently, this developmental lateralization could be a phenotypically plastic response to environmental drivers rather than the consequence of the individual genotype, as previously suggested in *Boops boops* (Mahé et al., 2019). Sagittal otolith size and shape for each species could

therefore be adaptive traits to different habitats and ecological niches (Lombarte et al., 2010; Lychakov and Rebane, 2000).

Our study suggests that herring larvae can cope with the marine environmental conditions predicted for 2100. The growth and shape of the otoliths studied here did not appear to be affected by the environmental conditions. Only the evolution of the relationship between otolith and fish sizes seems to be environment-dependent. This information is important to evaluate the age and the growth information for herring used in fisheries management.

General Discussion

Global change is threatening life on Earth. Many modifications of the environment are happening simultaneously, for instance in the oceans which are warming and acidifying at an alarming rate. In addition to challenging marine organisms' survival, development, and reproduction, environmental changes may lead to modifications of the ecosystem structure, food web productivity, and dynamics. Anticipating the consequences of these multiple changes on species, like herring (*Clupea harengus*), with a central place and ecological role in the food web, associated with a high socio-economic importance for humans, is a priority. The survival through early life stages of fish is a key factor determining recruitment. Although research on climate change impact on fish larvae started decades ago, most studies tested the resilience of organisms towards individual stressors. Conversely, few studies have investigated the resilience of herring larvae toward combined warming and acidification, and no study has yet combined this with the importance of food availability. Therefore, the goal of this thesis was to assess the potential of herring larvae to cope with levels of warming and acidification predicted for 2100 (OWA; +3°C and -0.4 pH) and to assess the importance of food quality (particularly the carbon to phosphorus ratio of the food, and the content of an important essential fatty acid: Docosahexaenoic acid), with a special focus on the ontology of early larvae and organ development.

To properly assess the effects of potentially stressful conditions on larval development, it was of utmost importance to first of all describe normal development and maturation of larvae. In the first chapter this was exactly what was done, and I characterized the complete ontogeny and maturation of the digestive system of herring larvae, associating morphological stages to distinct anatomical organisation. The main result of this chapter was the identification of two

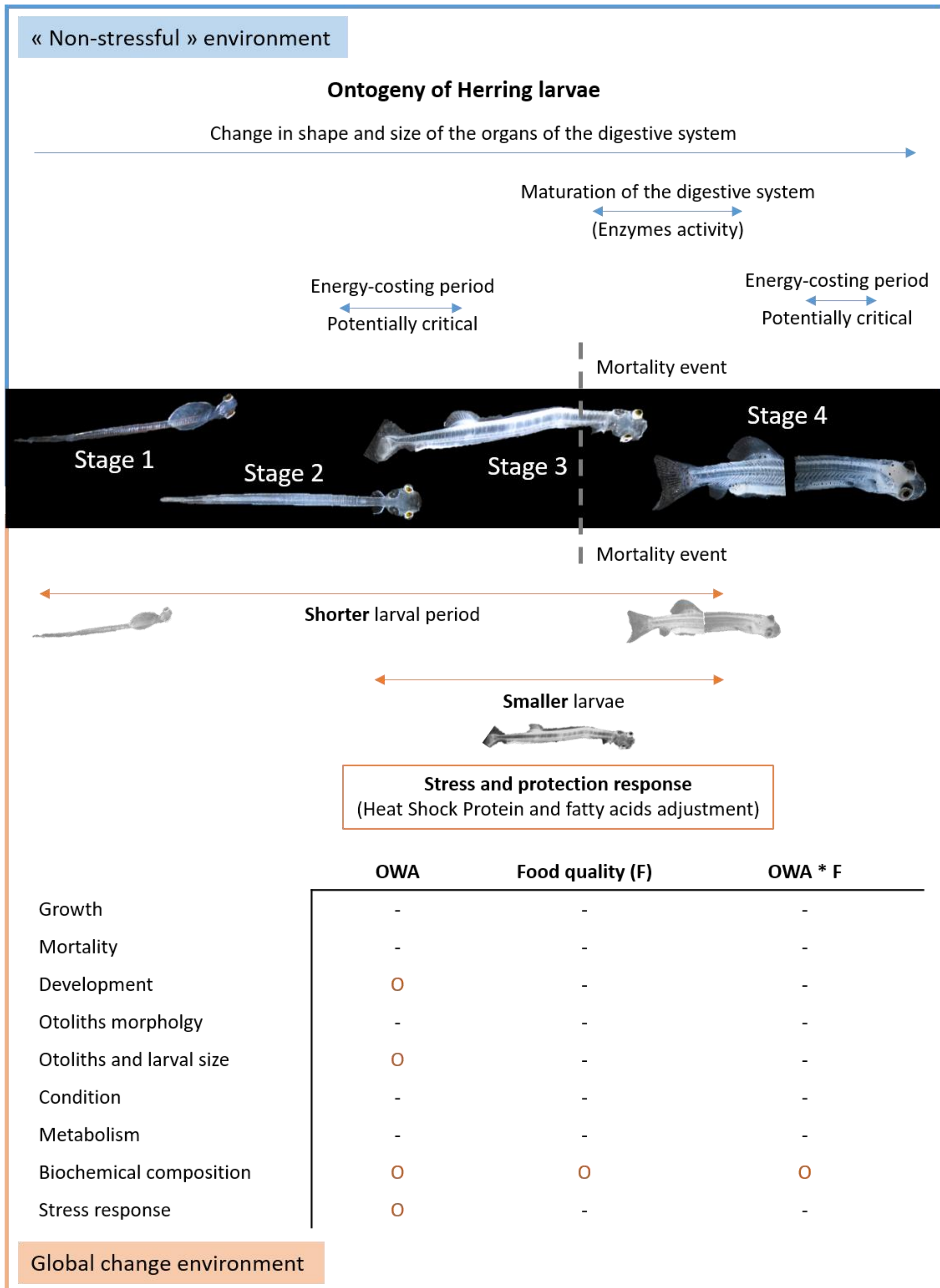
energy-costing periods, at the transition between pre-flexion and flexion stage and at the end of the larval phase, leading to an energy depletion in the liver, even if the food supply is ad-libitum. These results were then used as a reference to investigate the different developmental stages and their specific response to global change. Chapters 2 and 4 investigated the individual response in terms of somatic growth and development of herring along with otoliths formation during the larval phase. They showed that, while the scenarios tested did not induce specific mortality and growth rates, the duration of the larval stage was reduced in OWA (regardless of the food content) resulting in smaller stage 3 larvae, with larger otoliths. In addition, Chapter 2 focused on the potential critical period identified in the first chapter, and highlighted that the energetic metabolism of stage 3 larvae was not perturbed at the transcriptional level but that the OWA treatment induced a cellular stress response as evidenced by the increase in the transcription of heat shock proteins. Finally, Chapter 3 focused on the histological and lipid condition of late herring larvae, which survived the larval phase. The results showed no strong differences within organ state and amounts of energy reserves, suggesting no carry-over effect at this level. Nevertheless, analyses of fatty acids profiles suggested a potential for DHA biosynthesis in herring larvae and that the dietary supplementation with DHA does not appear to be beneficial to the larvae, particularly in view of intriguing changes in the fatty acids involved in the immune and stress response.

In the following discussion, I aim to integrate the responses found at different level of biological organisation to assess the vulnerability of herring larvae toward global change.

Herring larvae are robust when faced to Ocean Warming, Acidification and predicted changes in food quality

After investigating different responses of herring larvae toward global change scenarios, I argue that herring larvae are robust to predicted change in warming, acidification and predicted changes in food quality, provided that food resource is not limiting. Of the many physiological indicators monitored, the few that showed changes to future environmental conditions had no direct consequences on mortality, which supports that herring larvae could be resilient to global change (Figure 22).

Ocean acidification and warming were not directly lethal to herring larvae, which was not surprising as the lethal temperatures for herring larvae are between 22-24°C (Blaxter, 1960) and the acidification range tested in experiments was never lethal (Frommel *et al.*, 2014; Sswat *et al.*, 2018; Sswat *et al.*, 2018). The mortality event that occurred at the end of the stage 3 might be related to a likely delay or disruption of the maturation process of the digestive system. In the first chapter the maturation of the digestive system, with the activity of the brush border enzymes, was measured close to the first observation of stage 4 larvae in the tanks. Ocean acidification has been shown to alter otolith shape at different levels of pCO_2 (Ishimatsu, Hayashi and Kikkawa, 2008;; Vignon, 2018), which was not the case in our experiment. This may indicate an efficient ionic-regulation mechanism. In addition, snapshot investigations at the physiological level in chapter 2 and 3 revealed two underlying regulation mechanisms to cope with warming and acidification. In particular, the potential activation of an anti-inflammatory response and protection to cellular oxidative stress could be suggested based on the expression of heat shock protein and change in EPA/ARA ratio in polar lipids. Taken together, these results indicate that herring larvae have physiological regulatory mechanisms that allow them to cope with the predicted reduction of DHA in the environment (Hixson and Arts, 2016), at least at the larval stage (Chapter 3).



	OWA	Food quality (F)	OWA * F
Growth	-	-	-
Mortality	-	-	-
Development	O	-	-
Otoliths morpholgy	-	-	-
Otoliths and larval size	O	-	-
Condition	-	-	-
Metabolism	-	-	-
Biochemical composition	O	O	O
Stress response	O	-	-

Figure 22 - Summary of the principal results obtained during the thesis. In the upper blue part is represented de main findings for herring larval development, and in the lower orange part are represented the modifications observed in the experiment of global change. In blue are represented the result observed in the non-stressful environment of the Chapter 1. In comparison in orange are the results observed during the Global Change experiment (Warming, Acidification and food quality) from the Chapters 2, 3 and 4. The table at the bottom of the figure indicates the different measurements realized during the global change experiment. A black line indicates no effect of the treatment tested and on orange circle indicates a difference with the control.

Smaller fish at higher temperature: the result of phenotypic plasticity?

Whereas the OWA treatment yielded smaller larvae in stage 3, the consequences of size differences are difficult to predict. As growth and development rates are largely temperature-dependent, and there is little evidence of a link between acidification and reduced larval size (Hurst et al., 2013; Munday et al., 2009b) the role of temperature on the diminution of size-at-stage need to be explored.

The body-size temperature relationship in ectotherms

Smaller body size with warming is a commonly observed pattern for ectotherms, which is described by the temperature-size rule (Atkinson, 1994). This was supported by Daufresne and co-authors (2009) who stated that smaller body size is a universal ecological response to warming in the aquatic environment. Decline in fishes sizes related to increase in temperature have already been reported, for example in the North Sea (Baudron et al., 2014), but is not always the case (Audzijonyte et al., 2020). Because larval growth and subsequent adult size are key parameters determining fitness (survival and reproduction), researchers have investigated potential underlying mechanisms to explain the reduction in size with temperature. For instance, the “Gill Oxygen Limitation” (Pauly, 1981), and the “Oxygen- and capacity- limited thermal tolerance” hypotheses (Pörtner et al., 2017; Pörtner and Knust, 2007) state that the increasing oxygen demands with elevated metabolic rate under warming may not be met because the gills have a limited and finite capacity of oxygen supply in relation to body volume and because of lowered performances of oxygen delivery to tissue with warming. In turn, insufficient oxygen supply may limit basal metabolism and somatic growth, which has been used to predict a general decrease of fish size (Cheung et al., 2012, 2009). However, the global importance of oxygen supply to explain changes in body size has been

questioned and the above-mentioned theories received criticism (Clark et al., 2013; Jutfelt et al., 2018; Lefevre et al., 2018, 2017). Indeed, body size is a specific trait resulting from interactions of intrinsic and extrinsic factors, not only depending on oxygen availability (Audzijonyte et al., 2018). Extrinsic factors such as insufficient food availability can reduce growth and size of individuals (Clemmesen, 1994). During the Global Change experiment (Axe 2), I observed a smaller size-at-stage of herring larvae (stage 3), suggesting a similar relationship between body-size and temperature as described by the Temperature Size Rule (TSR) (Atkinson, 1994). However, although I did not measure the aerobic capacity of individuals as these were not yet using gill respiration which prevents me from excluding oxygen limitation as a cause of size reduction, the high oxygen concentration during the experiment and the absence of metabolism alteration at the transcriptional level do not support direct oxygen limitation as a cause for size reduction. The same applies to food limitation since the larvae were fed *ad libitum*. In Chapter 2, I showed that the difference in size-at-stage was not due *per se* to a reduction in growth rate, but a decoupling between developmental rate and somatic growth rate (Figure 23.A). This indicates that, when sampling by time (day), the size of the larvae would have been the same in both environments, but the stage different (Figure 23.C). However, results from the first chapter demonstrated that the morphological stages are associated with very specific anatomical organisation of the digestive system (maturation of organs and enzymes), which highlights the importance and usefulness of thermal time (degree-day) for adjusting the sampling protocol when measuring physiological traits to compare larval performance.

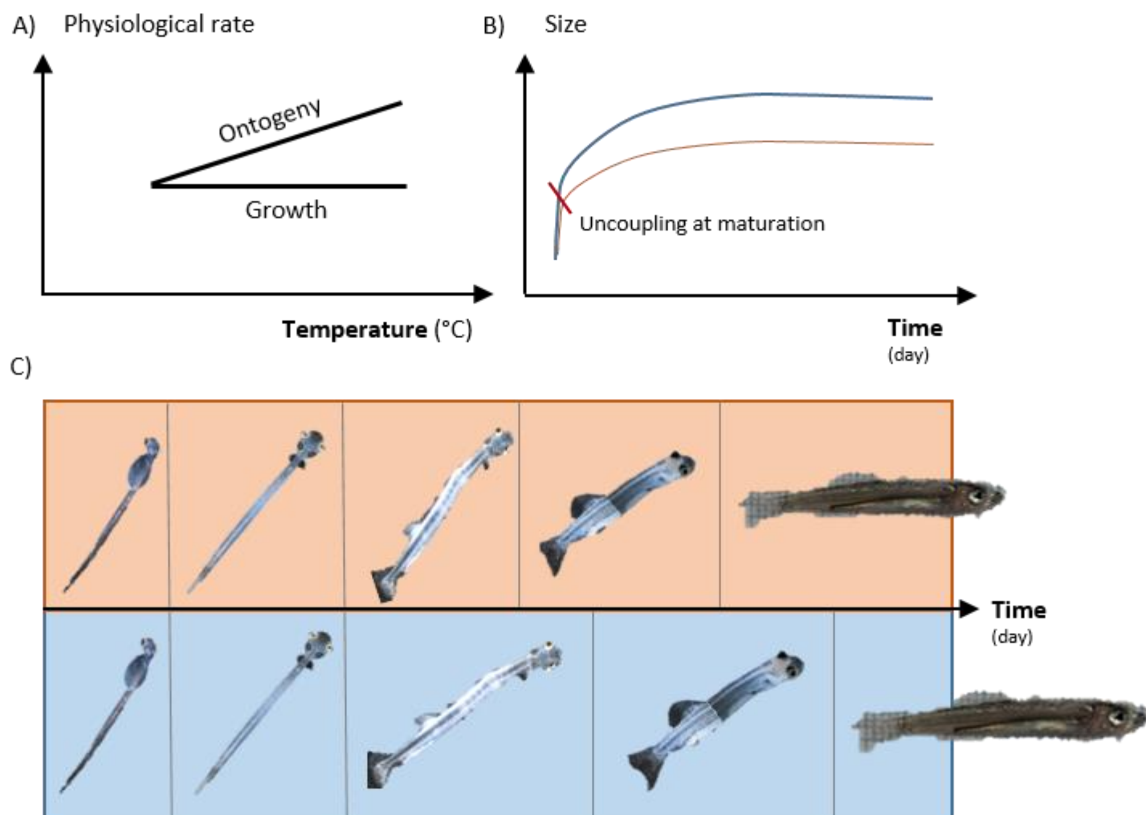


Figure 23- A) Representation of the relationship between growth rate and development rate and the temperature. B) Representation of the growth of herring and how reaching the maturity earlier in the Ocean Warming and Acidification (orange) can lead to smaller adult body size than in the Control environment (blue). C) Succession of the different herring stages in function of time in days.

The decoupling between development and growth rates is one of the first hypotheses that described Temperature Size Rule explained by a different thermal-dependence of growth and development (Forster et al., 2011). Despite numerous empirical data reporting Temperature Size Rule in various taxonomic groups, there is no general unifying explanation, and the underlying mechanisms of the temperature-body size relationship remain overall poorly understood (Audzijonyte et al., 2018). Nevertheless, as adult body size is determined by growth and development rate, I argue that the observed decrease in size-at-stage could also lead to a reduction of adult body size, because maturity will still be reached at a smaller size if the growth rate does not increase later in ontogeny (Figure 23.B, C).

Acclimation capacity of herring larvae

Both growth and development rates depend on the rate of biochemical reactions. Generally, warming increases metabolism for temperature-dependent traits, reflecting molecular thermodynamics and biochemical constraints (Havird et al., 2020). This 'passive' plasticity, or acute response, is not regulated by the individual (Ghalambor et al., 2007; Gotthard et al., 1995). Conversely, 'active' plasticity is a modification of a trait by the organism, including changes at different level of organisation, such as gene expression or membrane composition (Hochachka and Somero, 2002). The plastic response to a change in temperature generally reflects both responses, which cannot be distinguished by the type of experimental design used in this thesis, which targets the acclimation response (Havird et al., 2020). Extensive research allowed to depict a general pattern of the impact of temperature on fitness (Figure 24.A), with the performance-trait measured first increasing with increasing temperature until a maximum, reached at the thermal optimum (Topt), then performance rapidly decrease with higher temperature (Huey and Kingsolver, 1989; Sinclair et al., 2016).

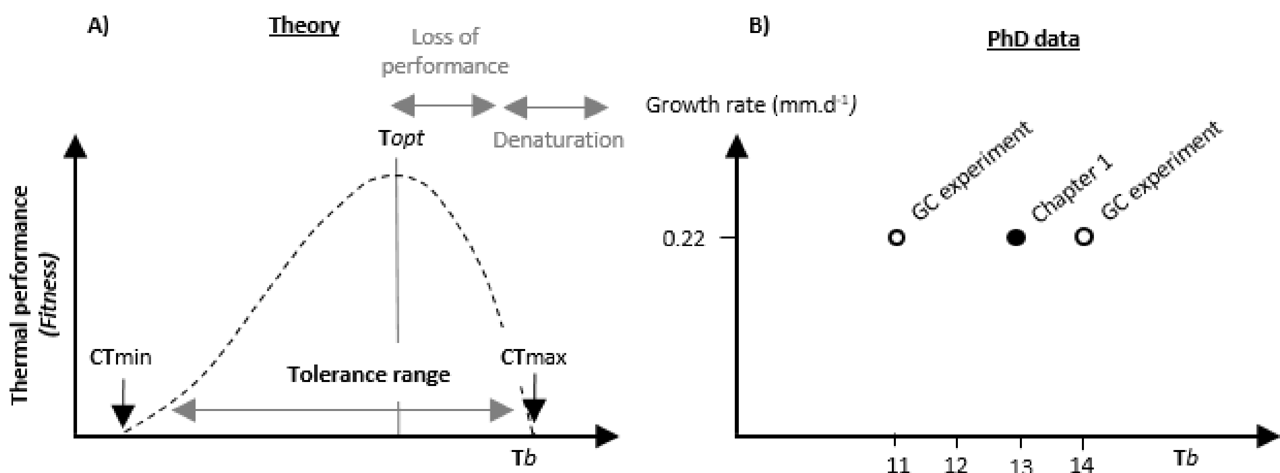


Figure 24 – A) Typical representation of a Thermal Performance Curve (TPC) (CTmin/CTmax: Critical Thermal minimum and maximum; Topt: optmal temperature; Tb: body temperature). B) Growth rate values obtained from both experiments.

The dynamic relation between a trait and temperature can be calculated as the Q_{10} coefficient, describing a factorial change of biological rate with temperature (Havird et al., 2020; Lefevre et al., 2017). A Q_{10} of 1 means that the trait is not affected by the temperature, while a value above 1 is indicative of an increase metabolism rates and a value lower than 1 imply a decrease in metabolism activity. During this PhD, herring larvae ware reared at three different temperatures with *ad libitum* feeding (Chapter 1: 13°C, Chapter 2, 3, 4: 11 and 14°C). The mean growth were all at 0.22 mm.d^{-1}, meaning a Q_{10} equal to 1 if we compare the growth between experimental temperatures (Figure 24.B). It shows that the growth metabolism was thermally independent in the temperature range tested, which suggests either that herring is a eurytherm species or that there was a complete compensation of the temperature effect. Such compensation happens when the initial passive response is counteracted and the acclimated rate returns to the original state (Havird et al., 2020). A study identified that the Critical Thermal Maximum (CTmax) of herring larvae increase with temperature (Moyano et al., 2017). These result suggest a potential acclimation which may shift horizontally the TPC curve, and modify the Topt (Angilletta and Dunham, 2003; Huey and Kingsolver, 1989; Izem and Kingsolver, 2005). Moreover, the growth of 0.22 mm.d^{-1} observed at each temperature tested in this thesis is close to the one often reported for different herring stocks or sub-components (Oeberst et al., 2009a). Experimentally reared Atlantic autumn spawner and Clyde herring larvae had a growth of 0.24 mm.d^{-1} at 8°C (Johannessen et al., 2011) and 0.22 at 9.5°C respectively (Ehrlich et al., 1976). In the field, western Atlantic spring spawning herring larval growth was of 0.22 mm.d^{-1} at 6°C (Campana and Moksness, 1991) and for Downs automn spawning of 0.165 mm.d^{-1} at 8°C (Hempel, 1960) and recently measured at 0.26 mm.d^{-1} in a water between 6.7 and 10.7°C in the study area (Denis et al., 2017). Overall, herring larvae may have an important capacity of phenotypic plasticity to cope with different

temperatures, suggesting that the early life stages of this species may be little affected by ocean warming.

Decoupling between development and growth rate: a trade-off in the energetic budget?

Energy budget: is the size reduced in advance or as a consequence?

Late herring larvae were smaller-at-stage, which may be caused by a reduction in energy allocation toward growth. Indeed in the second chapter I showed that the decoupling between growth and development happened between the stage 2 and 3. When looking at the thermal time (i.e. at the same physiological timescale), in the Control treatments the growth was increased between stage 2 and 3, and it was not the case in the OWA treatments. The period of transition between stage 2 and 3 is particularly energy demanding, and has been identified as potentially critical in the first chapter. As during larval ontogeny, energy allocation is mainly divided between maintenance and somatic growth, I suggest that in OWA more energy was directed toward basal metabolism and particularly cell and organism protection. Indeed, we found in the chapter 3 that larvae in the OWA environment expressed high level of heat shock protein transcripts and in the chapter 4 that the fatty acid composition of the membranes suggested an inflammatory response. Hence, I suggest that the reduction in growth is the result of a trade-off between growth and health maintenance. Combined warming and acidification could then be responsible for the mismatch between development and growth rate at the larval stage, and further compromised herring population.

Linking field and experimental ecology: is the transition between pre-flexion and flexion stage the most critical period of herring larval ontogeny?

As previously highlighted, the transition between the developmental stage 2 and 3 was identified as critical in the chapter 1 because of energy consumption and in chapter 2 because of the potential trade-off in energy allocation between growth and cellular protection. Linking chapter 2 and 4 (Figure 25) highlights that the decoupling between growth and development rate was associated with the decoupling between otolith area and fish size. Studies have shown that otolith growth is more conservative and regular than somatic growth (Neuman et al., 2001; Reznick et al., 2011). The transition between stage 2 and 3 (pre-flexion and flexion stage) was marked in each experiment (at each temperatures) around 13 and 14 mm. This is particularly interesting since the size of 13 mm was identified for field Downs herring to be critical (Denis et al., 2017).

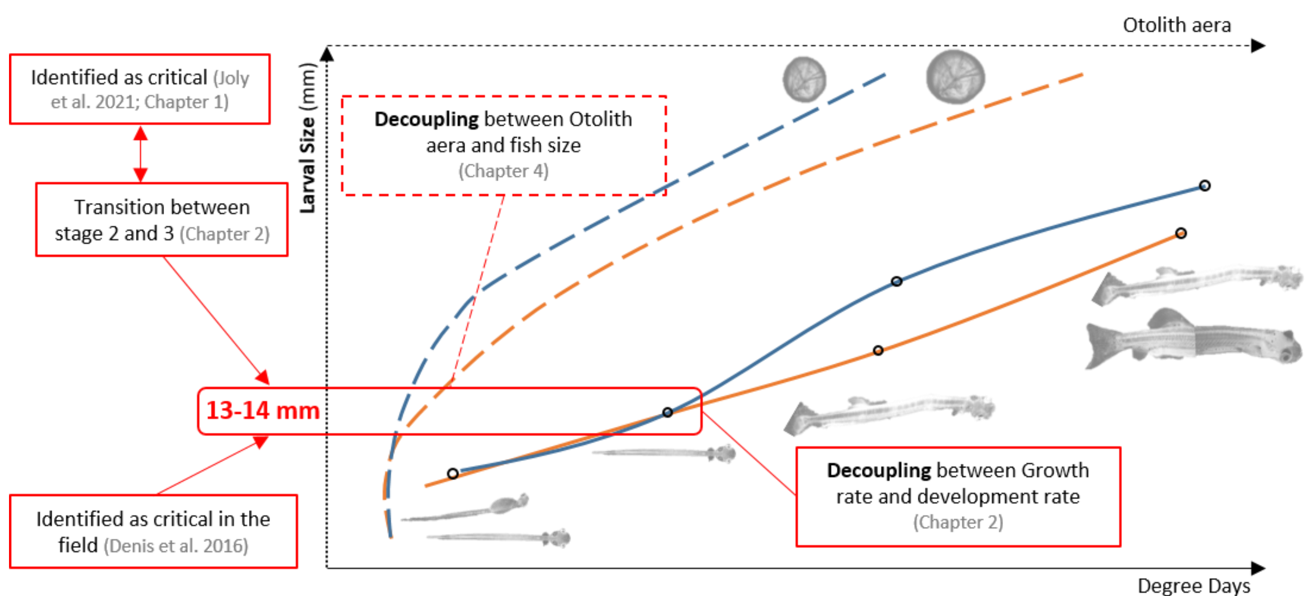


Figure 25 – Representation of the result of the chapter 1, 2, 4 and a field study to highlight the role of the transition between the pre-flexion and flexion stage

Another study on field western Baltic herring larvae obtained similar results, and identified a significant period of mortality between the pre-flexion and flexion stage (Dodson et al., 2019). The flexion stage is the period where the fin development is intensified, especially for the caudal and the dorsal ones (Doyle, 1977). Anatomical study on herring larvae also showed that this period corresponds to the development of new white muscle fibres (Johnston and Cole, 1998). At the organ level during this stage, the pancreas and liver increase in size, the stomachal cells start to develop and the villi of the intestine is more pronounced (Chapter 1). The sum of these physical changes likely yields a high energetic demand in a stage at which the digestive system, which supports energy acquisition, is not fully mature (Chapter 1).

The transition between pre-flexion and flexion stage is certainly a bottleneck period for herring. This period is not usually defined as critical for fish larvae. Indeed, research has mainly be directed toward Hjort's critical period hypothesis at the transition from endogenous to exogenous nutrition (Hjort, 1914b). Although his work has laid the foundation for understanding recruitment in fish, there is a need to move forward the idea that one short period is determining the entire recruitment success (Houde, 2008). We suggest based on our results, that for a better understanding of herring larval recruitment, the condition of herring larvae in relation to the abiotic and biotic environment at stage 2 and 3 must be investigated. As the condition in stage 3 should be partly the result of the condition at the previous stage, a more integrative approach of larval condition in the field could help to identify key parameters for recruitment success and thus to better estimate the vulnerability of the larvae toward global change.

Ecological relevance and implication of the findings

Herring larvae reared in warming, acidification and lower food quality treatments were resilient, only presenting a reduction in size-at-stage for the stage 3, which I attribute to the stress cellular responses of the organisms. While I focused on developing herring larvae, previous work on herring eggs incubated in warming and acidification conditions showed reduced size at hatch (Leo et al., 2018). This implies that by only subjecting the larvae to different environmental conditions, the results may underestimate the consequences of OWA. Furthermore, a recent phylogenic study showed that smaller herring are found in warmer waters, suggesting that size change is an adaptive response toward warming environment (Avaria-Llautureo et al., 2021). Verberk and co-authors (2021) stated that under favorable experimental conditions (unrestricted food) fish could have evolved to express a canalized growth response to prevent oxygen limitation, suggesting that conditions experienced by previous generations could lead to selection for adaptive change in growth trajectory when facing warmer condition. It is also possible that the observed global change effects may be stronger when food is insufficient. Indeed, feeding level influenced the growth response of organisms exposed to warming and/or acidification (Cominassi et al., 2020; Stiasny et al., 2019; Thomsen et al., 2013), and herring growth in the field has been reported to be limited by food supply (Paulsen et al., 2017). Although growth rates were not different than those quantified in the field (Denis et al., 2017; Oeberst et al., 2009b), *ad libitum* feeding conditions in my experiments might have allowed the larvae to respond to the stress and still develop without significant loss of performance. I suggest that further studies should investigate the growth response and condition of herring larvae at different restricted feeding levels or with more natural feeding level in mesocosm, it could shed light on the strategy of energy allocation and further constraint on fitness. This is particularly relevant in the context

of a recent study indicating that, as phytoplankton productivity and cell size decreased, the energy expenditure directed toward feeding increased which was linked to size reduction and overmortality of sardine (Queiros et al., 2021). Reduction in size and survival of herring larvae could have severe repercussion on herring recruitment as a small size is linked with a high predation pressure and mortality (Meekan et al., 2006). Decrease of size at the individual level, could lead to decrease in size at the population level and have repercussion on the entire ecosystem structure (Daufresne et al., 2009). Trophic interactions within the ecosystem could be modified as well as the herring population abundance, and it could lead to change in fisheries catch, compromising human food supply and causing socio-economical issues (Cheung, 2018).

In conclusion, herring larvae seem well equipped to cope with the combination of warming, acidification and reduced DHA availability. The strong compensation revealed at the molecular, biochemical and physiological level may suggest a fragile trade-off. While further research must focus on the restricted availability of preys to assess whether herring larvae would still be able to cope, field research need to be conducted in parallel to better understand which parameters are currently affecting recruitment. The combination of experimental and field research could improve our understanding of recruitment dynamics and better predict the impact of global change on recruitment and the future of the North Sea herring population.

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