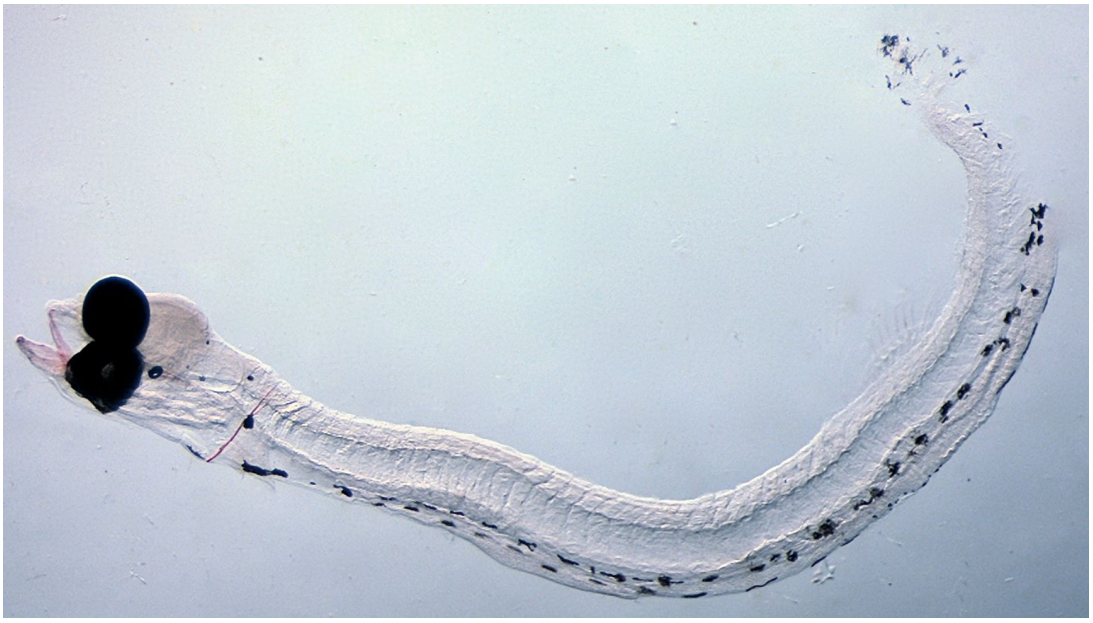
GEOMAR
Helmholtz-Zentrum für Ozeanforschung Kiel

Master Thesis:

The impact of ocean acidification on the skeletal ossification in herring larvae (*Clupea harengus*, L.)



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List of abbreviations:

$CaCO_3$ ..... *calcium carbonate*
CH_4 ..... *methane*
cm ..... *centimeter*
CO_2 ..... *carbon dioxide*
CO_3^{2-} ..... *carbonate*
CTD ..... *conductivity-temperature-depth-casts*
DD ..... *degree-days*
DIC ..... *dissolved inorganic carbon*
dph ..... *days post hatch*
H^+ ..... *proton*
H_2CO_3 ..... *carbonic acid*
HCO_3^- ..... *bicarbonate*
IPCC ..... *Intergovernmental Panel on Climate Change*
IWS ..... *integrated water sampler*
KOH ..... *potassium hydroxide*
kPa ..... *kilopascal*
m ..... *meter*
mm ..... *millimeter*
N_2O ..... *nitrous oxide*
OA ..... *ocean acidification*
pCO_2 ..... *carbon dioxide partial pressure*
ppm ..... *parts per million*
SD ..... *standard deviation*
SL ..... *standard length*
TA ..... *total alkalinity*
µatm ..... *mikro atmosphere*
µm ..... *mikrometer*

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1. Abstract:

As burning of fossil fuels and changes in land use are on-going, anthropogenic carbon dioxide is accumulating and causes the pH of the world`s oceans to decrease, and the partial pressure of carbon dioxide (pCO_2) in surface waters to increase. Adult teleost fishes are believed to be largely unaffected by this process known as ocean acidification. Their well-developed gills and kidneys are very effective in acid-base regulation and thus are capable of coping with strong changes of pCO_2. However, unlike the adult stages, larval stages with not yet developed gills and kidneys are believed to be very sensitive to changes in pH. Many studies examined detrimental effects of ocean acidification on the development, growth and survival of larval fishes. However, there is a lack of understanding on how their skeleton, which is composed of calcium phosphate, develops and ossifies under elevated pCO_2.

The present thesis intends to close this knowledge gap by examining the growth and skeletal development of Atlantic herring larvae (*Clupea harengus*, L.) in three completely different attempts. Herring larvae were exposed in three experiments (KOSMOS 2015, Espegrend 2010 and Kristineberg 2013) to distinctively different levels of pCO_2 (2200, 1830 and 1000 µatm) and prey densites (5, 2000 and 131 - 600 prey L^{-1}). The results showed that positive growth of herring larvae depended largely on sufficient food availability and the intensity of pCO_2 values. Elevated pCO_2 levels of 1830 µatm and 2200 µatm result in diminished growth, the lowest level of 1000 µatm had no negative effect on larval growth, assuming that they were already adapted to similar natural pCO_2 levels. However, all levels of pCO_2 impacted the skeletal development of the shoulder girdle and the upper jaw of the larvae, which could have negative consequences on foraging and survival. Further this study could show that somatic growth and bone growth differed in their responses to food and pCO_2 stress, assuming developmental decoupling.

All results indicated, although statistically not significant, that ocean acidification has the tendency to negatively affect growth and skeletal development in herring larvae, which could further lead to severe negative consequences on survival and stock recruitment.

1. Zusammenfassung:

Das Verbrennen fossiler Rohstoffe und die Intensivierung der Landnutzung führte dazu, dass der menschengemachte CO_2 Ausstoß den pH Wert der Ozeane herabsenkte und den pCO_2 Wert in den oberen Wasserschichten erhöhte. Es wird angenommen, dass adulte Teleostei bei diesem Prozeß der Ozeanversauerung nicht beeinträchtigt werden. Sie besitzen gut ausgebildete Kiemen und Nieren, die den Säure-Base-Haushalt effizient regulieren und starke Schwankungen im pCO_2 ausgleichen können. Doch larvale Stadien, die noch keine Kiemen oder Nieren ausgebildet haben, gelten als besonders anfällig für Ozeanversauerung. Viele Studien zeigten bereits nachteilige Effekte der Ozeanversauerung auf das Wachstum, die Entwicklung und das Überleben der Larven. Es gibt allerdings noch kaum Studien, die sich mit dem Einfluss von Ozeanversauerung auf die larvale Entwicklung und Verknöcherung des Skeletts, welches aus Calciumphosphat besteht, beschäftigen.

Die vorliegende Arbeit versucht diese Wissenslücke zu schließen, indem das Larven- und Knochenwachstum des Herings (*Clupea harengus*, L.) in drei unterschiedlichen Versuchsansätzen untersucht wird. In dem KOSMOS 2015-, dem Espegrend 2010- und dem Kristineberg 2013 Experiment wurden Heringslarven in unterschiedlichen pCO_2 Konzentrationen (2200, 1830 und 1000 µatm) und Futterdichten (5, 2000 und 131 – 600 Beute L^{-1}) gehalten. Die Ergebnisse zeigten, dass positives Wachstum stark von ausreichender Futterdichte und der Intensität der pH-Wert Veränderung des Wassers abhängt. Bei den pCO_2 Werten 1830 µatm und 2200 µatm wurde negatives Wachstum, bei 1000 µatm hingegen positives Wachstum ermittelt, was darauf hindeutet dass die Larven bereits an ähnliche Werte angepasst sind. Das Wachstum des Schultergürtels und des Oberkiefers ist bei allen pCO_2 Werten vermindert, was negative Auswirkungen auf die Beweglichkeit und auf die Nahrungsaufnahme haben könnte. Des Weiteren konnte eine gewisse Entkopplung festgestellt werden, da das somatische Wachstum und das Knochenwachstum unterschiedich auf Nahrungslimitation und Ozeanversauerung reagierten.

Alle Ergebnisse weisen darauf hin, wenn auch nicht statistisch signikant, dass Ozeanversauerung das Larven- und Knochenwachstum in Heringen mindert und somit negative Auswirkungen auf das Überleben und die Rekrutierung hat.

2. Introduction:

2.1 Ocean Acidification:

Human influence on earth´s climate system is distinctive and recent anthropogenic emissions of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are the highest in history (IPCC, 2014). The burning of fossil fuels, changes in land use and population growth especially lead to an increase of atmospheric CO_2 concentration from pre-industrial levels of about 280 parts per million (ppm) to levels of 400 ppm in 2014 (Le Quere *et al*., 2009; www.esrl.noaa.gov/gmd/ccgg/trends/). Estimates based on the Intergovernmental Panel on Climate Change (IPCC) business-as-usual emission scenarios suggest an even further increase of 0.5% per year over the next 100 years to a level of 800 mikro atmosphere (µatm) at the end of the century (Figure 1; Kleypas *et al*., 2006; Feely *et al*., 2009a, b).

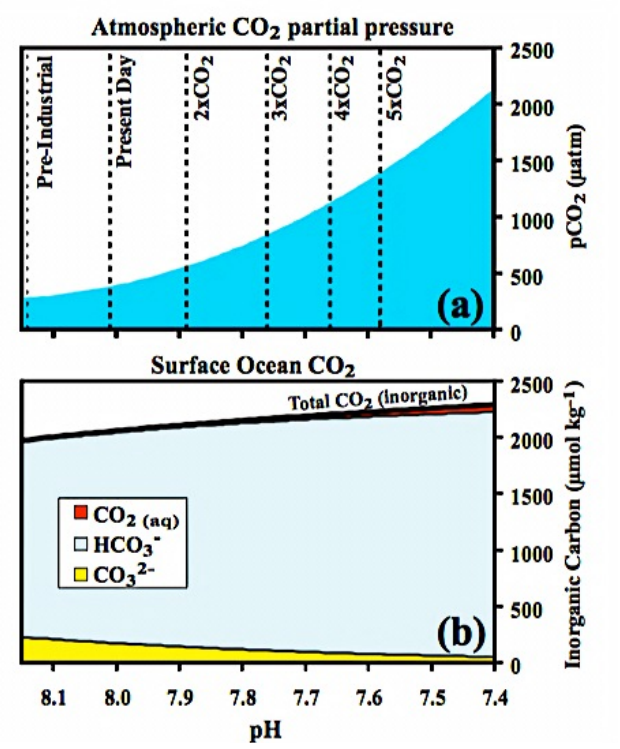


Figure 1: Increasing atmospheric CO_2 (a) partial pressure and (b) associated changes in the surface ocean carbonate chemistry (after Kleypas *et al*. 2006).

Only half of this excess anthropogenic CO_2 remains in the atmosphere and 20% of it are taken up by the terrestrial biosphere. The rest of about 30% is absorbed by the world´s oceans and is drastically changing the seawater chemistry (Feely *et al*. 2004). Model projections (IPCC A2 scenario) indicate that seawater pH will drop from pre-industrial means of 8.2 to 7.8 (pCO_2 - 1000 µatm) by the year 2100 and even further to 7.4 (pCO_2 - 2000 µatm) by the year 2300

(Figure 1; Caldeira and Wickett, 2003, Sabine *et al.*, 2004; 2005; Feely *et al.*, 2009a, b; Kleypas *et al.*, 2006; IPCC, 2014).

Carbon dioxide reacts naturally with seawater and forms carbonic acid (H_2CO_3), which dissociates to bicarbonate (HCO_3^-) and further to carbonate (CO_3^{2-}). Each process generates one free hydrogen ion (H^+), which, if free available, lowers the pH value and reacts with CO_3^{2-} to HCO_3^-. This in turn lowers the carbonate concentration and the calcium carbonate saturation state of the biologically important $CaCO_3$ minerals calcite and aragonite in a process commonly referred to as ocean acidification (OA) (Caldeira and Wickett, 2003, 2005; Kleypas *et al.*, 2006; IPCC, 2014; Feely *et al.*, 2009, Orr *et al.*, 2005). This furthermore results in an increase of total alkalinity (TA) in the upper ocean, favored by the extent dissolution reaction of marine carbonates, like biogenic magnesium calcites (from coralline algae), aragonite (from corals and pteropods) and calcite (from coccolithophorids and foraminifers) (Figure 2; Feely *et al.*, 2009; Melzner *et al.*, 2011).

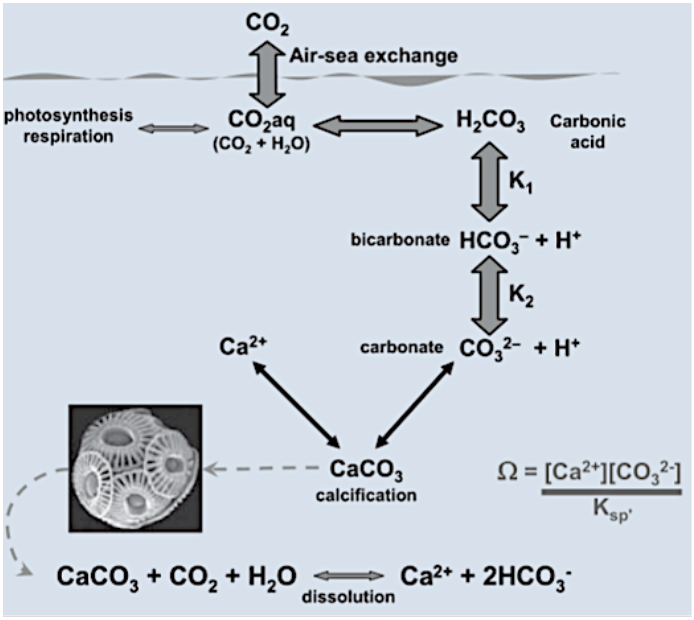


Figure 2: Scheme of the carbonate system in seawater, K_1 and K_2 are the dissociation constants for H_2CO_3 and HCO_3^- (after Kleypas *et al.*, 2006).

The decline of the calcium carbonate saturation state with decreasing ocean water pH will lead to under-saturated oceans with respect to aragonite. The largest projected decreases in aragonite saturation between 1865 and 2095 are predicted in warm tropical and subtropical waters (Figure 3; Feely *et al.*, 2009b). This implies that future low latitude conditions could stress tropical species by significantly altering the conditions to which they have adapted. However, large changes in carbonate chemistry relative to historical conditions (aragonite and

calcite saturation states) are highest in the high latitudes, which might have large impacts on high-latitude calcifying planktonic and benthic organisms. Particularly cold waters, like the Arctic Ocean, with high primary productivity and melting of sea ice are affected by future ocean acidification (Bellerby *et al*., 2005; Fabry *et al*., 2009; Denman *et al*., 2011).

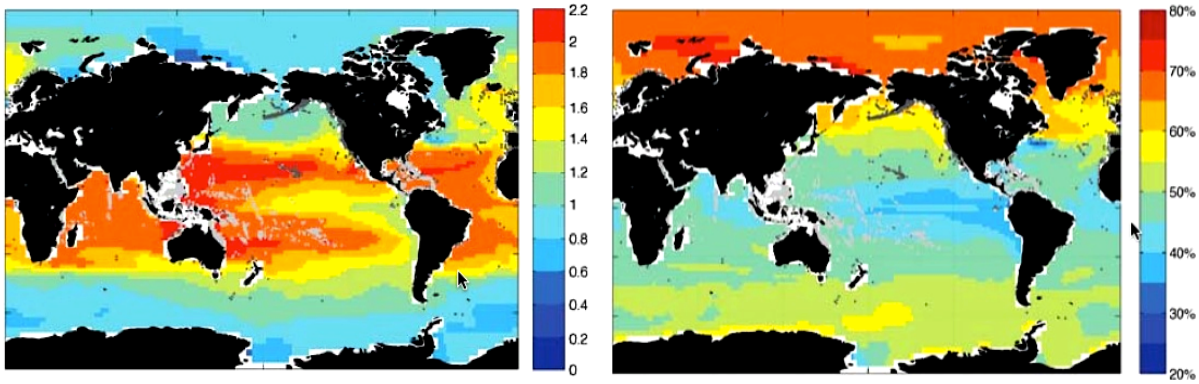


Figure 3: For the Intergovernmental Panel on Climate Change Special Report on Emissions Scenarios A2 scenario, (left) the CCSM-modeled decrease in surface aragonite between the decades centered around the years 1875 and 2095 and (right) the CCSM-modeled percent decrease in surface aragonite between the decades centered around 1875 and 2095. Deep coral reefs are indicated by darker gray dots; shallow-water coral reefs are indicated with lighter gray dots. White areas indicate regions with no data (after Feely *et a*l., 2009b).

All these scientific results of modeled elevated CO_2 uptake in the ocean show that the reduction of pH and lowering the $CaCO_3$ saturation states of aragonite and calcite in the upper ocean are ongoing already and have led to a decrease in ocean pH by 0.1 units since pre-industrial times. Knowing the chemical consequences of CO_2 uptake and ocean acidification, it has to be considered as a major threat to marine organisms, but the consequences of ocean acidification are not fully understood for most physiological processes and species to date. It is also unclear whether or not the declining carbonate saturation state or the decreasing ocean pH is causing the physiological impairments observed in experiments. Marine calcifying organisms, in particular, such as coccolithophores, foraminifers corals and pteropods, which construct elements of their body out of calcium carbonate have to cope with negative effects of OA, such as reduced calcification (Langdon, 2002; Fabry *et al*., 2008; Kuffner *et al*., 2008). Elevated pCO_2 reduces the ability to calcify particularly in reef building corals, crustose coralline algae, mussels, echinoderms, crustaceans and other benthic invertebrates that possess skeletal elements made of $CaCO_3$ (Kurihara and Shirayama, 2004; Gazeau *et al*., 2006; Langdon, 2002; Fabry *et al*., 2008; Fabricius *et al*., 2011; Kuffner *et al*., 2008; Dupont *et al*., 2008). Owing to their highly soluble aragonite shells, pteropods may be even more sensitive to ocean acidification. Severe shell dissolution within 48h after exposure to

undersaturated water conditions projected for Southern Ocean surface waters in the year 2100 was observed (Feely *et al.,* 2004; Orr *et al*., 2005). Nevertheless, species-specific responses are likely and it is possible that calcification rates of some species may also be insensitive to elevated pCO_2, as has been found for coccolithophores (Riebesell *et al*., 2000; Langer *et al.,* 2006). Recent data showed for several species a general capacity to evolve a tolerance to OA (Munday, 2009; Reusch, 2014). In addition to calcification, a number of other physiological parameters appear to go hand in hand with the capacity of acid-base balance (Fabry *et al*., 2008). Many recent studies on non-calcifying marine animals agree upon, that elevated pCO_2 can have detrimental effects on survival, growth, development, metabolism, pH-balance, and respiratory physiology (Seibel and Walsh, 2001; Rosa and Seibel, 2008; Munday *et al*., 2009a, b). Other studies find no such effect, showing that species- and even population-specific responses are likely.

2.2 Impact on marine larval fish:

Adult marine teleost fishes, in contrast to calcifying protists and invertebrates, have developed an effective acid-base regulatory mechanism, which allows them to accumulate bicarbonate and exchange ions across gills under hypercapnic conditions (Pörtner *et al*., 2005; Ishimatsu *et al*., 2008; Melzner *et al*., 2009; Gilmour and Perry, 2009). However, early life stages have not yet developed gills and kidneys in order to regulate and maintain their internal ionic environment, but have ionregulatory chlorid cells in the yolk sac membrane and body skin (Morris, 1989; Sayer *et al*., 1993, Katoh *et al*., 2000; Ishimatsu *et al*., 2004). Gas and ion exchange in embryos and larvae initially takes place on the surface of the skin (Rombough, 2004). Therefore, early larval stages are expected to be highly sensitive, which could lead to an inability to cope with changes in the pH and as a consequence may constitute to a reallocation of energy resources away from vital processes such as growth and development (Frommel *et al*., 2014). This would also have severe consequences on population size and further on stock recruitment.

However, studies revealed that effects of OA on marine fish can be very variable, depending on the fish species, the stage in life history, the fish's habits and exposures to natural fluctuations in CO_2 from upwelling or eutrophication (Miller *et al*., 2012; Bignami *et al*., 2013). Several studies reported minor or no effects of ocean acidification on the larvae of some fish species (Munday *et al.,* 2009a, 2011, 2015; Frommel *et al.* 2013; Franke and Clemmesen, 2011; Hurst *et al.,* 2013, 2015; Harvey *et al*., 2013; Flynn *et al*., 2015; Perry *et al*., 2015). For others, elevated pCO_2 had a direct major negative effect on hatching, survival,

growth, metabolism, behavior and development with even severe caudal vein angio-architecture changes (Baumann *et al*., 2012; Ishimatsu *et al*., 2004; Frommel *et al*., 2012, 2014; Bignami *et al.,* 2013; Pimentel *et al*., 2014a; Munday *et al.*, 2014; Ahnelt *et al*., 2015). Frommel *et al*. (2013), for instance, showed that eggs and larval stages of the commercially important Baltic cod, *Gadus morhua*, are robust and probably already adapted to high levels of OA (1400 ppm and 4000 ppm), by showing no effects on hatching, survival, development and otolith size. Whereas in another study by Frommel *et al.* (2012) Atlantic cod larvae exposed to OA (1800 ppm and 4200 ppm) showed severe tissue damage in many internal organs and gained more weight than under ambient conditions. In the same study, Maneja *et al.* (2013) found increased otolith growth, which could affect the acoustic functionality of the fish, such as sensitivity, temporal processing and sound localization.
The impact in pCO_2 on other commercially important fish like the Atlantic herring (*Clupea harengus*, L.) was examined only in few studies, which will be introduced in the next chapter.

2.2.1 Impact on Atlantic herring larvae:

The Atlantic herring (*Clupea harengus,* L., Clupeiformes, Clupeidae) is a demersal spawner, which lays sticky eggs on gravel/rocks or macroalgae on the seabed near coasts or on offshore banks (Blaxter 1990). The hatching of herring larvae occurs usually up to 3 weeks after the fertilization process (ICES, 2009) and depends on the water temperature, which is expressed in degree-days (DD= the number of days multiplied by the temperature experienced). In herring hatching occurs at about 120 DD, which means at a water temperature of 8°C that herring will hatch after 15 days (Arild Folkvord, personal communication). The schooling of the herring larvae and juveniles is mostly pelagic but also close inshore, while adults are found more offshore (Whitehead, 1985). Adult herring schools have a wide distribution throughout the temperate northern Atlantic (80°N-33°N, 79°W-70°E), in which they perform regular seasonal migrations as part of their spawning and feeding cycle in coastal and respective open water regions (Whitehead, 1985; Maravelias *et al*., 2000). Hence, the herring have various (sub-) population groups, which are characterized by these distinct spawning seasons and locations (Geffen, 2009). These distinct spawning, hatching, and wintering grounds of the Atlantic herring are in areas, which are predicted to be severely affected by ocean acidification. The effect of OA in coastal hypoxic regions was shown for example for the Kiel Bight, which will face higher pCO_2 values above 0.4053 kilopascal (kPa), as heterotrophic degradation of organic material is related to the carbon dioxide production

(Melzner *et al*., 2012). Therefore, it can be stated that nearly the whole life cycle and especially the very critical early life stages of the Atlantic herring may be affected by OA.

Frommel *et al.* (2014) showed that high CO_2 concentrations (1800 and 4000 ppm) can negatively affect herring larvae by inducing stunted growth and decreasing development and severe conditions by damaging the tissues in kidney, pancreas, liver and in the external structures of the fins, like finrays. An earlier study by Franke and Clemmesen (2011) states that elevated pCO_2 neither affects the embryogenesis nor the hatching rate of Baltic herring, which could very well be an adaption to natural high pCO_2 levels (2300 µatm) in surface waters during spring spawning seasons. In the same study they revealed almost no effects on the hatching rate, but negative effects on the RNA/DNA ratio, which furthermore could lead to a decreased protein biosynthesis.

The herring is characterized as a facultative zooplanktivorous filter feeder, which migrates vertically at daylight in order to feed mostly on copepods like *Calanus* spp., *Temora* spp., copepodites, nauplii larvae, cyclopoids but also on microzooplankton such as ciliates, dinoflagelates and tintinnids (Maravelias *et al.,* 2000; Gamble *et al*., 1985; de Fugueiredo *et al*., 2005). Thereby the dietary preferences change in relation to the size and age of the larvae during ontogeny, but are mostly in a size range of 68-350 mikrometer (µm) (Blaxter, 1965; Houde, 2008). Shifts in phytoplankton abundances under acidified conditions, as shown for diatoms (decreasing) and coccolithophores (increasing) (Riebesell, 2004) can alter the phytoplanktons fatty acid composition, which can be translated into higher trophic levels like copepods, leading to a reduction in growth rate and egg production (Jonasdottir *et al*., 2009; Rossoll *et al.*, 2012). This in turn could also have negative effects on the growth of herring larvae and adults.

2.3 Impact on the skeletal development and ossification in fish larvae:

Ontogenetic data and information about the skeletal development and ossification for many species are still poorly explored or are lacking, in case of the Atlantic herring, entirely. The skeleton in general is composed of calcium phosphate, in the form of hydroxyapatite and cartilaginous material (Lall *et al*., 2007). Under acidified conditions additional buffering of the tissue pH with bicarbonate and non-bicarbonate ions is expected, which may interfere with larval skeletal development (Pimentel *et al*., 2014b). To our knowledge, Pimentel *et al.* (2014b) is the only study that comments on the effect of OA on the skeletal development. They found that early life stages of *Solea senegalensis* exposed to combined future conditions

like warming and elevated pCO_2, suffer from several types of malformations of the vertebral column, which probably declines the species fitness immensely. They also report, independently from the skeletal deformities, a massive decline in hatching success and larval survival, which would have severe consequences for the adult fish population.

Among the first bones to appear in the ontogeny of fishes is the cleithrum (shoulder girdle), which is a dermal bone (Hilton, 2011; Mattox *et al*. 2014). A dermal bone is a type of bone, which is not preformed in cartilage, but develops directly in contact with the dermis. The cleithrum is a thin and medially curved vertical bony splint, which extends from the level above the notochord to the ventral margin of the body and gets more robust with growth (Mattox *et al*., 2014). The cleithrum serves as the attachment site of the pectoral fins, which are important for the first swimming movements and mobility to approach suitable prey at the critical first-feeding stage as postulated by J. Hjort (Houde, 2008).

The jaws are also dermal bones and among the first ones to develop. They consist of the maxilla and premaxilla, both in the upper jaw, and the dentary and the anguloarticular bone, both in the lower jaw (Hilton, 2011). The maxilla starts to ossify as a toothless splint in the lateral region of the upper jaw and is connected by a ligament with the dentary (Cubbage and Mabee, 1996; Mattox et al., 2014). The dentary first appears around the anterior end of Meckel`s cartilage and starts to ossify by forming a sheath of bone (Cubbage and Mabee, 1996; Mattox *et al*., 2014).

A well-developed and functional jaw plays also an important role at the first-feeding stage of herring larvae (Houde, 2008).

2.4 Questions and Hypotheses:

This thesis intends to close the knowledge gap on possible consequences of ocean acidification on the ossification of larval Atlantic herring. Is there a direct negative effect of elevated pCO_2 on the first developing bony structures, like the cleithrum, maxilla and dentary? Thus, what are further consequences for their growth and development?
The resulting hypotheses are as follows.

Hypotheses I: Growth of herring larvae (*Clupea harengus*, L.) under elevated CO_2 concentrations (pCO_2 – 1000 to 2200 µatm) is impacted.

Hypotheses II: The skeletal development (ossification) of herring larvae (*Clupea harengus*, L.) under elevated CO_2 concentrations (pCO_2 – 1000 to 2200 µatm) is impacted.

In order to investigate these hypotheses and questions, Atlantic herring larvae from three different CO_2 experiments (KOSMOS 2015, Espegrend 2010, Kristineberg 2013) were analyzed.

3. Material and Methods:

The following chapter describes the material and methods used for the mesocosm experiments in 2015 (KOSMOS 2015), the land-based mesocosm experiments in 2010 (Espegrend 2010) and the laboratory experiments in 2013 (Kristineberg 2013).

3.1 KOSMOS 2015:

3.1.1 Study site and experimental set up:

The KOSMOS Experiment 2015 was conducted at the University of Bergen`s Marine Biological Station near Espegrend (Norway) next to the Raunefjorden (60°16´18" N, 5°10`26" E) from 3th of May 2015 to 30th of June 2015 (Figure 4). Research groups of Prof. Dr. Ulf Riebesell (Geomar, Kiel) and Dr. Catriona Clemmesen-Bockelmann (Geomar, Kiel), especially Michael Sswat, collaborated in this experimental approach.

Figure 4: Location of the Raunefjorden in West Norway (top) and Location of the mesocosm facilities in the Raunefjorden (bottom). Small numbers indicate the water depth in meter. Colored circles indicate mesocosms M1-M8 (blue: ambient mesocosms; red: high CO_2 mesocosms; modified after Riebesell, 2015).

The Kiel Off-Shore Mesocosm for Future Ocean Simulations (KOSMOS) is a mobile sea-going mesocosm facility designed to establish natural conditions in which environmental factors can be manipulated and closely monitored (Riebesell *et al*., 2013). Each mesocosm unit consists of a floatation frame, the mesocosm bag, a bottom shutter, a sediment trap, a dome-shaped hood covered with metal spikes on top of the floatation frame, and weights (Figure 5). The latter are at the bottom of the floatation frame and at the lower end of the bags to maintain an upright position when exposed to wind and wave activity. The mesocosm bag enclosures a 21m deep-water column of the surrounding fjord with a diameter of 2m (~63m^3 in volume) without disturbing the vertical structure or manipulating the natural plankton community.

In this particular experimental approach, eight mesocosms were used in which the manipulated environmental factor was CO_2. As seen in Figure 4, the eight mesocosms were deployed in the Raunefjord just outside of the Marine Biological Station, which is a wind and wave protected area. Four mesocosms served as a ambient treatment (pCO_2 350 - 400 µatm) with the replicate numbers 1, 2, 4, 7 and the other four served as a high CO_2 treatment (pCO_2 2200 µatm) with the replicate numbers 3, 5, 6, 8.

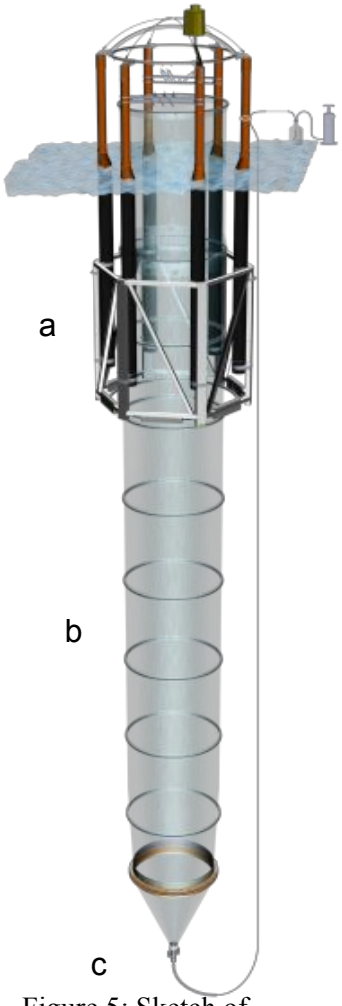


Figure 5: Sketch of flotation frame (a) with mesocosm bag (0-19 m) (b) and the sediment trap at the bottom (19-21m) (c) (modified after Riebesell *et al*., 2013).

3.1.2 General sampling and seawater manipulation (CO_2 addition):

The sampling schedule is based on the t-day system, in which t0 (12th of May 2015) marks the first CO_2 addition and t49 (30th of June 2015) marks the end of the experiment. In order to investigate the main approach of KOSMOS 2015 to monitor the plankton community responses to ocean acidification, special sampling days were conducted. Every second day was a sampling day starting at t0. For each mesocosm carbonate chemistry parameters like total alkalinity (TA), dissolved inorganic carbon (DIC), pH, chlorophyll, and phytoplankton, data were collected by integrated water samplers (IWS), sediment traps and conductivity-temperature-depth casts (CTD). The development and distribution of zooplankton and larval

fish was assessed with respective nets every eight days. The sampling schedule of KOSMOS 2015 is depicted in the following Figure 6.

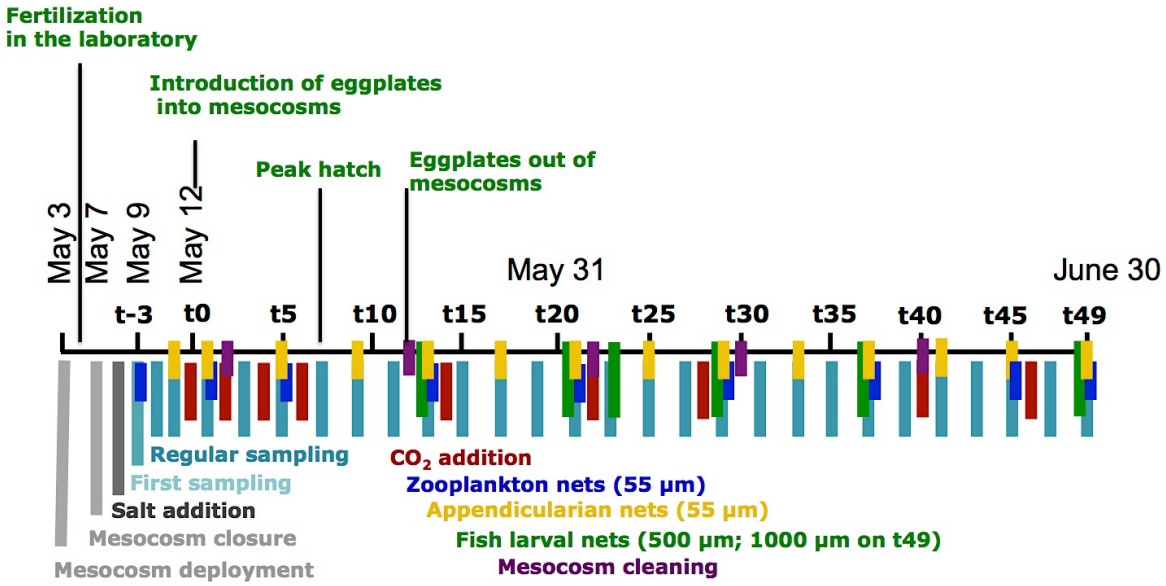


Figure 6: Scheme of KOSMOS 2015 sampling schedule (modified after Riebesell, 2015) Introduction of herring egg and larval sampling are added.

To establish the target pCO_2 of 2200 µatm, three CO_2 additions were carried out by adding the same amount of CO_2-enriched fjord water into each of the four respective mesocosms (M3, M5, M6, M8) at t0, t2 and t4. The addition of CO_2-enriched seawater increases DIC while leaving TA constant, which is perfectly mimicking on-going ocean acidification (Schulz *et al*., 2009; Gattuso *et al*., 2010). Due to outgassing and natural biological processes, six more CO_2 additions were conducted throughout the experiment (t6, t14, t22, t28, t40, t46; Figure 6). To assure an equal distribution of the enriched seawater, the so-called „CO_2 spider device“ was lowered and heaved inside the mesocosms (Riebesell *et al*., 2013). To have the same turbulences caused by the device in the CO_2 manipulated group, the same amount of filtered seawater was added into the control group.

3.1.3 Fertilization:

The brood stock herring used in this experiment originated from the Fens Fjord, being caught on May, 5t^h 2015 at a depth of approximately 6m with a gillnet (mesh size: 36mm), at 60°34`795 N, 5°0`759 E in cooperation with Prof. Dr. Arild Folkvord (Department of Fisheries Ecology and Aquaculture at the University of Bergen). Eggs from these ready-to-spawn Norwegian Coastal herring (two females, three males) were strip spawned onto 20

plastic plates as “mixed families” (crossing every female with every male) on May 5th (see Figure 6). All egg plates were placed in photo trays, fertilized in ambient seawater and afterwards photographed to count the fertilized and unfertilized eggs in order to calculate the fertilization success (Figure 7, left). After the pictures were taken, the plates were placed into a raceway with a fresh seawater flow through system in order to keep the eggs in nearly natural conditions for seven days in the laboratory. In order to compare between the mesocosms and the two treatments, it was necessary to have a similar number of larvae in each mesocosm. Therefore, after four days (t-3) each plate was photographed again and every egg was counted with respect to live and dead ones. On t0, the selected egg plates, with ~3000 alive eggs attached, were transferred into specially built egg incubators (Figure 7, middle; developed by Dr. Daniela Storch, AWI), that enabled incubation in the mesocosms and the newly hatched larvae to swim independently out of the incubators into the water. The egg incubators were suspended by ropes at 8m depth in the respective eight mesocosms (Figure 7, right).

Figure 7: Strip spawned herring eggs on plastic plates, fertilized in ambient seawater with sperm (left); plastic plate with attached fertilized herring eggs placed into egg incubators (middle), which were suspended by ropes into each mesocosm (right) (right picture by Kosmas Hench).

3.1.4 Hatching:

Peak hatching of the herring larvae occurred approximately on t7 (May 19th, 2015), which is 14 days post fertilization (seven days in the laboratory in ambient seawater and seven days in the respective treatment water), after approximately 120 DD. In order to monitor the development of the herring eggs and to calculate the DD in the mesocosms, temperature data from CTD casts were used. Temperature at the start of the experiment in May was 9°C and

reached 16°C when the experiment was terminated in June (unpublished data from the KOSMOS 2015 team). In the laboratory, temperatures were around 9°C (thermometer).
Five days (t12) after the approximated peak hatch, the egg incubators were removed from the mesocosms, and the egg plates were counted again. The number of possibly hatched herring larvae was calculated by comparing the initial number of alive eggs on t3 and the number of open or dead eggs on t12, as well as subtracting the number of counted dead herring larvae from the incubators.
Until the end of the experiment on t49, larval herring stayed in the mesocosms and fed on the natural zooplankton community establishing in each mesocosm.

3.1.5 Fish larval sampling:

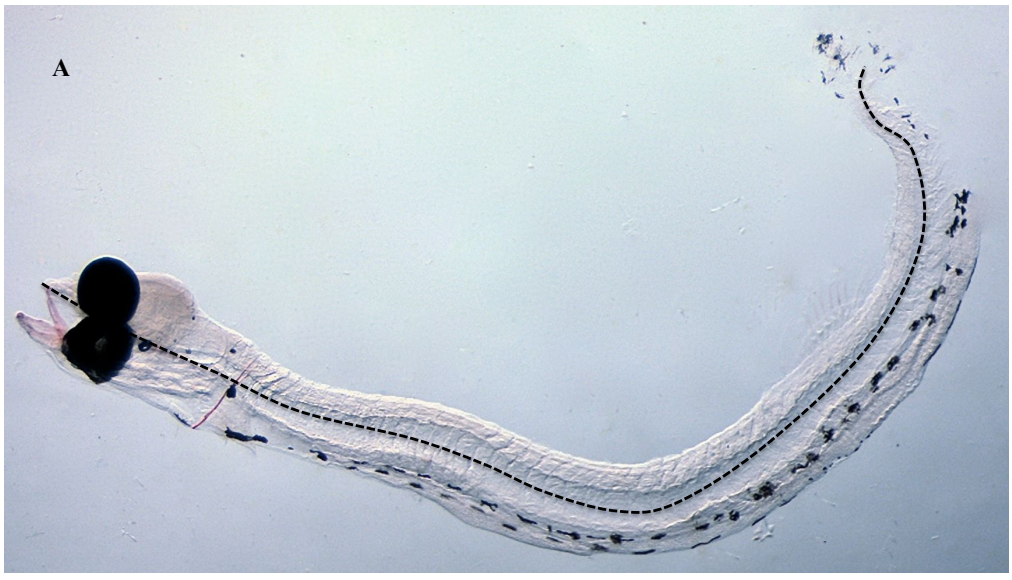
In order to sample living larvae, vertical plankton net (500µm, 50cm in diameter) hauls were performed four times (t13, t23, t29, t37) during the experiment. Newly hatched larvae are attracted to light (Hernandez and Shaw, 2003) and therefore a light was attached to the net and the hauls were performed at night.
Since regular sampling with the plankton net was more difficult than expected, dead larvae from the sediment were sampled every two days to monitore larval growth and mortality. This proved to be a well suited method for sampling dead fish and simultanously avoid major disturbances in the mesocosm system, as no net was dragged through it. Dead larvae sank down into the sediment trap at the bottom of each mesocosm, where they could be collected, and counted and preserved (in 95% ethanol or frozen at -80°C) afterwards.
At the end of the experiment (t49) the big fish net (1000µm, 2m in diameter) was deployed in order to catch all larvae from the entire water column from the bottom to the top of each mesocosm. Larvae were either fixed in 95% ethanol or frozen at -80°C.

3.1.6 Visualizing ossified structures and bone measurements:

Identification and quantification of possible effects of a decreasing seawater pH on the ossification of the first developing bony elements, which are the shoulder girdle (cleithrum), the upper jaw (maxilla) and the lower jaw (dentary), is the focus of this thesis.
The standard method for anatomical studies especially for larval fishes is called clearing and double staining (Dingerkus and Uhler, 1977; Potthof, 1984; Taylor and Van Dyke, 1985). With this method bone is stained red and cartilage blue, while muscle is macerated with

trypsin. For this study, the standard protocols have been modified. The larvae were fixed in 95% ethanol, according to the protocol by Schnell *et al*. (accepted). Each larva was stained with Alizarin Red S (CAS: 130-22-3, Sigma-Aldrich) in a 0.5% KOH (Potassium hydroxide, VWR 26669.290) solution for 24 hours, highlighting bony structures in the larvae's bodies. At the same time the weakly developed muscle tissue of the very young larvae was cleared to a certain degree in the KOH solution (no trypsin has been used). At 18 dph (days post hatch), first bony elements have been observed in the larvae, younger larvae were too small and their bony structures were not yet that well developed to stain properly. Therefore, larval samples from 18 dph until 42 dph (18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40 and 42) have been used in this study.

After the staining, the specimens were transferred into a 70% glycerol (1.04092.1000, MERCK) solution for dissection and long-term storage. Cleithrum, maxilla and dentary of each larva were dissected out and digitally documented using a stereomicroscope (Leica $MZ9_5$, Leica) attached to a camera (Qimaging 32-01017A-338, MicroPuplisher 3.3. RTV). The standard body length (SL) as well as the three bones were measured using imageJ64 (Rasband, 1997-2015). As the different bones are slightly curved, two different measurements were applied: 1) straight measurement from one end to the other, and 2) consideration of certain bending of each bony structure to the nearest of 0.01mm (Figure 8). This measurement required up to 5 manual set points in order to compute the measuring line.



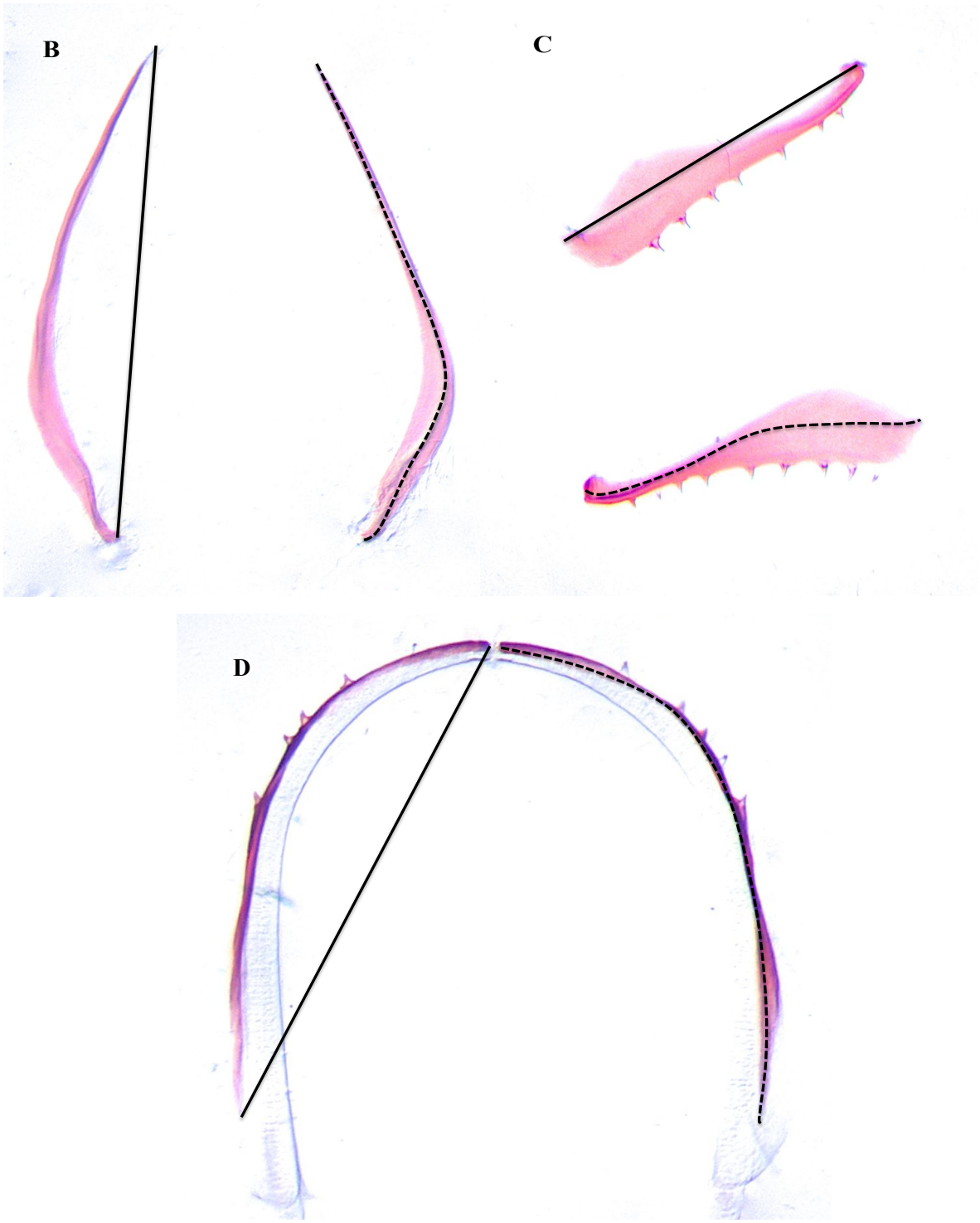


Figure 8: Examples of stained herring larvae (with Alizarin Red S) and dissected bony structures. A: herring larvae with SL measured once by considering bending (pointed black line); B: cleithrum measured straight (black line on the left) and considering the bending (pointed black line on the right); C: maxilla measured straight (black line on top) and considering the bending (pointed black line on the bottom); D: dentary measured straight (black line on the left) and considering the bending (pointed black line on the right). Larvae and structures depicted in different magnifications.

The length of the cleithrum and the maxilla was calculated by measuring the length of the right and the left respective bone in order to compute the mean. To relate the measurements with other studies, the straight measurement was chosen.

The length of the dentary is not considered in this thesis because of uncertainties during the measurement. The dentary was in most of the samples poorly ossified and it was difficult to determine the beginning and end of this bone.

3.1.7 Data analyses KOSMOS 2015:

Statistical analyses of the dataset were not possible due to very low and uneven sample sizes in the mesocosms and between the treatments. However, the mean and standard deviation (SD) of SL, cleithrum length, maxilla length, cleithrum to standard length ratio and maxilla to standard length ratio for the two treatments over time was only calculated for sample sizes ≥ 3.

In order to analyze the relation between the cleithrum length and maxilla length with the size of the respective larvae in each treatment, a Simple Linear Regression Model was performed using the software Rstudio (R Core Team, 2013). Assumptions of normality and homogeneity of variance were confirmed beforehand, using Fligner-Killeen-Test and Shapiro-Wilk-Test.

3.2. Espegrend 2010:

The following experiment was also conducted at the University of Bergen`s Marine Biological Station near Espegrend (Norway) next to the Raunefjorden fjord (60°16´18" N, 5°10`26" E) from 28th of March, 2010 to 25th of May, 2010. In this experimental approach the Department of Biology from the University of Bergen represented by Audrey Geffen and Arild Folkvord cooperated with Dr. Catriona Clemmesen-Bockelmann, Andrea Y. Frommel, Rommel Maneja and Uwe Piatkowski from the Geomar, in Kiel and with David Lowe and Cristine K. Pascoe from the Plymouth Marine Laboratory. For details see Frommel *et al*. (2013).

3.2.1 Experimental set up:

In this study, Atlantic herring larvae were exposed to three levels of pCO_2 (a control group*:* pCO_2 385 µatm; medium group: pCO_2 1830 µatm; high group: pCO_2 4260 µatm) from fertilized eggs to 39 dph. The experiment followed a balanced randomized design of three different pCO_2 levels with 3 replicates per level. The resulting nine land-based, outdoor mesocosms, each hold 2300 liter of seawater with a depth of 1.5m. They were maintained at each of the three pH levels by dissolving pure CO_2 into the water with fine diffusers in the treatment. The control was not manipulated at all. The tank numbers were 2, 6, and 12 for the control group, 1, 3 and 10 for the medium group and 4, 7 and 8 for the high treatment group. Natural conditions were ensured by a flow through of fresh seawater pumped from 40m depth and the addition of natural plankton from the fjord as feed for the herring larvae.

3.2.2 Fertilization and rearing:

Eggs from wild caught Norwegian Coastal herring (11 males and 11 females) were strip spawned onto 20 X 20 cm glass plates. After the fertilization with respective ambient or CO_2-treated seawater, the glass plates were suspended by ropes to mid-depth of the mesocosm tanks. Peak hatch occurred 23 days post fertilization (135 DD) in extra-arranged floating buckets. Afterwards, they were mixed proportionally from the different families and placed into the replicated mesocosm. During the experiment, the larvae were fed once a day *ad libitum* with a prey density of 2000 zooplankton items per liter. The zooplankton had a respective size range of 80-250µm and 350-500µm, as larvae grew larger.

3.2.3 Fish larval sampling and bone staining:

40 fish from each tank were sampled each week using a tube that reached to the bottom of the mesocosm in order to sample larvae over the entire water column. After three weeks the larvae had to be fished with plastic ladles in addition to the tube, because they then were able to avoid the tube. Sampled larvae were either stored at -80°C or preserved in 4% buffered formaldehyde for several analyses. At the end of the experiment, all tanks were drained and the remaining larvae sampled.

In order to compare this experiment with the KOSMOS 2015 project and under consideration of low sample size, 3 larvae per tank and date (27.04/ 11 dph, 4.05/ 18 dph, 18.05/ 32 dph, 25.05.2010/ 39 dph) from the control and medium treatment were used, because the medium treatment (1830 µatm of CO_2) is more comparable to the high treatment (2000 µatm of CO_2) of KOSMOS 2015 (Table 1). Also DD were used and calculated by multiplying the number of days with the temperature experienced at that time. In order to stain larvae from this experiment, some frozen samples were fixed in ethanol (95%) afterwards. The staining of the first developing bony structures was conducted in the same way like for the specimens from the KOSMOS 2015 experiment.

Table 1: Number of stained herring larvae from the Espegrend 2010 experiment out of the ambient and the medium treatment with respective sample dates, DD and dph:

Date (dd/mm/yy)	DD	dph	Ambient treatment: Number of larvae in total (tank: 2, 6, 12)	High CO_2 treatment: Number of larvae in total (tank: 1, 3, 10)
27.04.2010	73	11	9	9
04.05.2010	126	18	9	9
18.05.2010	251	32	6 (tank: 6, 12)	8
25.05.2010	310	39	9	9

3.2.4 Data analyses Espegrend 2010:

All statistical analyses were performed using the software Rstudio (R Core Team, 2013). The mean and standard deviation of the larval standard length, the cleithrum length, the cleithrum to larval size ratio, the maxilla length, the maxilla to larval size ratio and the ratio between cleithrum and maxilla, for each tank in each DD, was computed for samples sizes ≥ 3. After that, all data were tested for normality using Shapiro-Wilk-Test and showed non normal distribution, possibly due to high variations within the tanks. This assumption was approved after examine for normality between the means of the two treatments over time. Homogeneity of variance was confirmed using Fligner-Killeen-Test. A two-way analysis of variance (Two-Way-ANOVA) was used to test for significant differences between the tanks of each treatment over DD. As no differences were found between tanks, all of the samples from the same treatment were pooled and analyzed together.

The response variable (standard length, cleithrum length, cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio, ratio of cleithrum to maxilla) was compared among the independent variables, treatment and DD (73, 126 and 310), using Two-Way-ANOVA. The 251 DD is not considered in this analyze, due to low sample sizes and replicates.

In order to analyze the relation between the cleithrum length and maxilla length with the size of the respective larvae in each treatment, a Simple Linear Regression Model was performed. Assumptions of normality and homogeneity of variance were again confirmed beforehand, using Fligner-Killeen-Test and Shapiro-Wilk-Test.

All statistical analyses were performed using a significance level of 0.05.

3.3 Kristineberg KOSMOS 2013:

In this experiment, the larval rearing was conducted in the laboratory of the Sven Lovén Centre for Ocean Research in Kristineberg (Sweden) from April until June 2013. Research groups of Prof. Ulf Riebesell (Geomar, Kiel) and Dr. Catriona Clemmesen-Bockelmann (Geomar, Kiel) collaborated in this experimental approach with Michael Sswat and Martina Stiasny being mostly involved and responsible.

3.3.1 Experimental set up:

In this study, herring larvae were exposed to two levels of temperature (10°C and 12°C) and pCO_2 (a control group*:* pCO_2 400 µatm and a high group: pCO_2 1000 µatm), which was combined with each combination and replicated thrice. In total there were three tanks for ambient cold (3, 6, 11), three tanks for high cold (2, 8, 10), 3 tanks for ambient warm (1, 5, 9), and three tanks for high warm (4, 7, 12), which makes 12 tanks in total.

3.3.2 Fertilization and rearing:

The brood stock, which was used in this experiment, originated from the Oslo-Fjord and was caught on April 22nd 2013 at a depth of approximately 30m with a gillnet. Eggs from five ready-to-spawn female herring were strip spawned onto 150 plastic plates with approximately 150 eggs per plate and fertilized with sperm from five males (25 families) under the two respective CO_2 treatments. The rearing water was pumped from 30m depth, was filtered and than manipulated with the respective CO_2 and temperature level. The light conditions were set according to natural hours of daylight in that region.
The larvae were fed three times a day with a prey density of 131-600 natural zooplankton items per liter in all tanks (calanoid, copepod, nauplii and copepodites), which had a respective size range of 70-200µm as the larvae grew larger.

3.3.3 Fish larval sampling and bone staining:

Dead larvae were collected on a daily basis by siphoning the tank floors and counted afterwards for survival analyses. Every five days, samples for further analysis were freeze storaged at -

80°C until the experiment ended on June 15th, 2013 (32 dph). In order to stain larvae from this experiment, some frozen samples were fixed in ethanol (95%) afterwards. The staining of the first developing bony structures was proceeded in the same way as for KOSMOS 2015.
The following Table 2 shows the larval sample size with respective degree-days and treatment. Only larval samples from 8.06.2013/ 25 dph were used, because of sufficient sample size.

Table 2: Number of stained herring larvae from the Kristineberg 2013 experiment out of the ambient and the high CO_2 treatment with respective sample dates, DD and dph.

Date (dd/mm/yy)	Temperature/ DD	dph	Ambient treatment: Number of larvae in total	High CO_2 treatment: Number of larvae in total
8.06.2013	Cold/345	25	15 (tank: 3, 6, 11)	15 (tank: 2, 8, 10)
8.06.2013	Warm/413	25	10 (tank: 1, 5)	15 (tank: 4, 7, 12)

3.3.4 Data analyses Kristineberg 2013:

All statistical analyses were performed using the software Rstudio (R Core Team, 2013). The mean and standard deviation of the larval standard length, the cleithrum length, the maxilla length and the ratio between cleithrum and maxilla for each tank in each treatment was computed.
Assumptions of normality and homogeneity of variance were confirmed beforehand, using Fligner-Killeen-Test and Shapiro-Wilk-Test. The response variable (standard length, cleithrum length, cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio, ratio of cleithrum to maxilla) was compared among the independent variable, CO_2, using one-way analysis of variance (ANOVA). This was only tested in the cold treatment and not within the warm treatment, due to sufficient sample size and replicates. A second ANOVA was performed using as response variable (standard length, cleithrum length, cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio, ratio of cleithrum to maxilla) and as independend variable, temperature. This was only tested in the high CO_2 treatments and not within the ambient ones, due to sufficient sample size and replicates.
All statistical analyses were performed using a significance level of 0.05.

4. Results:

4.1 KOSMOS 2015:

4.1.1 Fertilization success and hatching rate:

After counting alive eggs and dead eggs on eight out of thirteen egg-plates, one day after fertilization, a fertilization success of ~89% was calculated.
Table 3 shows the number of hatched larvae in each mesocosm and the respective hatching rates, which is in the mean a rate of ~0.33.

Table 3: Number of alive eggs before deployment and number of hatched herring larvae after deployment, for each mesocosm and respective hatching rate.

Mesocosm number	Treatment	Egg-plate number	Number of alive eggs on t-3	Number of hatched larvae	Hatching rate
1	Ambient	72	2937	1538	0.38
2	Ambient	90/93	3658	2247	0.34
3	CO_2	78/87	3408	2835	0.36
4	Ambient	70/73	3406	2136	0.30
5	CO_2	74/80	3475	2038	0.35
6	CO_2	79	2769	1405	0.28
7	Ambient	84/91	3356	2512	0.37
8	CO_2	71/82	3474	2078	0.38

An unequal amount of larvae hatched in each mesocosm, but the differences are tolerable. In the two treatments nearly the same amount of larvae hatched (ambient treatment= 8433; high CO_2 treatment= 8356).

4.1.2 Sampling:

Table 4 shows the number of dead herring larvae sampled from the sediment, which were preserved in ethanol in order to be cleared and stained later. The rest of the sediment samples were frozen at -80°C for further analyses by Michael Sswat.

Table 4: Number of herring larvae sampled from the sediment and preserved in ethanol.

t	DD	dph	Ambient treatment (mesocosm: 1, 2, 4)	High CO_2 treatment (mesocosm: 3, 5, 6, 8)
25	172	18	2	2
27	195	20	3	2
29	219	22	1	0
31	244	24	2	3
33	270	26	1	3
35	295	28	2	2
37	322	30	0	5
39	350	32	1	3
41	377	34	4	5
43	407	36	4	13
45	436	38	0	2
47	465	40	0	3
49	497	42	0	3
50	505	42	2	3

Overall, a very low number of herring larvae in both treatments from t25 to t50 was found. The sampling day t50 was added in order to sample larvae, which escaped the 1000µm net at the end of the experiment. However, a higher number of larvae could be sampled in the high CO_2 mesocosms (3, 5, 6, 8) than in the ambient mesocosms (1, 2, 4). Mesocosm 7 had no larvae from t25 until the end of the experiment. Sampling day's t45, t47 and t49 showed no herring larvae in the ambient mesocosms. The 505 DD was changed from 500 DD due to an easier visualization in the later graphs.

Table 5 shows the number of herring larvae sampled at only two dates (t29 and t37) with a plankton net.

Table 5: Number of herring larvae fished with a 500µm net and fixed in ethanol.

t	DD	dph	Ambient treatment (mesocosm: 1, 2, 4)	High CO_2 treatment (mesocosm: 3, 5, 6, 8)
29	220	22	2	3
37	323	30	1	2

Again, the overall number of fished herring larvae during the night in both treatments and on both dates (t29, t37) is very low. However, slightly more were sampled from the manipulated high CO_2 treatment.

The Table 6 shows the number of herring larvae taken as the "survivors" with the final fish net and preserved in ethanol in each mesocosm at t49/ 42 dph. The rest were frozen at -80°C.

Table 6: Number of herring larvae at t49/ 42dph fished with 1000µm net and fixed in ethanol.

DD	Mesocosm number	Treatment	Number of fished herring larvae
500	1	Ambient	0
500	2	Ambient	2
501	3	CO_2	16
500	4	Ambient	1
501	5	CO_2	12
501	6	CO_2	10
500	7	Ambient	0
501	8	CO_2	12

The number of caught surviving herring larvae in each mesocosm is very low. In the ambient mesocosms 1 and 7 no herring larvae survived at all. In mesocosm 2 are two survivors and in mesocosm 4 only one single survivor, which gives three survivors in total for the ambient treatment. The manipulated mesocosm 3 is the one, which has the most herring larvae left (16 survivors). The other three mesocosms (5, 6, 8) show similar survival numbers of respective 12, 10 and 12 herring larvae. In total, there are 50 larvae in the high CO_2 treatment

Apart from herring larvae, also juveniles from gadoid fish species, the pelagic polychaete *Tomopteris sp.* (J.F. Eschscholz, 1825) and different jellies, e.g. *Aglantha digitale* (O.F. Müller, 1776), *Clythia* sp. (Lamouroux, 1812), *Sarsia tubulosa* (M. Sars, 1835), *Obelia geniculate* (Linnaeus, 1758), were caught in all mesocosms at the end of the experiment (Figure 9) (for details see Spisla, 2015).

Figure 9: Jellyfish with herring larvae in the stomach (left) and gadoid juveniles caught with 1000 µm net at t49 (right).

4.1.3 Standard length of herring larvae:

The standard lengths of the preserved herring larvae from sediment samples and net samples (500µm and 1000µm) were measured and are depicted with the respective degree-days (DD) in Figure 10.

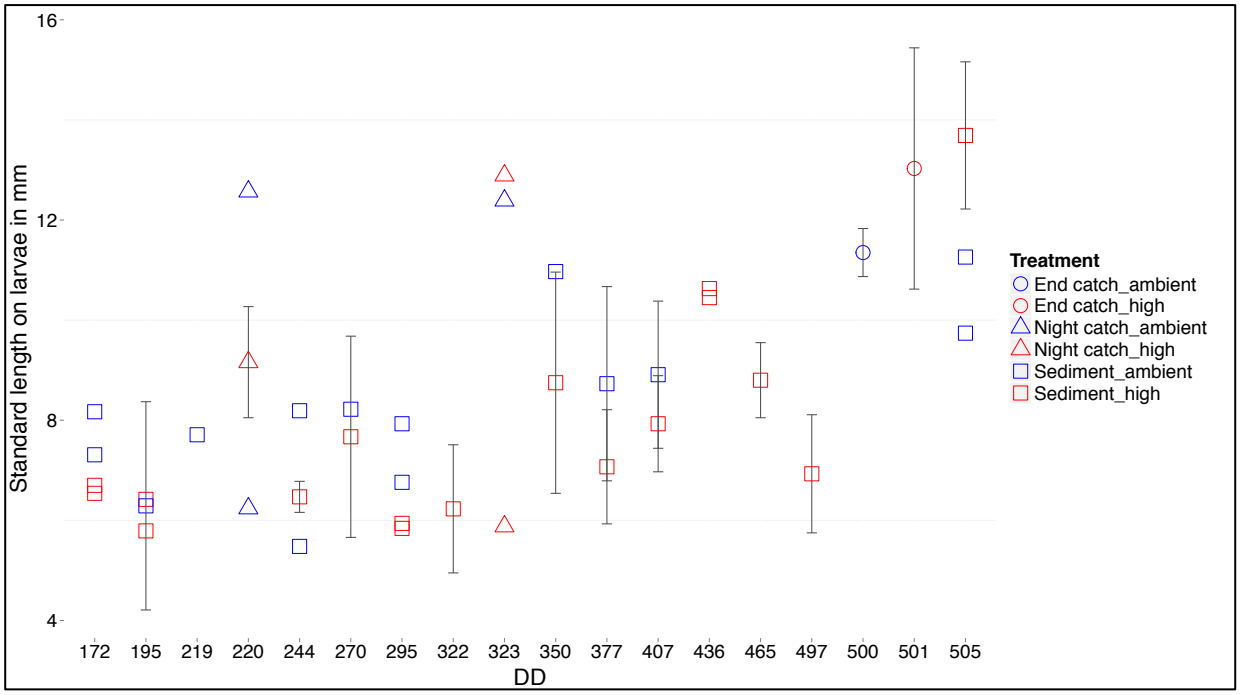


Figure 10: Individual standard length (mean and standard deviation only calculated for sample size ≥ 3) of herring larvae from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

Overall, a low growth in larval length was observed in all treatments during this experiment. Standard length varied between 5 to 8mm at the beginning of the experiment and reached 9.74 to 13.7mm ± 1.5mm (mean ± SD) at the end of the experiment.

In the sediment samples, the herring larvae from the ambient mesocosms are larger (~8 to 11mm) than the larvae from the high CO_2 mesocosms (~7 to 8.7mm ± 2.2mm) until 407 DD. After that, the larvae from the high CO_2 treatment are larger than the ones from the ambient treatment, which emerge only at 505 DD with 10 to 11mm in the ambient treatment and 13.7mm ± 1.5mm in the high CO_2 treatment.

Larvae caught with the 500µm net at 220 DD and 323 DD show in both treatments a large variation in length with 5.8 to 12.9mm, but no treatment effect is visible. The larvae from the ambient night catch are larger (6.24 – 12.6mm) than the single dead larva (7.7mm) sampled at the same day in the sediment, 220 and 119 DD, respectively. The same pattern is valid for larvae from the high CO_2 treatment, with 5,8 – 12.9mm in the sediment to 6.2mm ± 1.3mm in the night catch (322 - 323 DD).

At 500 and 501 DD respectively, the larvae from the high treatment, caught with the 1000µm net, are bigger (13mm ± 2.4 mm) than the ones from the ambient treatment (11.4mm ± 0.48mm), although the high CO_2 mesocosms show a large variation in size.

4.1.4 Length of cleithrum:

The length of the cleithrum, of each preserved larva, which was sampled in the sediment and in the net catches (500µm and 1000µm), was measured and is shown in Figure 11 with the respective DD over time.

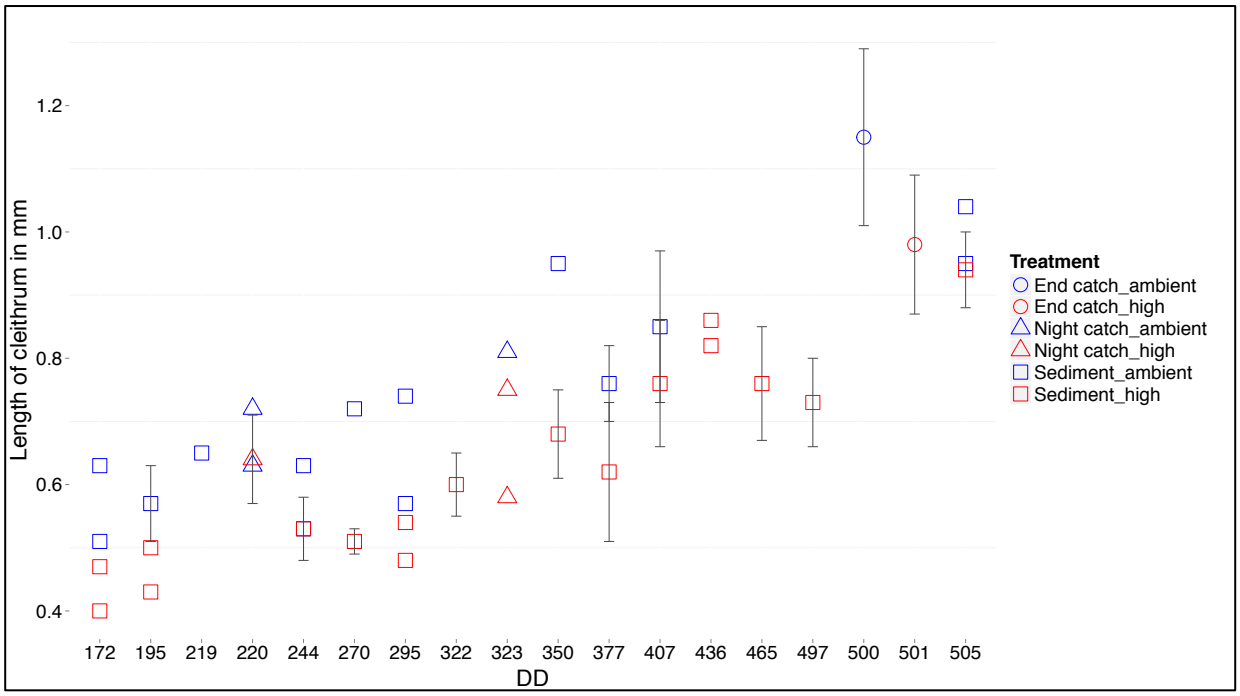


Figure 11: Individual length of cleithrum (mean and standard deviation only calculated for sample size ≥ 3) from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

The cleithrum lengths are overall increasing over time. The cleithrum is larger in all samples of the ambient treatment (end catch, night catch and sediment) at all degree-days than in the larvae from the high CO_2 treatment.

The cleithra from the sediment samples of the ambient treatment have a length of 0.55 to 0.65mm at the beginning and increase with larval growth to 0.94 – 1.04mm at the end. In contrast the cleithra from the high CO_2 treatment larvae are smaller with a length of 0.4 to 0.47mm at the beginning and increase, with variations in length, to a mean of 0.94mm ± 0.06mm at the end. This corresponded with the overall smaller size of the larvae from sediment samples of the high CO_2 treatment. An exception can be seen at 505 DD, when ambient larvae are smaller but show larger cleithra than larvae from the high CO_2 treatment.

The cleithra from the ambient night catch larvae are bigger than the ones from the high CO_2 larvae. At 220 DD, the cleithra in the ambient treatment larvae are 0.63 to 0.72mm long and in

the high CO_2 treatment larvae 0.64mm ± 0.07mm long. At 323 DD, the difference is even more distinct, with 0.81mm in the ambient and 0.58 to 0.75mm in the high CO_2 mesocosms. The cleithra from the ambient night catch larvae are bigger, with 0.63 to 0.72mm, than the cleithrum (0.65mm) from the dead ambient sediment larva sampled at the same day. In the high CO_2 treatment, the cleithrum from the night catch larvae are with 0.58 – 0.75mm larger than the ones from the respective sediment larvae (0.6mm ± 0.05mm).

The cleithra from the ambient end catch samples are in the mean larger (1.15mm ± 0.14mm) than the cleithra (0.98mm ± 0.11mm) from the high CO_2 end catch samples, although the ambient larvae are smaller than the high CO_2 larvae.

4.1.4.1 Relation between cleithrum and standard length:

Figure 12 shows the relation between the standard length of each larvae and the length of their respective cleithrum in both treatments.

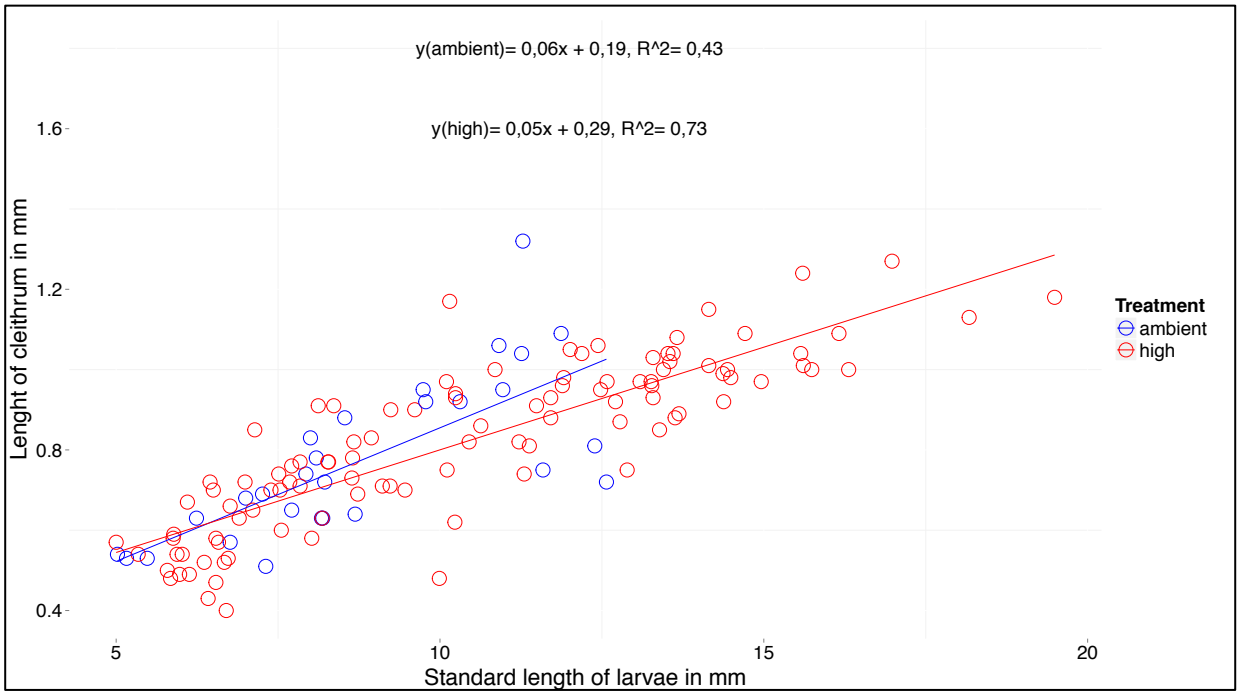


Figure 12: Cleithrum length in relation to size of herring larvae from combined samples (sediment and net sampling) between treatments Blue circle represent ambient treatment and red circle represent high CO_2 treatment. Ambient= 400 µatm, High= 2200 µatm.

There is a positive correlation between the size of the larvae and the length of their respective cleithrum for both treatments. This is true, considering growing bones in association with growing larval body size. However, same sized larvae (10mm) develop larger cleithra in the

ambient treatment (~0.9mm) than in the high CO_2 treatment (~0.8mm). This shows a delayed development of the cleithrum in larvae from the high CO_2 treatment.

4.1.4.2 Ratio of cleithrum to standard length:

In order to analyze if the cleithrum and standard length of the larvae grow in a constant proportion to each other and to investigate possible deviations from this relationship, a ratio of both measurements was computed (Figure 13).

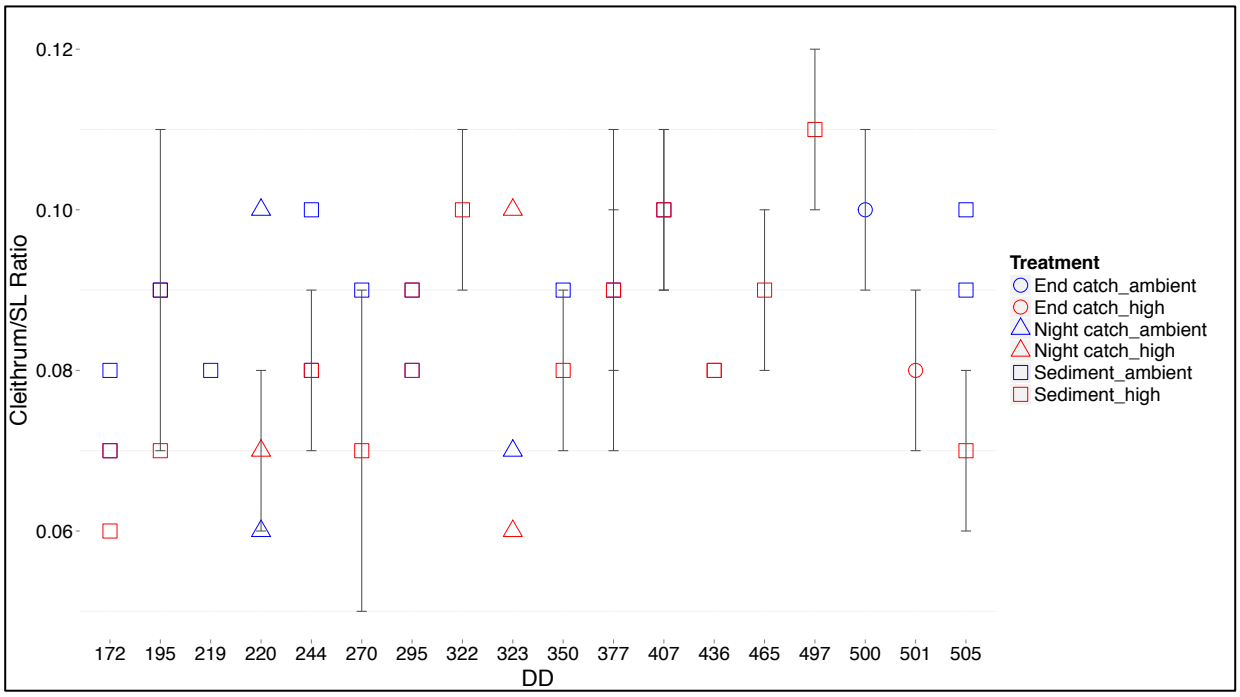


Figure 13: Ratio (mean and standard deviation only calculated for sample size ≥ 3) of cleithrum to standard length from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

The ratio of the cleithrum length to the larval size in the ambient sediment samples show an oszilating ratio of 0.07 from the beginning of the experiment to 0.1 at the end. In the high treatment, sediment samples show also an oszilating ratio, but on a lower level than the ambient ones, of 0.06 at the start to 0.11 at the end of the experiment.

This indicates larger cleihtra in relation to it´s larval size in the ambient treatment than in the high CO_2 treatment.

4.1.5 Length of maxilla:

The length of the upper jaw, the maxilla, of each larva that was sampled in the sediment and in the net catches (500µm and 1000µm) was measured and is shown in Figure 14 with the respective DD.

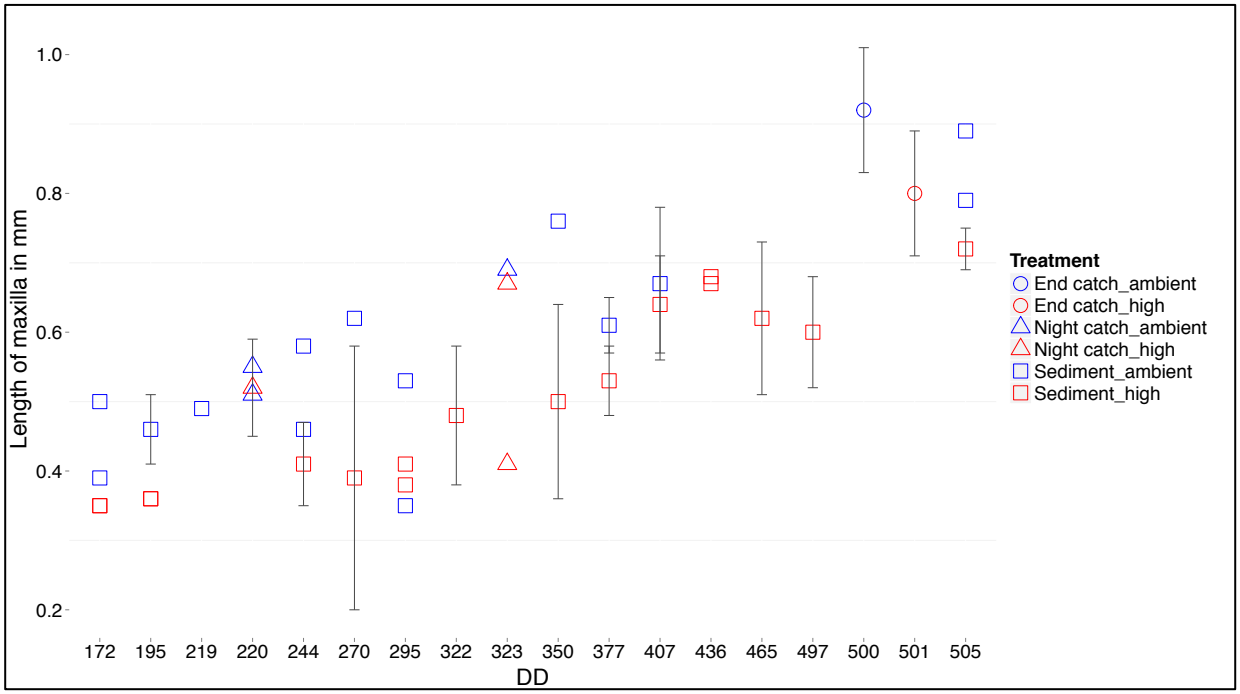


Figure 14: Individual length of maxilla (mean and standard deviation only calculated for sample size ≥ 3) from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

The lengths of the maxillae are larger in the ambient treatment larvae of all samples (sediment, night catches, end catches) at all degree-days than in the high CO_2 treatment larvae.

The maxillae of the ambient sediment larvae are 0.39 to 0.5mm long at the beginning and increase with larval size to 0.79mm and 0.89mm at the end of the experiment. The maxillae of the high CO_2 sediment larvae are in contrast to that 0.35mm long at the beginning and increase also with variations in length, but on a lower level, to a mean of 0.72mm ± 0.03mm at the end.

The maxillae from the two ambient night catch larvae are bigger (220 DD: 0.51 – 0.55mm; 323 DD: 0.69mm) than the ones from the respective high CO_2 larvae (220 DD: 0.52mm ± 0.07mm; 323 DD: 0.41- 0.67mm). The maxillae of the ambient night catch larvae are larger with 0.51 – 0.55mm than the maxilla from the ambient sediment larva (0.49mm). The maxillae from the high CO_2 night catch ones are larger (0.41 – 0.67mm) than the maxillae from the respective sediment larvae (0.48mm ± 0.1mm).

Similar to the cleithrum, the maxillae from the small ambient end catch larvae are larger (0.92mm ± 0.09mm) than the ones from the large high CO_2 treatment larvae (0.8mm ± 0.09mm).

4.1.5.1 Relation between maxilla and standard length:

Figure 15 shows the relation between the standard length of each larvae and the length of their respective maxilla for both treatments and all sampling days.

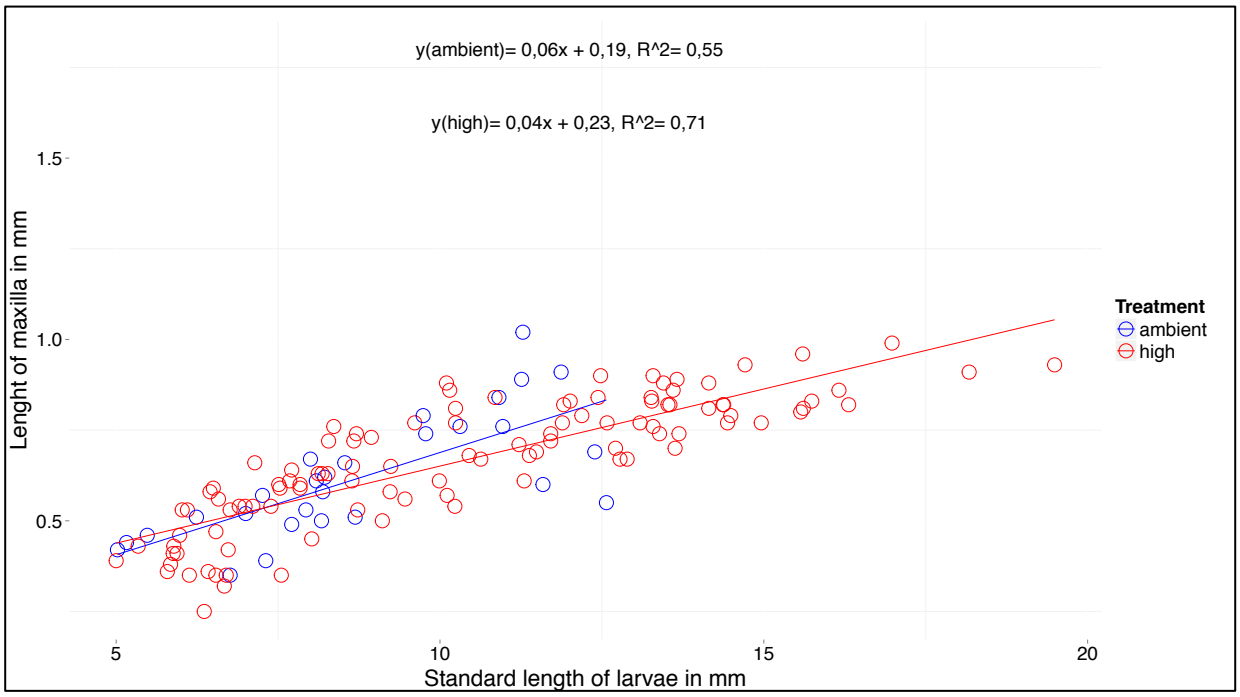


Figure 15: Maxilla length in relation to size of herring larvae from combined samples between treatments Blue circle represent ambient treatment and red circle represent high CO_2 treatment. Ambient= 400 µatm, High= 2200 µatm.

The maxilla length and the size of the herring larvae show a positive relationship for the two treatments. Same sized larvae (10mm) develop larger maxillae in the ambient treatment (~0.6mm) than in the high treatment (~0.55mm), which shows a delay in maxilla development in the high CO_2 treatment.

4.1.5.2 Ratio of maxilla to standard length:

In order to analyze if the maxilla and standard length of the larvae grow in a constant proportion to each other and to investigate possible deviations from this relationship, a ratio of both lengths was computed (Figure 16).

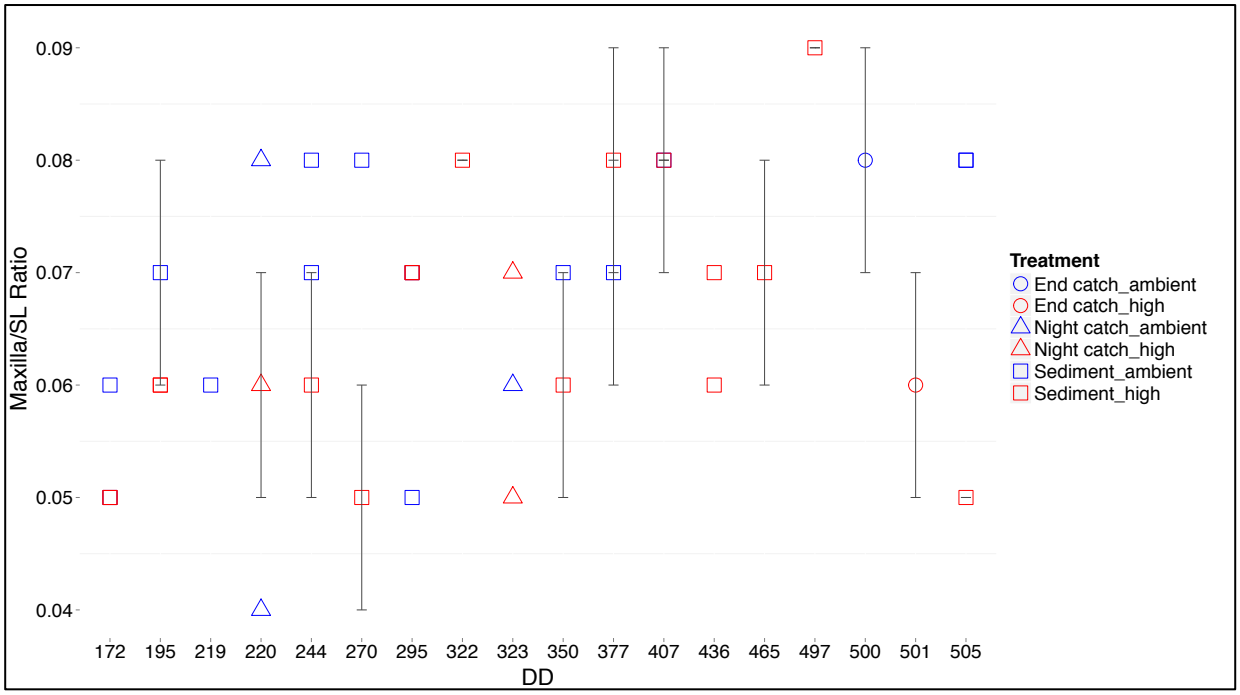


Figure 16: Ratio (mean and standard deviation only calculated for sample size ≥ 3) of maxilla to standard length from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

The ratio of the maxilla length to the larval size deviates in both treatments over time. In the ambient treatment, sediment samples show an oszilating ratio of 0.04 from the beginning of the experiment to 0.08 at the end. In the high treatment, sediment samples show also an oszilating ratio, but on a lower level than the ambient ones, of 0.05 at the start to 0.09 at the end of the experiment.

This indicates, although with large variations, larger maxilla in relation to it´s larval size in the ambient treatment than in the high CO_2 treatment.

4.1.6 Ratio of cleithrum to maxilla:

In order to analyze if the cleithrum and maxilla grow in a constant proportion to each other and to investigate possible deviations from this relationship, a ratio of both bones was computed (Figure 17).

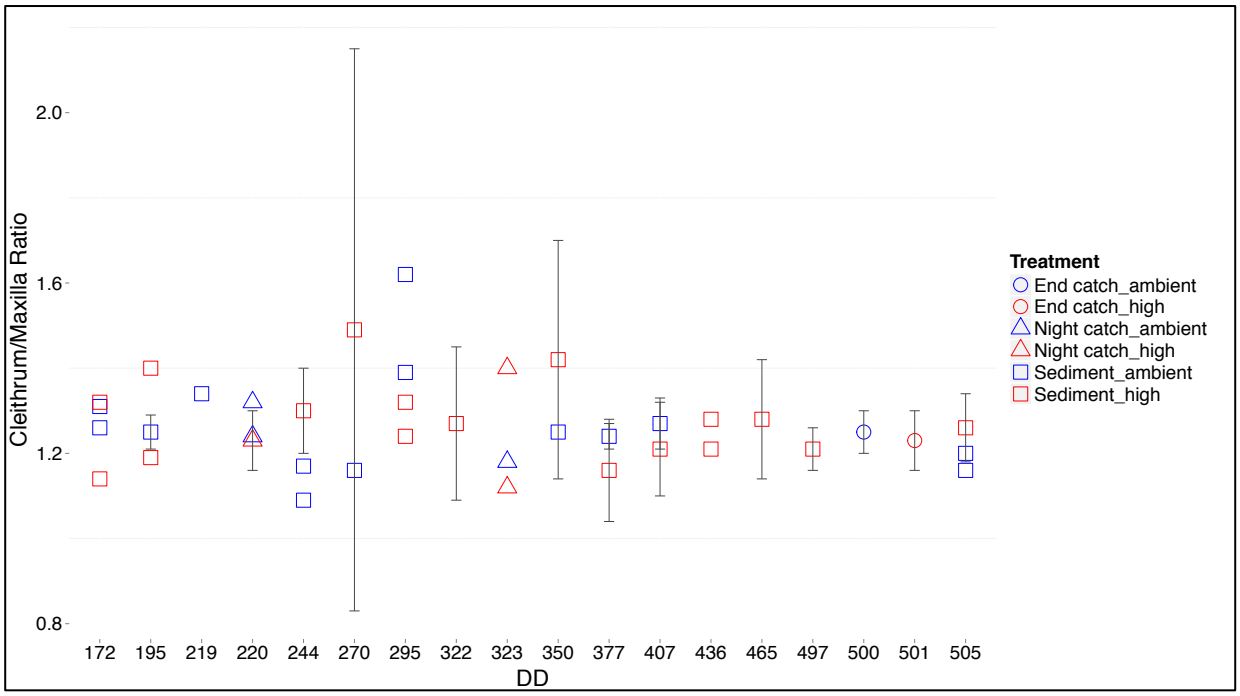


Figure 17: Ratio (mean and standard deviation only calculated for sample size ≥ 3) of cleithrum to maxilla from sediment samples (square) and net hauls (Night catch with 500µm net: triangle; End catch with 1000µm net: circle) over time represented by degree-days (DD). Blue shapes represent ambient treatment and red shapes represent manipulated/high CO_2 treatment. Number of larvae for each treatment at the respective DD, see table 4, 5, 6. Ambient= 400 µatm, High= 2200 µatm.

The ratio of cleithrum to maxilla is relatively constant over time (1 to 1.6). This shows that both bones grow at a constant proportion to each other over time. Some relationships in the sediment samples of the high CO_2 treatment are deviating from the main ratio at 270 DD and 350 DD.

Table 7 shows the effects of the two treatments on the larval size, the cleithrum length cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio and the ratio of cleithrum to maxilla.

Table 7: Comparisons between the effects of the two treatments (n.s= not significant). Response variables are SL, cleithrum length and maxilla lenght, cleithrum/SL ratio (C/SL), maxilla/ SL ratio (M/SL) and cleithrum/maxilla ratio (C/M).

Ambient treatment	High CO_2 treatment
SL ≥ SL (dead) n.s	
SL ≤ SL (survivors) n.s	
Cleithrum ≥ Cleithrum n.s	
C/SL ≥ C/SL n.s	
Maxilla ≥ Maxilla n.s	
M/SL ≥ M/SL n.s	
C/M = C/M n.s	

4.2 Espegrend 2010:

4.2.1 Standard length of herring larvae:

The length of the herring larvae per treatment was measured and is depicted with the respective DD in Figure 18.

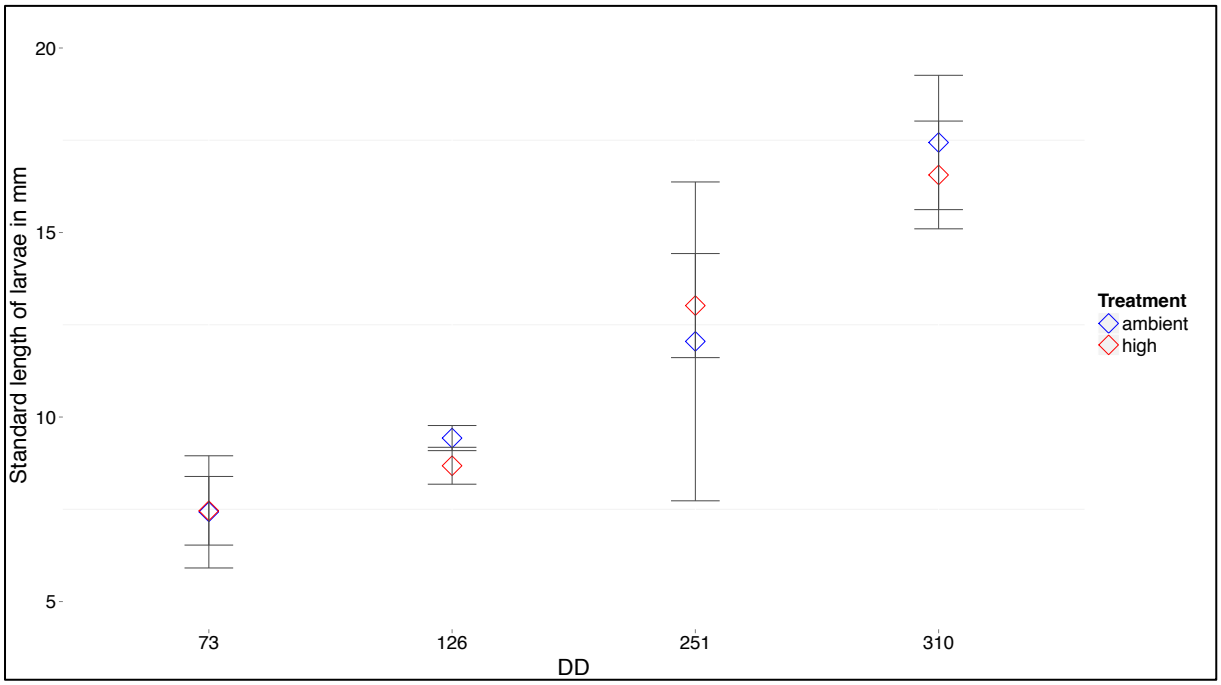


Figure 18: Standard length (mean and standard deviation) of herring larvae per treatment (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

The overall mean standard length of the herring larvae is significantly increasing over time for both treatments (ANOVA, $p < 0.05$) from 7.4mm ± 1.5mm (ambient) and 7.4mm ± 0.93mm (high CO_2) at the beginning of the experiment to up to 17.4mm ± 1.8mm (ambient) and 16.6mm ± 1.5mm (high CO_2) at the end of the experiment. There is no significant difference in larval size between treatments at respective DD (ANOVA at 73, 126 and 310 DD, p-value= 0.34). However, at 126 DD and 310 DD, larvae from the ambient tanks are in the mean larger (9.4mm ± 0.3mm and 17.4mm ± 1.8mm) than the ones from the high treatment (8.7mm ± 0.5mm and 16.6mm ± 1.5mm). The mean size of larvae in the ambient treatment shows the largest deviation at 251 DD (12mm ± 4.3mm), probably due to a low sample size.

4.2.2 Length of cleithrum:

The mean length of the cleithrum of each preserved larvae per treatment was measured and is shown in Figure 19 with the respective DD.

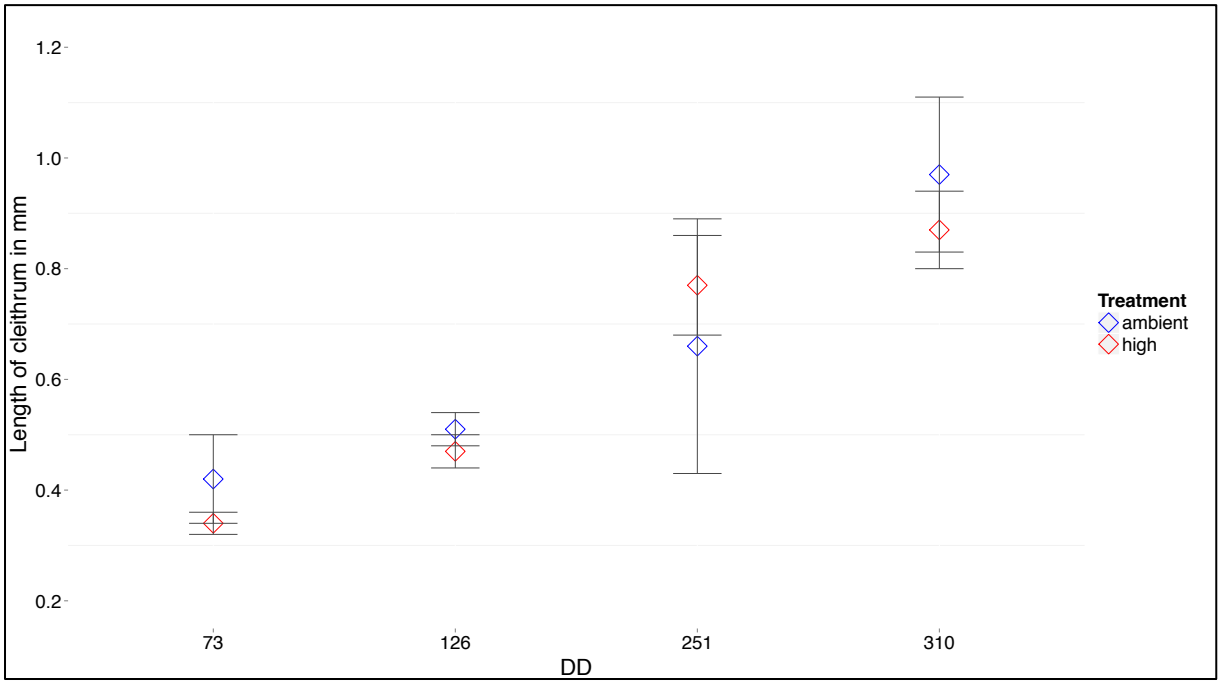


Figure 19: Length (mean and standard deviation) of cleithrum per treatment (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

The cleithrum lengths are overall significantly increasing over time (ANOVA, $p < 0.05$). There is a significant treatment difference in the length of the cleithrum over time (ANOVA at 73, 126 and 310 DD, p-value= 0.04), although a post hoc t-test shows no significant difference (p-values at 73 DD= 0,20; 126 DD= 0.19; 310 DD= 0.35). However, at 73 DD, 126 DD and 310 DD the ambient cleithra are in the mean larger with respective 0.42mm ± 0.08mm, 0.51mm ± 0.03mm and 0.97mm ± 0.14mm than the high CO_2 cleithra, with 0.34mm ± 0.02mm, 0.47mm ± 0.03mm and 0.87mm ± 0.07mm, although the deviations within treatments are large. At 251 DD the CO_2 cleithra are bigger, with 0.77mm ± 0.09mm than the ambient ones, with 0.66mm ± 0.23mm, which show a large variation within treatment.

4.2.2.1 Relation between cleithrum and standard length:

Figure 20 shows the relation between the standard length of each larvae and the length of their respective cleithrum in both treatments.

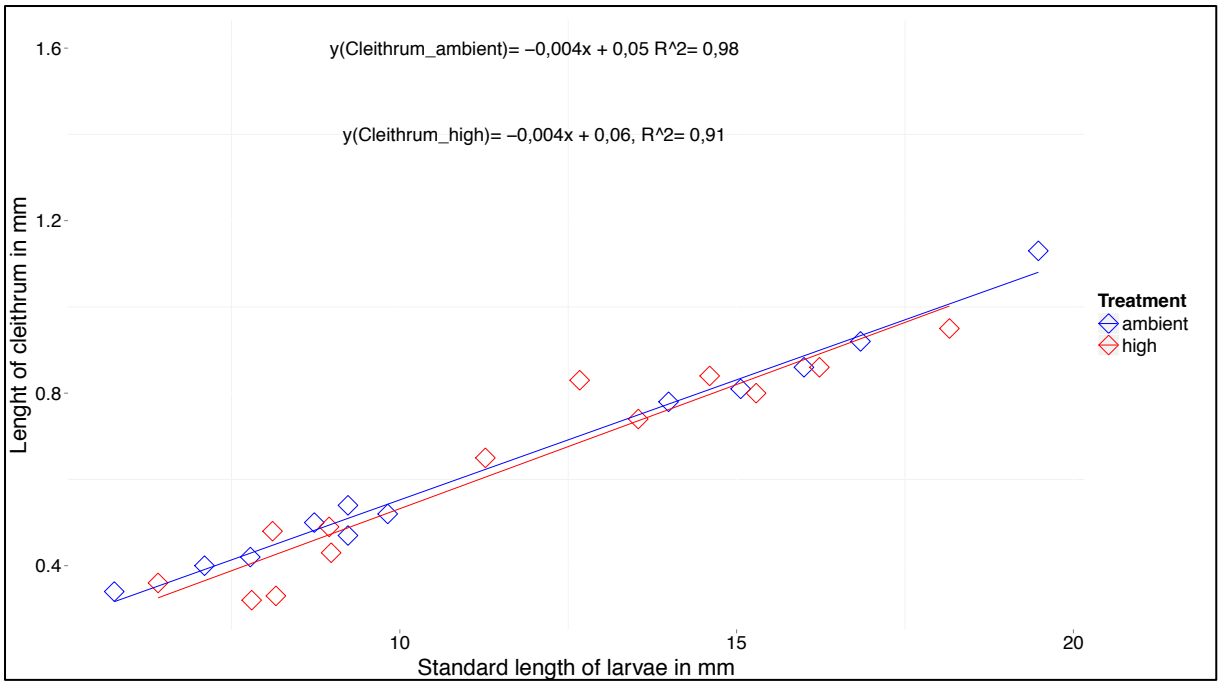


Figure 20: Cleithrum length in relation to size of herring larvae between treatments (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment). Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

There is a positive correlation for the ambient (R^2= 0.98) and high CO_2 treatment (R^2= 0.91), between the size of the larvae and the length of their respective cleithrum. Same sized larvae (10mm) develop same sized cleithra in the ambient treatment (~0.55mm) and in the high CO_2 treatment (~0.53mm) shown by minor difference in the regression lines. No clear treatment effect on the development of the cleithrum was found.

4.2.2.2 Ratio of cleithrum to standard length:

The ratio of cleithrum to larval size over time is depicted in Figure 21.

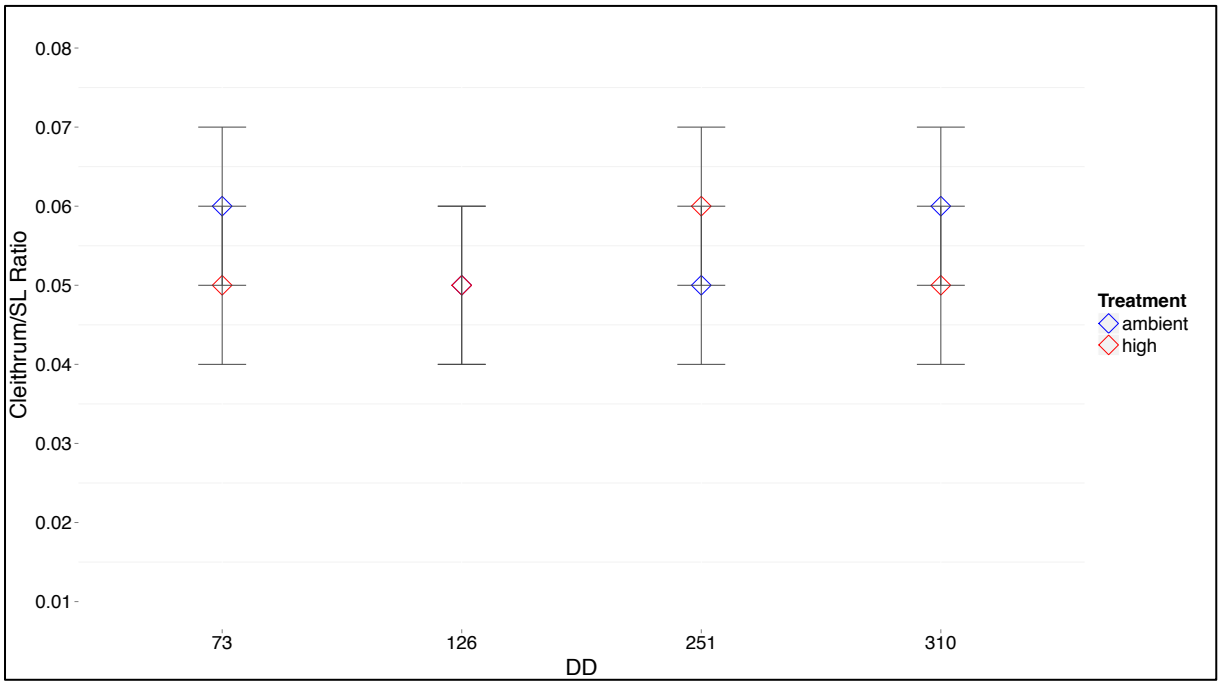


Figure 21: Ratios (mean and standard deviation) of cleithrum to SL per treatment (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. At 126 DD treatment values overlay. Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

The cleithrum to standard length ratio is not changing, indicating constant proportional growth over time (ANOVA, p-value= 0.07).

The ambient treatment shows larger ratios of 0.06 at 73 DD and 310 DD than the respective ratios of 0.05 from the high CO_2 treatment. This depicts, although not significant, larger cleithra in relation to it´s larval size in the ambient treatment than in the high CO_2 treatment (ANOVA, p-value= 0.34).

4.2.3 Length of maxilla:

The mean length of the maxilla of each preserved larva per treatment was measured and is shown in Figure 22 with the respective DD.

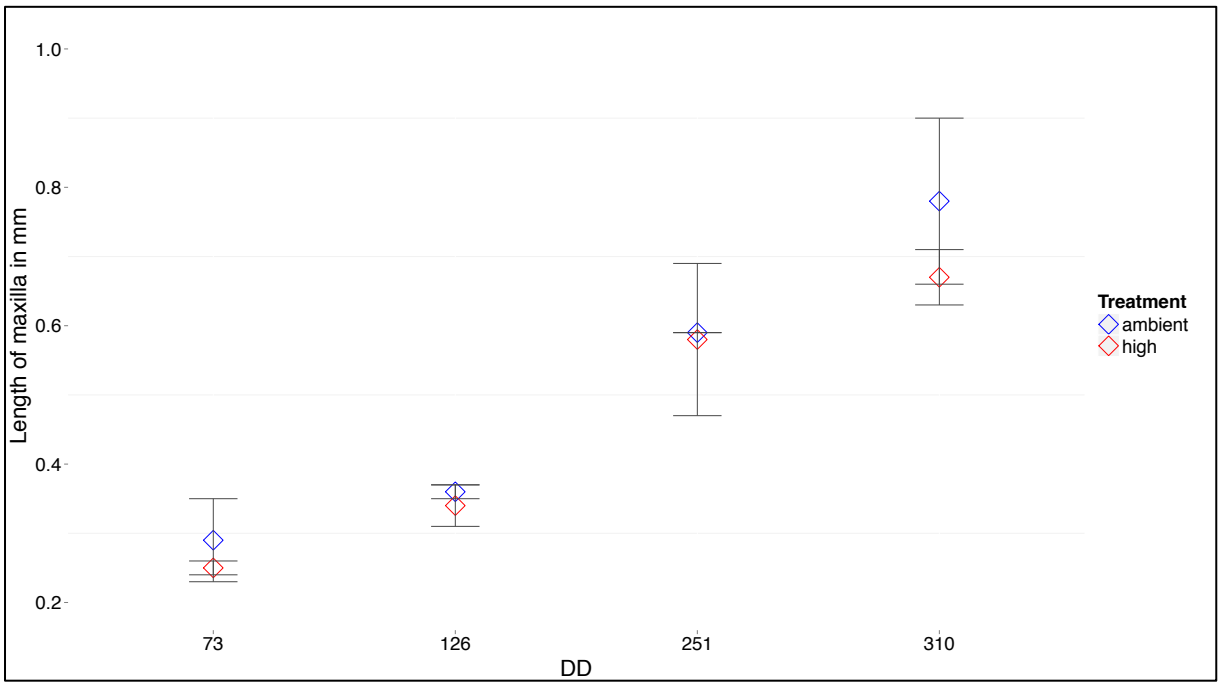


Figure 22: Length (mean and standard deviation) of maxilla per treatment per treatment (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

The mean maxilla length is again significantly increasing with increasing larval size over time (ANOVA, $p < 0.05$). There is no significant treatment difference in the length of the maxilla over time (ANOVA at 73, 126 and 310 DD, p-value= 0.06). However, the mean maxillae from the ambient treatment are larger in all degree-days than from the maxillae from the high CO_2 treatment, although the variations within are high. The differences between the means of both treatments are especially distinct at 73 DD with 0.29mm ± 0.06mm (ambient) and 0.25mm ± 0.01mm (high CO_2) and at 310 DD with 0.78mm ± 0.12mm (ambient) and 0.67mm ± 0.04mm (high CO_2).

4.2.3.1 Relation between maxilla and standard length:

The relation between the larval size and respective maxilla length is depicted in Figure 23 for both treatments.

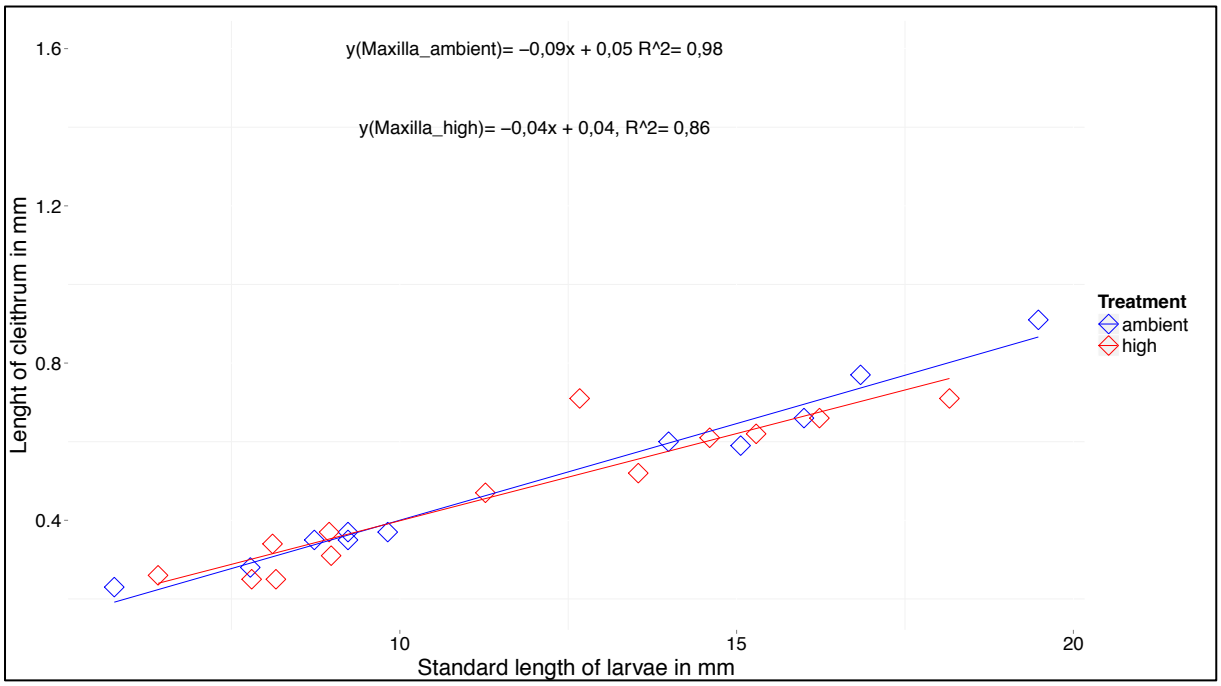


Figure 23: Maxilla length in relation to size of herring larvae between treatments (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment). Red diamonds depict the high CO_2 treatment. Blue diamonds depict the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

There is also a positive correlation, for the ambient (R^2= 0.98) and high CO_2 treatment (R^2= 0.86), between the size of the larvae and the length of their respective cleithrum. Both treatments show no distinct difference in maxilla length, as they develop in the same pattern. At larger larval sizes (17mm), differences between maxilla length from ambient (0.7mm) and high CO_2 treatment (0.6mm) become more distinct.

4.2.3.2 Ratio of maxilla to standard length

The ratio of maxilla to larval size over time is depicted in Figure 24.

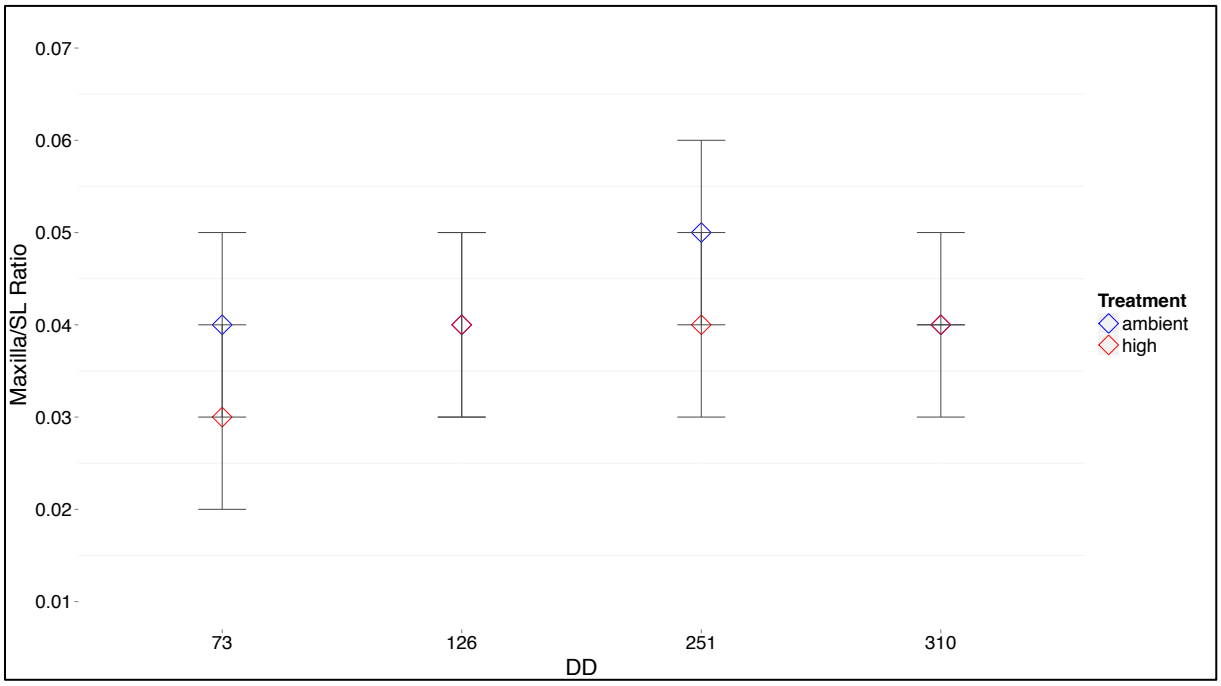


Figure 24: Ratios (mean and standard deviation) of maxilla to SL per treatment (n= 9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. At 126 and 310 DD treatment values overlay. Red diamonds depict the high CO_2 treatment. Blue diamonds depicts the ambient treatment. Ambient= 385 µatm, High= 1830 µatm.

The maxilla to standard length ratio is not changing, indicating significant constant proportional growth over time (ANOVA, p-value= 0.09).

The ambient treatment shows larger ratios of 0,04 at 73 DD and 0.05 at 251 DD than the respective ratios of 0.03 and 0.04 from the high CO_2 treatment. This depicts, although not significant, a CO_2 effect with larger cleithra in relation to it´s larval size in the ambient treatment than in the high CO_2 treatment (ANOVA, p-value= 0.22).

At 126 DD and 310 DD, the ratio in both treatments is the same (0.04).

4.2.4 Ratio of cleithrum to maxilla:

The ratio of cleithrum to maxilla over time is depicted in Figure 25.

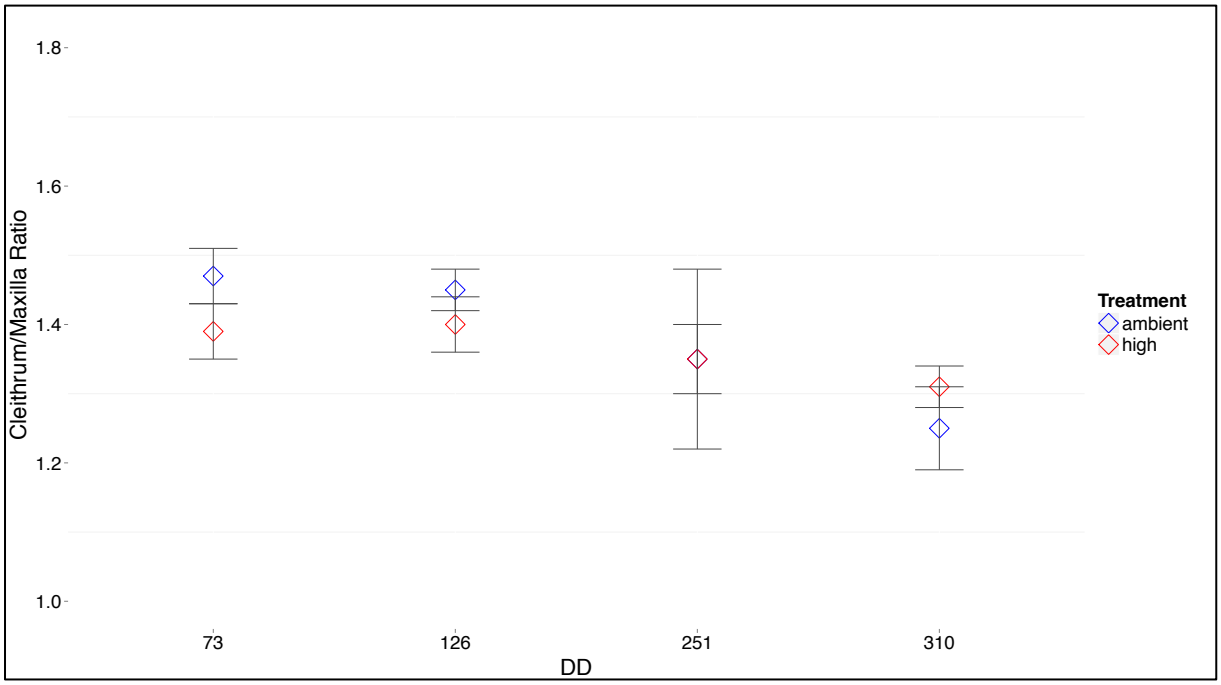


Figure 25: Ratios (mean and standard deviation) of cleithrum to maxilla per treatment (n=9 in each treatment, except at 251 DD: n= 6 in ambient and n= 8 in high CO_2 treatment) over time represented by respective DD. Red diamonds depict the high CO_2 treatment. Blue diamonds depicts the ambient treatment. Ambient= 385µatm, High= 1830µatm.

The ratio between the two bones declines significantly over time from around 1.4 to 1.3, indicating that the maxilla was growing faster than the cleithrum (ANOVA, p-value $p < 0.05$). No significant treatment effect over time was detected (ANOVA at 73, 126 and 310 DD, p-value= 0.40).

Table 8 shows the effects of the two treatments on the larval size, the cleithrum length cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio and the ratio of cleithrum to maxilla.

Table 8: Comparisons between the effects of the two treatments (n.s= not significant). Response variables are SL, cleithrum length and maxilla lenght, cleithrum/SL ratio (C/SL), maxilla/ SL ratio (M/SL) and cleithrum/maxilla ratio (C/M).

Ambient treatment	High CO_2 treatment
SL ≥ SL n.s	
Cleithrum ≥ Cleithrum n.s	
C/SL ≥ C/SL n.s	
Maxilla ≥ Maxilla n.s	
M/SL ≥ M/SL n.s	
C/M ≥ C/M n.s	

4.3 Kristineberg 2013:

4.3.1 Standard length of herring larvae:

The mean standard length of the herring larvae was measured per tank and is depicted with the respective treatment combinations (ambient/cold, ambient/warm, high/cold, high/warm) in Figure 26.

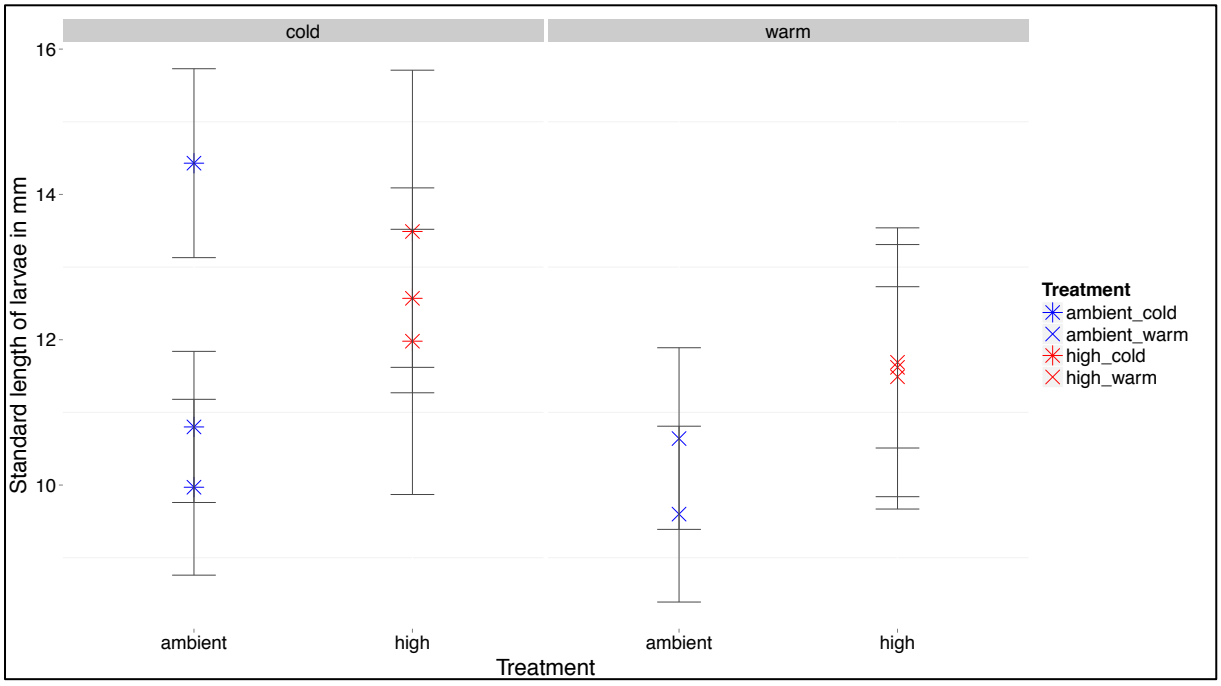


Figure 26: Standard length (mean and standard deviation) of herring larvae (25dph) per tank. Blue stars depict three tanks of ambient cold treatment and red stars depict three tanks of high CO_2 cold treatment; Blue crosses depict two tanks of ambient warm treatment and red crosses depict three tanks of high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

The means of the herring length from the ambient/cold (9.9mm ± 1.2mm to 10.8mm ± 1mm) and ambient/warm tanks (9.6mm ± 1.2mm to 10.6mm ± 1.3mm) are smaller than the larvae from the two respective high CO_2 tanks (11.9mm ± 2.1mm to 13.5mm ± 2.2mm and 11.5mm ± 1.8mm to 11.7mm ± 1.85mm). Therefore, with exception of tank 11 in the ambient/cold treatment (14.4mm ± 1.3mm), the CO_2 treatment shows a positive CO_2 effect for both temperatures, although this is not statistically significant (ANOVA in cold treatment, p-value= 0.55).

The temperature treatments show no distinct differences in larval size, especially between each of the two ambient treatments. However, the mean larval sizes of the high/warm tanks are smaller with 11.6mm ± 1.1mm, though not significant (ANOVA, p-value= 0.07) than the mean sizes of the high/cold treatment with 12mm to 13mm.

4.3.2 Length of cleithrum:

The mean lengths of the cleithrum of the larvae were measured per tank and are depicted with the respective treatment combinations (ambient/cold, ambient/warm, high/cold, high/warm) in the following Figure 27.

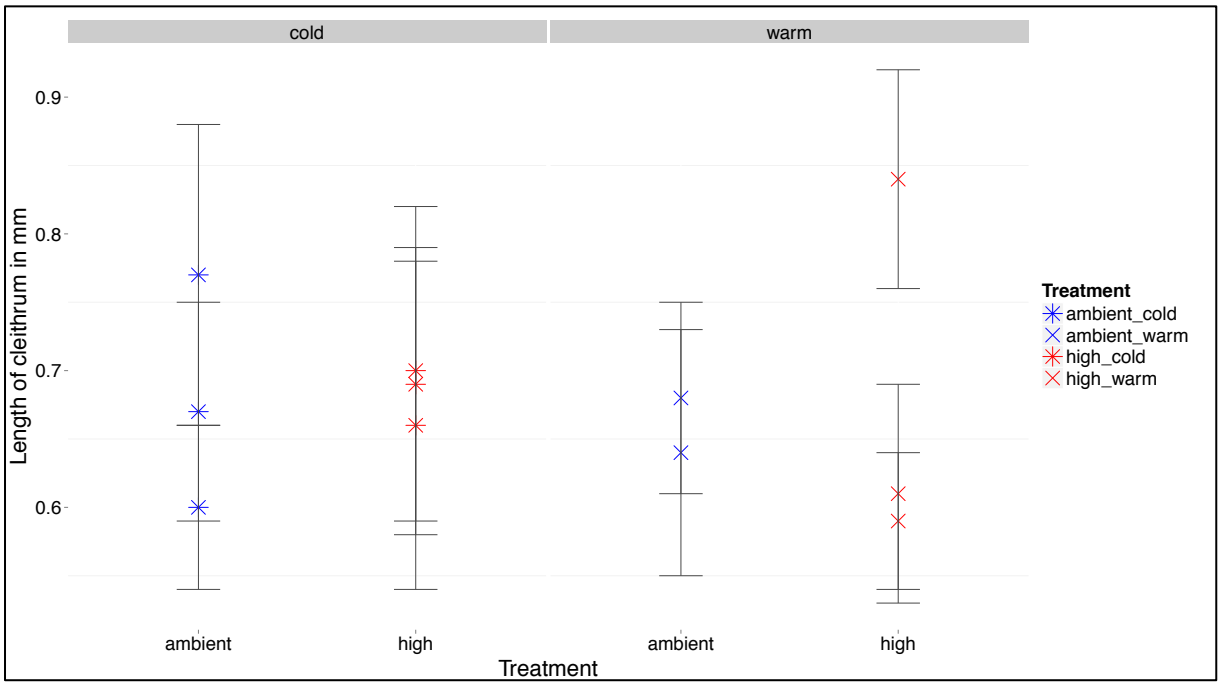


Figure 27: Length (mean and standard deviation) of cleithrum in herring larvae per tank. Blue stars depict three tanks of ambient cold treatment and red stars depict three tanks of high CO_2 cold treatment; Blue crosses depict two tanks of ambient warm treatment and red crosses depict three tanks of high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

The cleithrum lengths do not differ between the four treatment combinations.

Within the cold treatment the mean cleithrum lengths of larvae from ambient (0.67mm ± 0.08mm to 0.60mm ± 0.06mm) and high CO_2 tanks (0.66mm ± 0.12mm, 0.7mm ± 0.12mm to 0.69mm ± 0.1mm) are in the same size range (ANOVA, p-value= 0.95). In the warm treatment, the mean cleithrum lengths of larvae from the ambient tanks (0.64mm ± 0.09mm, 0.68mm ± 0.07mm) are larger, with exception of tank 4, than the mean lengths out of the high CO_2 tanks (0.59mm ± 0.05mm, 0.61mm ± 0.08mm).

The mean lengths of the cleithrum from the high/warm treatments are distinctly smaller (0.59mm ± 0.05mm, 0.61mm ± 0.08mm), with one exception (tank 4), than the ones from the high/cold CO_2 treatment (0.66mm ± 0.12mm, 0.7mm ± 0.12mm, 0.69mm ± 0.1mm). However, this temperature effect is not significant (ANOVA, p-value= 0.97).

4.3.2.1 Ratio of cleithrum to standard length:

The ratio of cleithrum to standard length in the four treatment combinations is depicted in Figure 28.

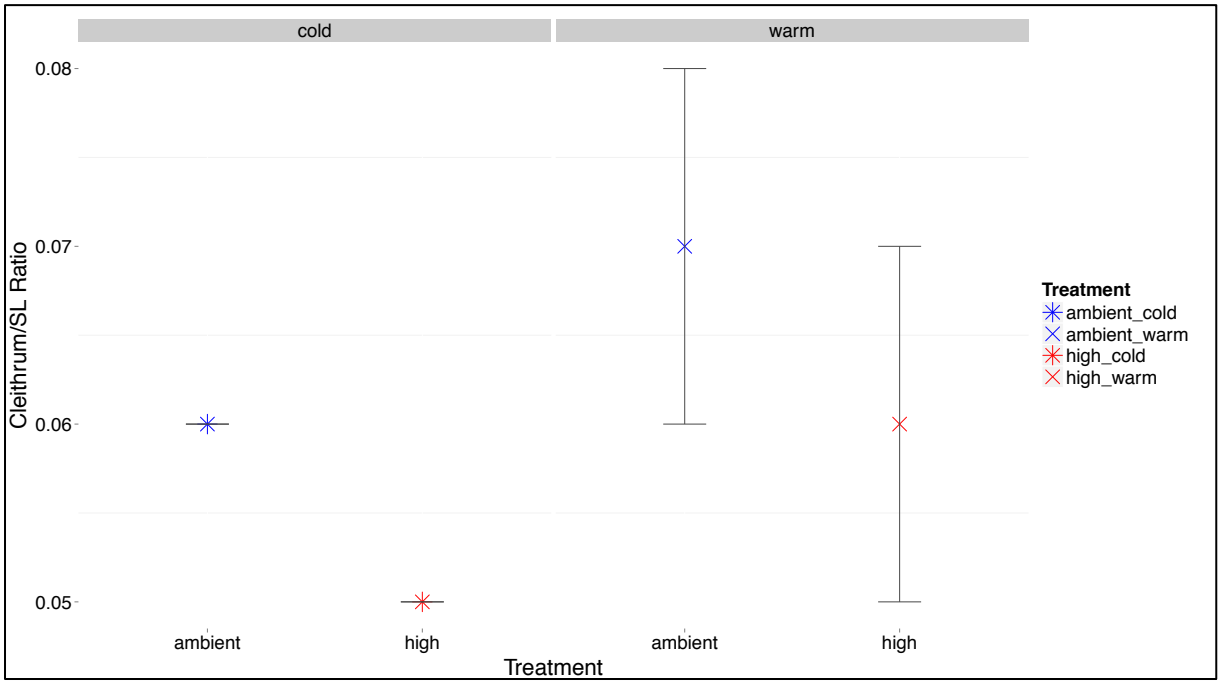


Figure 28: Ratios (mean and standard deviation) of cleithrum to larval standard length per treatment. Blue stars depict mean of three tanks in ambient cold treatment and red stars depict mean of three tanks in high CO_2 cold treatment; Blue crosses depict mean of two tanks in ambient warm treatment and red crosses depict mean of three tanks in high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

The ratio of cleithrum to larval size varies between temperature and CO_2 level.

In the cold treatment, the cleithra of the ambient treatment are in relation to larval size larger (0.06) than the ones from the high CO_2 treatment (0.05). This is not statisticaly significant (ANOVA, p-value= 0.52). In the warm treatment, the ambient ratio is also larger (0.07), although with large variation, than the high CO_2 ratio (0.06).

The ratios in the cold treatment are smaller than the ratios in the warm treatment, which depicts a temperature effect on the relation of cleithrum to larval size. The ambient/cold ratio is with 0.06 smaller than ambient/warm with 0.07. The same trend is detectable between the high/cold (0.05) and high/warm ratios (0.06). However, this is not statistically significant (ANOVA, p-value= 0.68).

4.3.3 Length of maxilla:

The mean lengths of the maxilla of the larva were measured per tank and are depicted with the respective treatment combinations (ambient/cold, ambient/warm, high/cold, high/warm) in Figure 29.

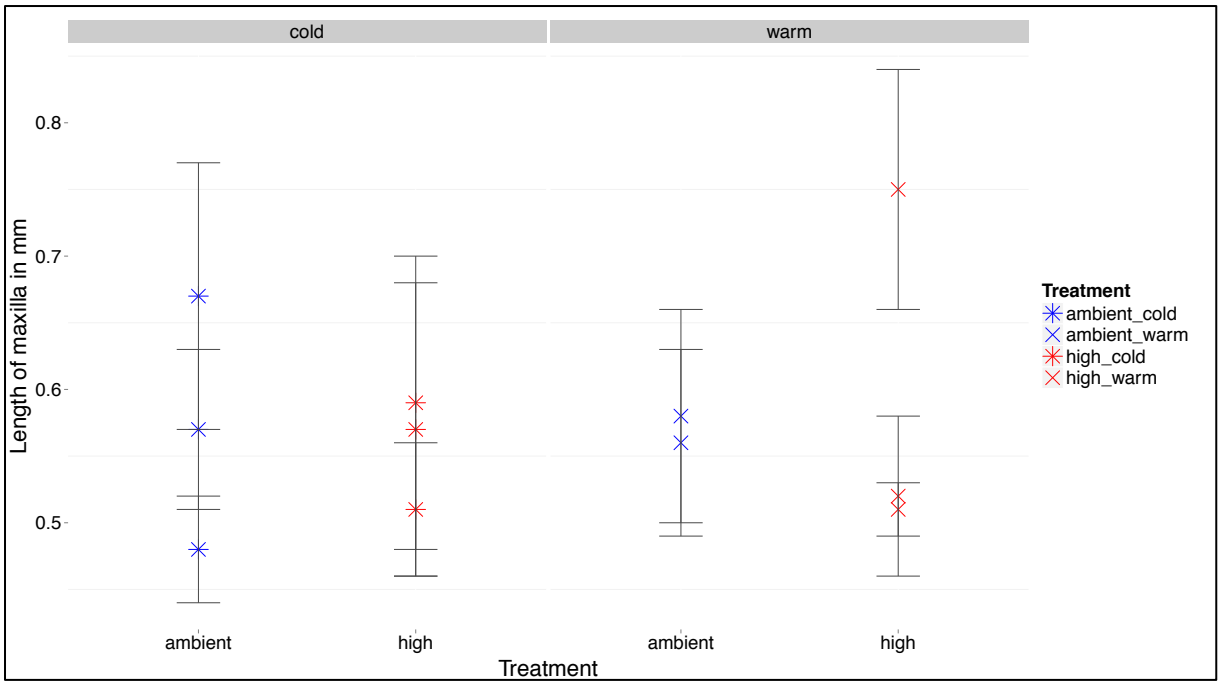


Figure 29: Length (mean and standard deviation) of maxilla in herring larvae (25 dph) per tank. Blue stars depict three tanks of ambient cold treatment and red stars depict three tanks of high CO_2 cold treatment; Blue crosses depict two tanks of ambient warm treatment and red crosses depict three tanks of high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

The mean maxilla lengths do not differ significantly within the four treatment combinations.

In the cold treatment, the mean maxilla lengths of larvae from ambient (0.48mm ± 0.04mm to 0.67mm ± 0.1mm) and high CO_2 tanks (0.51mm ± 0.05mm to 0.59mm ± 0.11mm) are about the same size (ANOVA, p-value= 0.79). In the warm treatment, the mean maxilla lengths out of the ambient tanks (~0.57mm ± 0.07mm) are larger, with exception of tank 4, than the mean lengths out of the high CO_2 tanks (~0.51mm ± 0.02mm).

The mean lengths of the maxilla from the high/warm CO_2 treatments have the same size (~0.51mm ± 0.02mm), except for tank 4, than the ones from the high/cold CO_2 treatment (0.51mm ± 0.05mm to 0.59mm ± 0.11mm). No significant temperature effect was found (ANOVA, p-value= 0.68).

4.3.3.1 Ratio of maxilla to standard length:

The ratio of maxilla to larval size in the four treatment combinations is depicted in Figure 30.

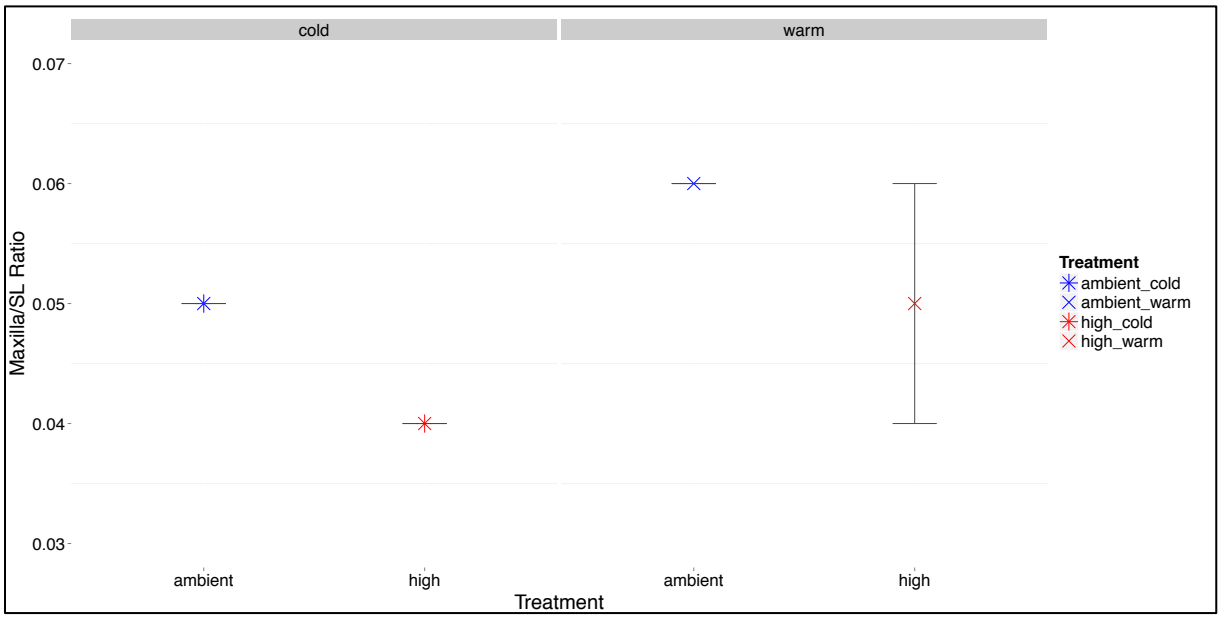


Figure 30: Ratios (mean and standard deviation) of maxilla to larval standard length per treatment. Blue stars depict mean of three tanks in ambient cold treatment and red stars depict mean of three tanks in high CO_2 cold treatment; Blue crosses depict mean of two tanks in ambient warm treatment and red crosses depict mean of three tanks in high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

The ratio of maxilla to larval size varies between temperature and CO_2 level.

In the cold treatment, the maxillae of the ambient treatment are standardized to larval size larger (0.05) than the ones from the high CO_2 treatment (0.04). This is not statisticaly significant (ANOVA, p-value= 0.12). In the warm treatment, the ambient ratio is also larger (0.06), although with large variation, than the high CO_2 ratio (0.05).

The ratios in the cold treatment are smaller than the ratios in the warm treatment, which depicts a temperature effect on the relation of cleithrum to larval size. The ambient/cold ratio is with 0.05 smaller than ambient/warm with 0,06. The same trend is detectable between the high/cold (0.04) and high/warm ratios (0.05). However, this is not statistically significant (ANOVA, p-value= 0.68).

4.3.4 Ratio of cleithrum to maxilla:

The ratio of cleithrum to maxilla is depicted in Figure 31.

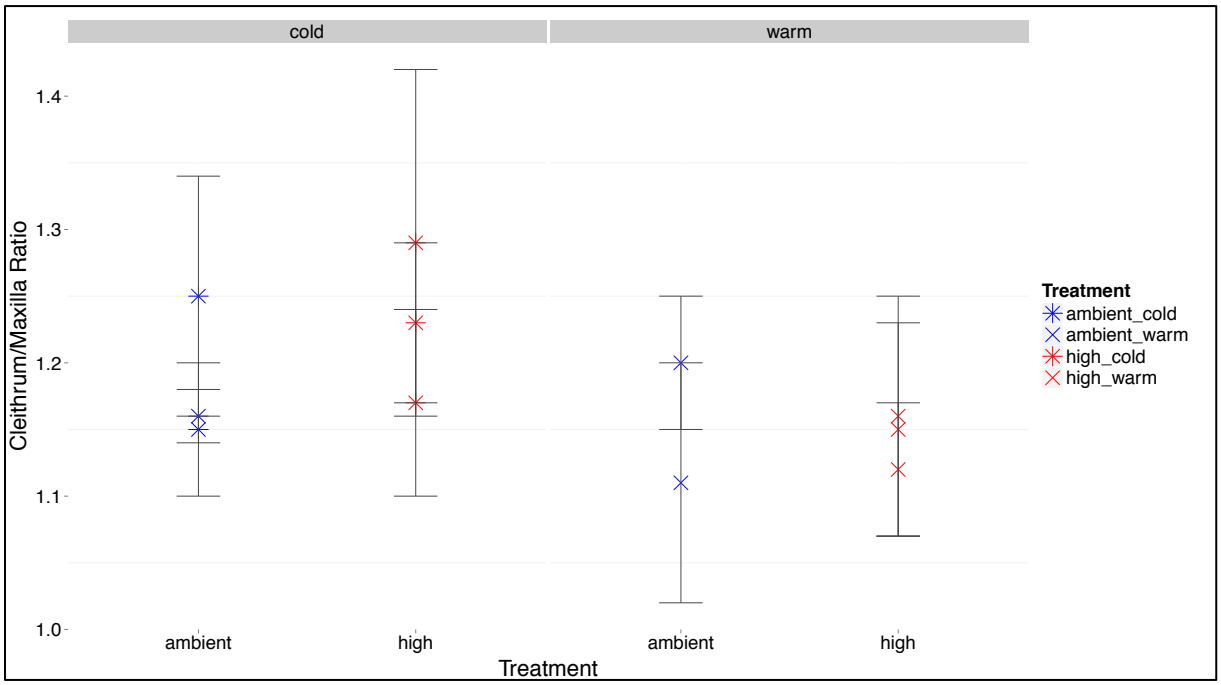


Figure 31: Ratios (mean and standard deviation) of cleithrum to maxilla per tank. Blue stars depict three tanks of ambient cold treatment and red stars depict three tanks of high CO_2 cold treatment; Blue crosses depict two tanks of ambient warm treatment and red crosses depict three tanks of high CO_2 warm treatment. Warm= 12°C, Cold= 10°C, Ambient= 400 µatm, High= 1000 µatm.

In the cold treatment, the mean ratios of the ambient and high tanks did not differ and are in the same range of 1.15 ± 0.05 to 1.25 ± 0.09 in the ambient and 1.17 ± 0.07 to 1.25 ± 0.13 in the high CO_2 tanks (ANOVA, p-value= 0.41). In the warm treatment, the mean ratios also show no difference between ambient and high CO_2 tanks (ambient: 1.11 ± 0.09 to 1.2 ± 0.05; high CO_2: 1.12 ± 0.05 to 1.16 ± 0.09).

The mean ratio of the cold/high tanks are smaller with 1.17 ± 0.07 to 1.25 ± 0.13 than the ratio of the warm/high tanks with 1.12 ± 0.05 to 1.16 ± 0.09. However, this temperature effect is not significant (ANOVA, p-value= 0.07).

Table 9 shows the effects of the two treatments on the larval size, the cleithrum length cleithrum to larval size ratio, maxilla length, maxilla to larval size ratio and the ratio of cleithrum to maxilla.

Table 9: Comparisons between the effects of the two treatments (n.s= not significant). Response variables are SL, cleithrum length and maxilla lenght, cleithrum/SL ratio (C/SL), maxilla/ SL ratio (M/SL) and cleithrum/maxilla ratio (C/M). ↑↓ symbolize larger and smaller length.

Cold treatment		Warm treatment	
Ambient	High CO_2	Ambient	High CO_2
SL ≤ SL n.s		SL ≤ SL n.s	
Cleithrum = Cleithrum n.s		Cleithrum ≥ Cleithrum n.s	
C/SL Ratio ≥ C/SL Ratio n.s		C/SL Ratio ≥ C/SL Ratio n.s	
Maxilla = Maxilla n.s		Maxilla ≥ Maxilla n.s	
M/SL Ratio ≥ M/SL Ratio n.s		M/SL Ratio ≥ M/SLRatio n.s	
C/M Ratio = C/M Ratio n.s		C/M Ratio = C/M Ratio	
No effect	SL, Cleithrum, C/M ↑ n.s	No effect	SL, Cleithrum, C/M ↓ n.s
C/SL, M/SL ↓ n.s	C/SL, M/SL ↓ n.s	C/SL, M/SL ↑ n.s	C/SL, M/SL ↑ n.s

4.4 All experiments:

4.4.1 Standard length of herring larvae:

In order to compare the three experiments with each other, the different sampling procedures of the KOSMOS experiment 2015 were considered as one, but are differentiable by DD. As well as the different tanks in the Kristineberg experiment of 2013 were combined to one value (mean ± SD) per treatment combination (ambient/cold= 345 DD, high/cold= 345 DD, ambient/warm= 413 DD, high/warm= 413 DD). The standard lengths of the herring larvae are depicted with the respective DD in Figure 32.

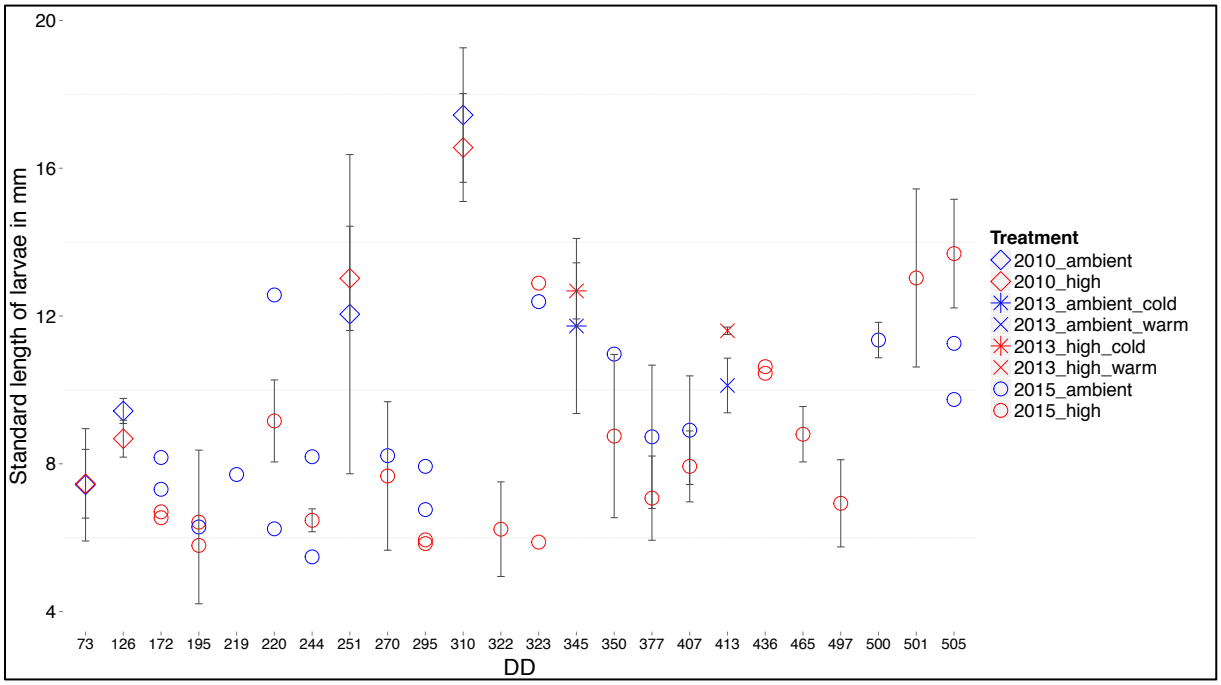


Figure 32: Standard length (mean and standard deviation) of herring larvae from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The larval sizes differ distinctly between the experiments and their respective DD over time. The 2010 experiment has, compared to the other experiments, the coldest water temperatures, which are represented by relatively small DD (73 – 310 DD). However, it depicts the largest and most-developed larvae. Already at 126 DD, the larvae show larger lengths of ~9mm than larvae from the 2015 experiment at later 172 DD (5 - 8mm). The larvae from the 2010 experiment also show higher growth (7,4mm to a maximum of ~17mm) under colder

temperatures than the ones from the 2015 experiment (5-8mm to a maximum of 14mm). The larvae of the 2013 experiment are smaller and less developed compared to those of 2010. At 345 DD, which represents the cold treatment in 2013, the 25 dph larvae are distinctly smaller (~13mm) than the 39 dph larvae from 2010 (~17mm) at 310 DD. However, larvae from 2013 experienced warmer temperatures shown by higher DD than the ones from 2013.

The larvae from the 2013 experiment show a delayed development at 345 DD by reaching about the same sizes (~13mm) than the night catch larvae from 2015 at 323 DD. However, the opposite is shown at 350 DD, where dead larvae from the sediment samples of 2015 are distinctly smaller (~10mm) than the ones from 2013 at 345 DD. The same pattern is valid for larval length (~8.5mm) from 2015 at 407 DD compared to larval length (~10mm) at 413 DD, which represents the warm treatment of 2013 experiment.

No clear treatment effect for all experiments was found. In 2015 experiment, larvae show higher growth in the ambient treatment until 407, which later changed into higher growth in the high CO_2 treatment. In 2010, the larvae are larger in the ambient treatment at 125 and 310 DD. At 73 and 251 DD larvae from the high CO_2 treatment show higher growth. Only in the 2013 experiment, larvae in the cold and warm tanks are larger in the respective high CO_2 treatments than in the ambient ones. This is mainly due to merged tanks in order to depict the experiments in Figure 25.

4.4.2 Length of cleithrum:

The lengths of the cleithrum with the respective DD for all experiments are depicted in the following Figure 33.

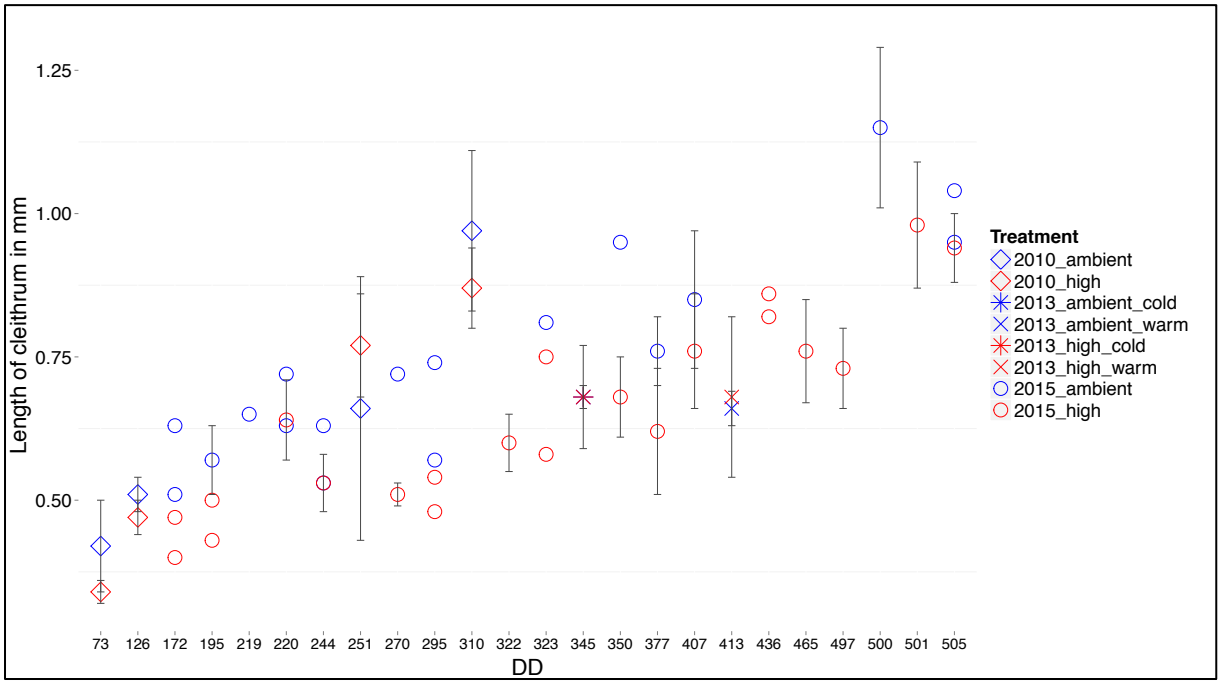


Figure 33: Cleithrum length (mean and standard deviation) of herring larvae from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The different cleithrum lengths from 2010 and 2015 match the growth pattern in size at respective DD as shown in Fig. 25. However, the cleithrum lengths of larvae from those ambient treatments show growth on a higher level over time than in the respective high CO_2 treatments. The cleithrum from the ambient treatments grow from ~0.4mm at the beginning to a maximum of ~1.1mm at the end. In contrast, the ones of the high CO_2 treatments grow from ~0.3mm to ~0.9mm. At 310 DD, the cleithrum length of the larvae from 2010 are distinctly larger (~0.9mm) in both treatments than the cleithrum (~0.6mm) of larvae from 2015 at comparable 295 DD, which shows a delay in the cleithrum development in larvae of 2015 at this distinct DD.

The cleithrum lengths of larvae from the two ambient treatments of the 2013 experiment do not fit into this overall growth pattern. They show in the cold (~0.7mm at 345 DD) and in the warm (~0.65mm at 413 DD) smaller growth than the cleithra from the 2015 ambient

treatment at comparable 350 and 407 DD (0.95mm and ~0.8mm). The high CO_2 treatments show in contrast similar lengths of the cleithrum (~0.7mm at 345 DD; ~0.7mm at 413 DD) compared to the ones of 2015 at 350 and 407 DD with 0.7mm and ~0.75mm respectively.

4.4.3 Ratio of cleithrum to standard length:

The ratio of cleithrum to larval size in all experiments over time is depicted in Figure 34.

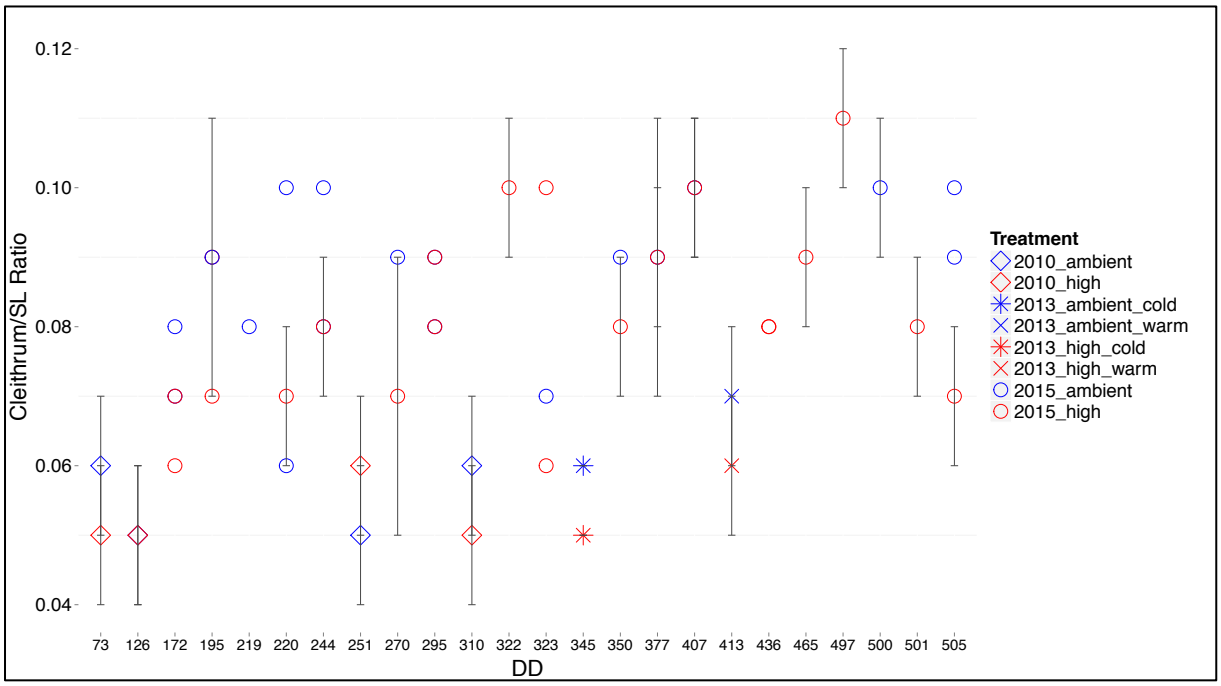


Figure 34: Ratio (mean and standard deviation) of cleithrum to larval standard length from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The ratios of cleithrum to larval size differ between the three experiments and depict distinct treatment effects.

The relationship between cleithrum length and larval standard length in the 2010 experiment is the smallest (0.05 – 0.06), but most constant one over time. The cleithra standardized to larval size are larger in the ambient treatment than in the high CO_2 treatment. The same treatment pattern is detectable in the 2013 experiment, and also on the same ratio level of 0.06 in the ambient treatment and 0.05 in the high treatment. The warmer treatment (413 DD) shows higher ratios, but depicts also a treatment effect with 0.07 in the ambient and 0.06 in the high CO_2 treatment.

The 2015 experiment shows the largest ratios compared to the other two. The cleithrum to larval size relationship oscillates distinctively from 0.06 to 0.11 over time. However, 2015 depicts also larger cleithra standardized to SL in the ambient treatment than in the high CO_2 treatment.

4.4.4 Length of maxilla:

The lengths of the maxilla with the respective DD for all experiments are depicted in the following Figure 35.

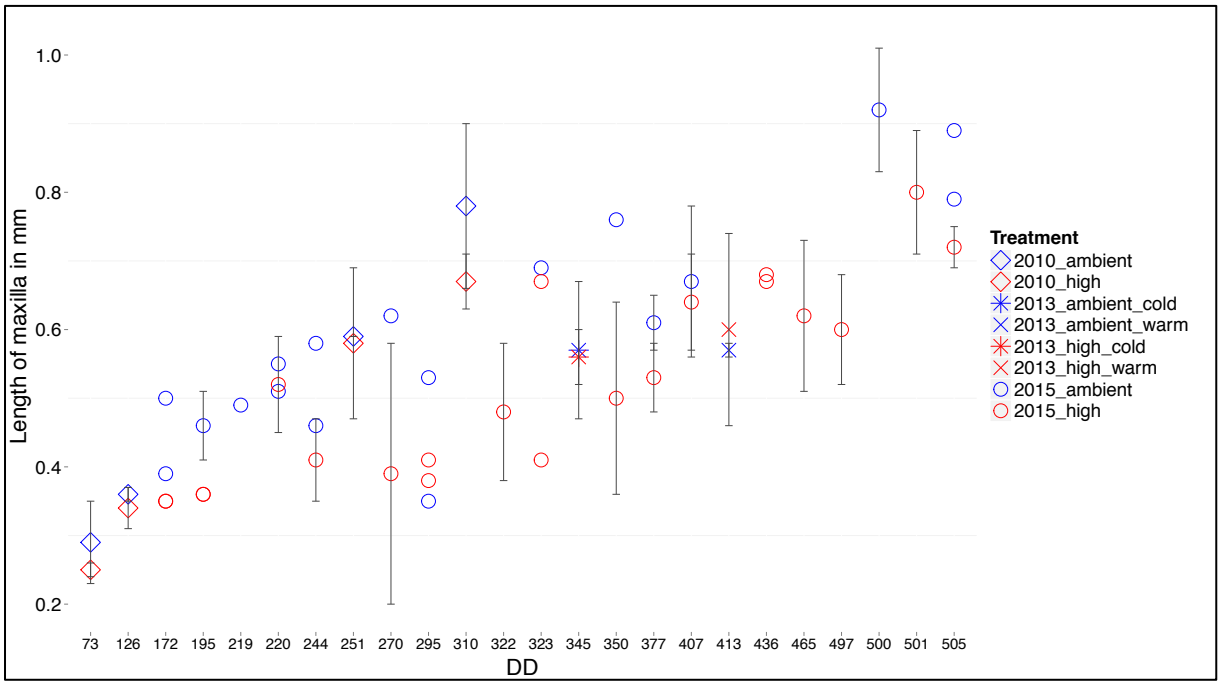


Figure 35: Maxilla length (mean and standard deviation) of herring larvae from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The same pattern like for the cleithrum growth is valid for the maxilla growth in the three experiments. Similar growth pattern for maxilla lengths of larvae from 2015 and 2010 over their respective DD are shown. The ambient treatment larvae of those two experiments depict, with deviations, larger maxilla than in the high CO_2 treatment larvae. They grow from ~0.29mm at the beginning to a maximum of ~0.9mm at the end, whereas the maxillae of the high CO_2 larvae start with ~0.25mm and reach a maximum of 0.75mm. At 310 DD, the

maxilla length of the larvae from 2010 are distinctly larger (~0.7mm) in both treatments than the cleithrum (~0.4mm) of larvae from 2015 at comparable 295 DD.

The maxilla lengths of larvae from the ambient treatments of the 2013 experiment, cold (~0.55mm at 345 DD) and warm (~0.55mm at 413 DD), show smaller growth than the maxilla from the 2015 ambient treatment at comparable 350 and 407 DD (0.76mm and ~0.65mm). The high CO_2 treatments show in contrast similar lengths of the maxilla (~0.55mm at 345 DD; ~0.6mm at 413 DD) compared to the ones of 2015 at 350 and 407 DD with 0.5mm and ~0.65mm respectively.

4.4.5 Ratio of maxilla to standard length:

The ratio of maxilla to larval size in all experiments over time is depicted in Figure 36.

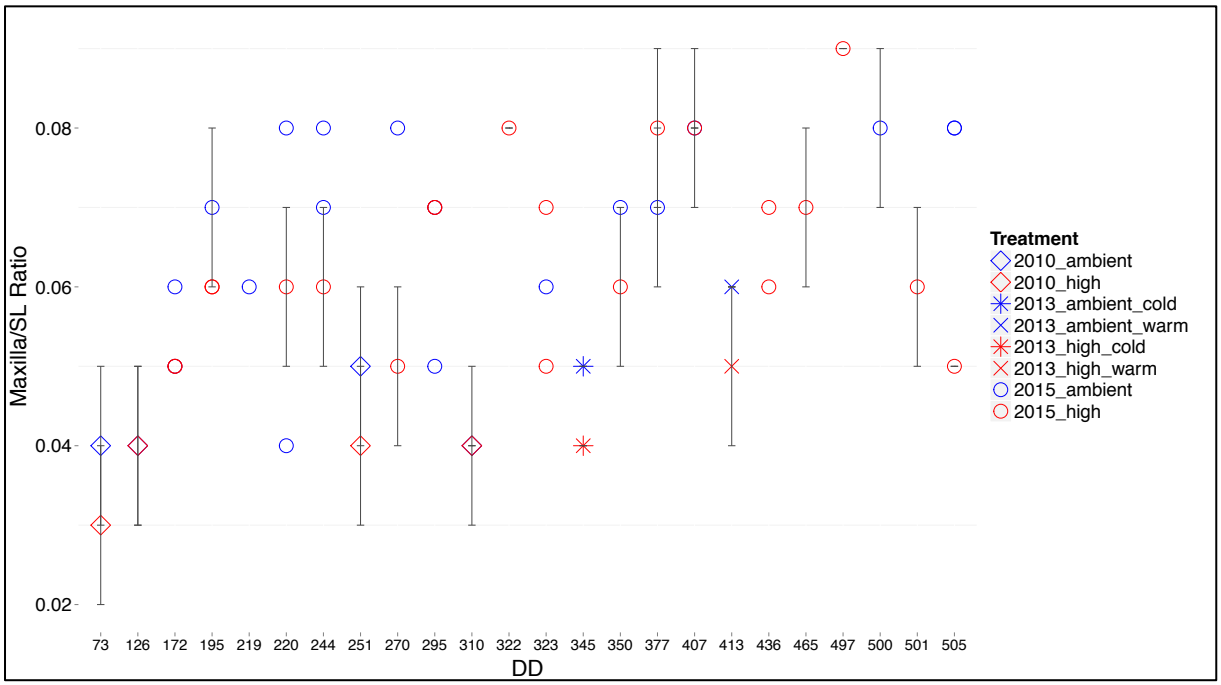


Figure 36: Ratio (mean and standard deviation) of maxilla to larval standard length from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The ratios of maxilla to larval size differ between the three experiments, but show relatively stable proportional growth over time and depict distinct treatment effects.

The relationship between maxilla length and larval standard length in the 2010 experiment is the smallest (0.03 – 0.05), but most constant one over time. The maxilla standardized to larval size is larger in the ambient treatment (~0.045) than in the high CO_2 treatment (~0.035). The

same treatment pattern is detectable in the 2013 experiment, but on a higher ratio level of 0.05 in the ambient treatment and 0.04 in the high treatment. The warmer treatment (413 DD) shows higher ratios, but depicts also a treatment effect with 0.06 in the ambient and 0,05 in the high CO_2 treatment.

The 2015 experiment shows the largest ratios, compared to the other two. The maxilla to larval size relationship oscillates distinctively from 0.04 to 0.09 over time. However, 2015 depicts also larger maxilla standardized to larval length in the ambient treatment than in the high CO_2 treatment.

4.4.6 Ratio of cleithrum to maxilla:

The ratio of cleithrum to maxilla in all experiments over time is depicted in Figure 37.

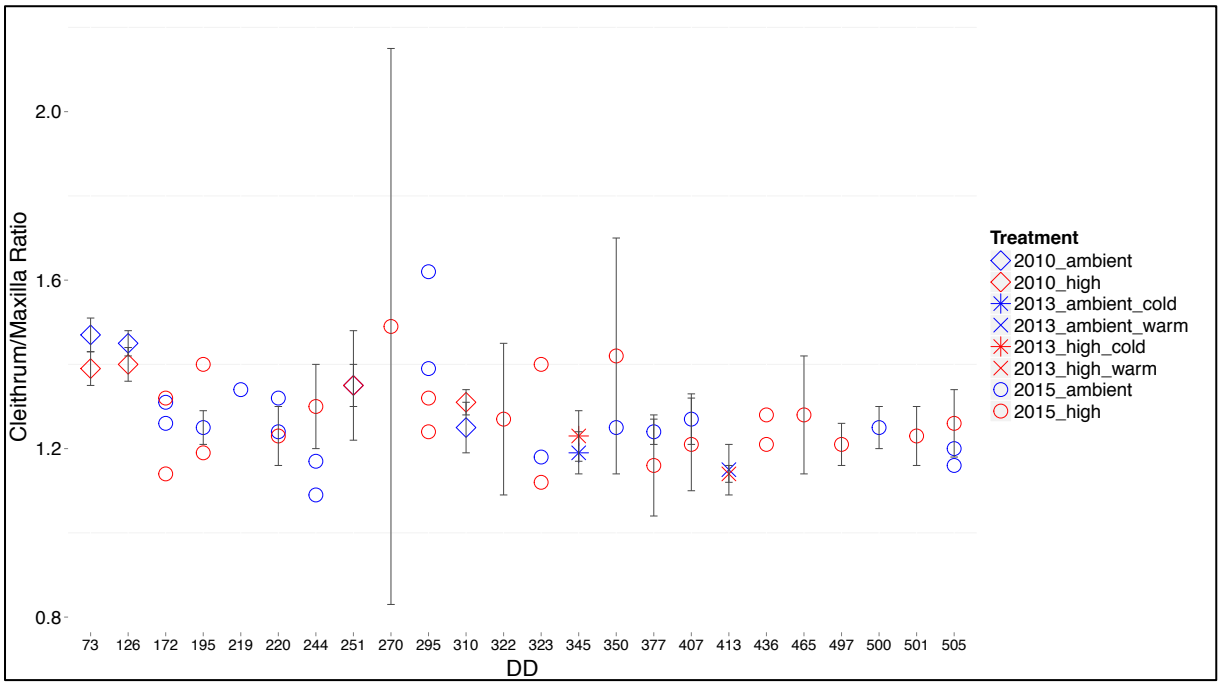


Figure 37: Ratios (mean and standard deviation) of cleithrum to maxilla from three different experiments over time represented by respective DD. **Experiment 2010:** Blue diamonds depict ambient treatment and red diamonds depict the high CO_2 treatment. Mean and standard deviation per treatment. **Experiment 2013:** Blue stars depict ambient cold treatment and red stars depict high CO_2 cold treatment; Blue crosses depict ambient warm treatment and red crosses depict high CO_2 warm treatment. Mean and standard deviation per treatment. **Experiment 2015:** Blue points depict ambient treatment and red points depict high CO_2 treatment. Mean and standard deviation only calculated for $n \geq 3$.

The different ratio data from 2010, 2013 and 2015 oscillate distinctively over time and show no treatment preference. A distinct difference between the experiments are not detectable, although the ratio of cleithrum to maxilla in larvae from both 2010 treatments show at 73 DD and 126 DD a larger length relationship (~1.5) than in the 2015 experiment (~1.3) at later 172 DD. This indicates, compared to the other cleithrum/maxilla ratios that the maxilla grows

faster than the cleithrum in larvae from 2010 at 73 and 126 DD. At 270 DD the ratio in larvae from the 2015 experiment show a large deviation, due to low sample size of n= 3.

5. Discussion

5.1 KOSMOS 2015

The KOSMOS 2015 experiment gave the unique opportunity to enclose a natural plankton community, manipulate the water to a future pCO_2 scenario of 2200 µatm (End of 23th Century) and examine the effects of ocean acidification on the predominant community over time (Riebesell *et al*., 2013).
The addition of Atlantic herring served in this experimental set up as a theoretical top down control in a progressing near natural environment, and was at the same time a great opportunity to focus on the effects of OA on larval growth and skeletal development (ossification).

5.1.1 Standard length:

The results showed an overall very low growth and development of herring larvae in all mesocosms over time. 42 dph larvae reached a maximum length of 13,7mm ± 1,5mm during respective 9°C to 16°C (505 DD). This shows a clear reduction of growth, compared to herring growth under controlled laboratory conditions (8°C, 40-1200 prey L^{-1}) by Folkvord *et al*. (2000), which showed maximum SL of 18mm at 40 dph. This poor development can be explained by naturally low abundances of nauplii and copepodites (*Calanus* spp., *Oithona* spp., *Temora* spp.) in all mesocosms, assuming no distinct treatment effect. In KOSMOS 2015, the nauplii abundance, which is considered to be the suitable food source especially at critical first larval feeding, was on a low concentration level. This nauplii fraction showed a distinct decrease in abundance throughout the experiment. With already low 4,9 prey L^{-1} at the start of the experiment to 1.7 prey L^{-1} at the end of the experiment in all mesocosms (Spisla, unpublished data 2015). The copepodite abundance, which is considered to be the suitable food source in the later stages of the larval development, was also on an extreme low concentration level. From 6.5 prey L^{-1} in the ambient and 5.3 prey L^{-1} in the high CO_2 treatment to respective 3.4 prey L^{-1} and 0.8 prey L^{-1} (Spisla, unpublished data 2015).
Assuming the low concentration of adequate food compared to minimum food density values of 10 prey L^{-1} at which fish larvae show sufficient growth (Rosenthal and Hempel, 1970; Werner, 2011), KOSMOS 2015 can be considered as a starvation experiment. The herring larvae suffered from diminished energy intake, which can be an additional stress factor to the treatment stress.

According to de Silva (1972), gill filaments with lamellae for branchial respiration do not develop in Atlantic herring until a larval size of 20mm SL. This developmental stage was never reached by herring in this experiment, implying that all of the larvae depended on cutaneous gas exchange. Therefore, a weak acid-base regulation, especially in the larvae of the high CO_2 mesocosms, can be assumed. Frommel *et al.* (2014) speculated that the energy needed for efficient osmoregulation leads to a decrease in energy available for growth. Hence, this leads to smaller herring larvae in the elevated treatment than in the ambient one (Frommel *et al*., 2014). The same results can be shown with the sediment samples of the KOSMOS 2015 experiment.

However, at the end of the experiment the high CO_2 treatment showed larger survivors than the ambient treatment. This was consistent the next day, were sediment samples also depict larger larvae in the high CO_2 mesocosms than in the ambient ones, assuming the loss of few larvae during the end catch procedure with the big 1000µm fish net. This switch of size can be explained by possible size selective mortality mechanisms throughout the experiment. Mortality rates usually decrease with increasing body size, because larvae that grow faster spend less time in critical size classes (Peterson and Wroblewski, 1984; Houde, 1987; Miller *et al*., 1988). This "bigger is better" hypothesis has a logical appeal of larger larvae being better adapted as smaller larvae to escape from predators, resist starvation and tolerate physiological extremes (Sogard, 1997). According to this hypothesis, larvae in the elevated mesocosms showed a size selection for larger larvae at the end of the experiment. This could depict an effect within the elevated pCO_2 mesocosms, assuming survival of the fittest under stressed conditions, whereas in the ambient treatment no selection occurred. Due to very low survival rates and therefore low sample sizes at the end of the KOSMOS experiment, this result is speculative. In the four ambient mesocosms only three out of ~12000 larvae (0.03%) survived, whereas in the high CO_2 mesocosms 50 out of ~12000 larvae (0.4%) survived. This apparently high mortality throughout the experiment is not reflected in high numbers of dead larvae in the sediment. Contrary low numbers were sampled, which was possibly due to high predation of larvae by juveniles from gadoid fish species, pelagic polychaetes like *Tomopteris sp.* (J.F. Eschscholz, 1825) and different jellies as e.g. *Aglantha digitale* (O.F. Müller, 1776), *Clythia* sp. (Lamouroux, 1812), *Sarsia tubulosa* (M. Sars, 1835), *Obelia geniculate* (Linnaeus, 1758*)*, which were caught in all mesocosms at the end of the experiment (Spisla, 2015).

5.1.2 Cleithrum and Maxilla lengths:

The larvae from the ambient treatment showed a trend of larger cleithra and maxilla than larvae from the high CO_2 treatment throughout the experiment. This could indicate a direct negative CO_2 effect on the skeletal ossification in herring larvae.

Fish skeleton is mainly composed of calcium phosphate (Lall and Lewis-McCrea, 2007), which performes additional buffering with bicarbonate and non-bicarbonate ions under low pH conditions. This could interfere with the development of bone tissue, especially at early life stages when cutaneous osmoregulation is predominant. Until now, only Pimentel *et al*. (2014b) found reduced skeletogenesis under hypercapnic conditions (pCO_2= ~1600 µatm) in 30 dph *Solea senegalensis* larvae.

Further, a distinct delay in the development of cleithrum and maxilla in larvae from the high CO_2 treatment compared to those in larvae from the ambient treatment was visible. The cleithrum to SL ratio and the maxilla to SL ratio were always smaller in the elevated treatment than in the ambient treatment over time, assuming decoupling of somatic growth and bone growth under stressed conditions. This decoupling was very distinct in the "survivors" from the high CO_2 mesocosms. They showed larger somatic growth at the end of the experiment, but substantial smaller cleithra and maxilla related to body size. This different response of somatic growth and bone growth to stress was also shown by Mosegaard *et al.* (1988). They found decoupling of somatic growth and otolith growth rates in arctic char (*Salvelinus alpinus*) as an effect of difference in temperature response and postulated a reconsideration of somatic growth as a development parameter.

No decoupling within the different bones of the skeleton was detectable as the length ratio of cleithrum to maxilla was relatively constant over time. Both bones grew at a constant proportion to each other over time, assuming same response on CO_2 stress over time. However, the ambient ratio was on a higher level than the ratio of high CO_2, because the maxilla grew faster than the cleithrum in the ambient treatment compared to the elevated ones.

Limitations in the development of important bony structures like the cleithrum and the maxilla could have negative consequences for the successful recruitment of herring. The cleithrum serves as the attachment site of the pectoral fins, which are important for the first swimming movements and mobility to approach suitable prey at the critical first-feeding stage as postulated by J. Hjort (Houde, 2008). A well-developed and functional jaw plays also an important role at the first-feeding stage of herring larvae (Houde, 2008). Larvae with small jaws might have immense feeding restrictions and less energy intake than larvae with normal

or bigger jaws. Hence, small cleithra and maxilla could have severe negative implements on foraging and survival.

5.2 Espegrend 2010

In the Espegrend 2010 experiment, herring larvae were reared at two different future ocean acidification scenarios and today´s levels under near-natural conditions in large outdoor tanks (Frommel *et al*., 2014). In order to compare the effects of OA on larvae between the different experiments, the end of the century scenario samples with a medium pCO_2 level of 1830 µatm were favored over the upwelling scenario samples with a high pCO_2 level of 4260 µatm.

5.2.1 Standard length:

The 39 dph larvae reached under *ad libitum* conditions (2000 prey L^{-1}) maximum lengths of 17.4mm ± 1.8mm, which does not match the maximum larval length of 20mm monitored by Frommel *et al.* (2014). Frommel et al. (2014) measured SL from photographs of live larvae, whereas in this thesis the SL was measured from photographs of larvae, which were already fixed in 95% ethanol. Considering a mean shrinkage of 4.1% ± 0.3% in 100% ethanol (Cunningham *et al*., 2000), maximum larval lengths of approximately 19.2mm can be reached. It can only be assumed that there was a certain size selection within the sampling and fixation procedure.

However, the treatment effect on larval length and growth was monitored in both studies. The larvae from the ambient treatment were larger than larvae from the high CO_2 treatment over time. At 251 DD, the opposite trend was monitored, but this is probably due to lower sample size. As all larvae did not reach 20mm (de Silva, 1974), they depended completely on cutaneous gas exchange and could propably not show efficient acid-base regulation. It can be assumed that they spend more energy on osmoregulatory processes than for somatic growth (Frommel *et al*., 2014). This observed reduction in growth under elevated pCO_2 could keep larvae for a longer time in early life stages, which are known to be a subject to very high rates of mortality and represents a bottleneck for the recruitment of fish (Bailey and Houde, 1989). Leaving the larval stages as fast as possible is crucial for fish in order to be less susceptible to predation, and survival because of the crucial match-mismatch with larval zooplankton prey (Cushing *et al.,* 1990; Suthers, 1992).

5.2.2 Cleithrum and Maxilla lengths:

The larvae from the ambient treatment showed throughout larger cleithra and maxilla than larvae from the high CO_2 treatment. However, the opposite trend was shown at one sampling day (251 DD), where the ambient treatment depicted smaller cleithra than the elevated pCO_2 treatment, assuming no clear effect due to smaller sample size. Despite the 251 DD, all results revelead a negative CO_2 effect on the skeletal ossification in herring larvae.

The results showed also certain decoupling of somatic and bone growth under stressed conditions, as the cleithra and maxilla related to body size were smaller in the high CO_2 treatment than in the ambient ones. Especially at 251 DD, as the larvae of the high CO_2 treatment showed larger somatic growth, but smaller maxilla in relation to it´s larval body size.

The ratio between the cleithrum and maxilla in both treatments declined significantly over time, indicating that the maxilla was growing faster than the cleithrum. This delay could also show a decoupling between bony structures. The fact that there is no treatment difference could reveal a certain adaption to the *ad libitum* food availability. Depending on the prey densitiy, either swimming or feeding could be favored. As there is no need for extended foraging trips, the cleithrum development delays and the maxilla development is promoted. Somarakis *et al.* (1997) found this kind of developmental instability (fluctuating asymmetry) only in bilateral traits like otoliths of larval fish. There, it is well used as a sensitive indicator of genetic and environmental stress and hence promoted as a fitness parameter (Parsons, 1990; Clark, 1995). To what extent developmental instability between two different bones can be used as a condition factor, is so far not clear and cannot be determined with the present material.

5.3 Kristineberg 2013:

In this experiment, herring larvae were not only exposed to two levels of pCO_2 (a control group*:* pCO_2 400 µatm and a high group: pCO_2 1000 µatm) but also to two levels of temperature (10°C and 12°C) in order to monitore the synergystic effects of ocean acidification and ocean warming under food limitation (131 - 600 prey L^{-1}).

5.3.1 Standard length:

The results indicated that the growth of herring larvae is not significantly affected by OA, with the combined differences in temperature. The 25 dph larvae were, in both temperature levels, in the ambient treatment smaller (~10mm) than in the high CO_2 treatment (12mm - 13mm). The response implies that the larvae might be robust to medium high pCO_2 of 1000 µatm, which could indicate a natural adaption to variations in ambient pCO_2 levels. Franke and Clemmesen (2011) showed that local Baltic herring populations are not significantly affected by OA because they are adapted to naturally high fluctuations of pCO_2 (2300µatm). The Atlantic herring has a wide distribution troughout the North Atlantic Ocean, where they experienced different conditions in terms of temperature, salinity, oxygen and present pCO_2 levels (Whitehead, 1985; Maravelias *et al*., 2000). Limborg *et al*. (2012) and Lamichaney *et al*. (2012) found indications of local adaption in genes of different herring stocks in the Baltic, North Sea and North Atlantic. The Atlantic herring of this study might also be adapted to naturally high fluctuation of pCO_2 in especially shallow spawning regions along the Norwegian coast down to the Western Baltic, where upwelling of oxygen-poor and CO_2-rich water with pCO_2 values of ~1000 µatm regularly occur in the summer months (Thomsen *et al*., 2010). Early life stages smaller than 20mm SL have not yet developed gills and kidneys in order to regulate and maintain their internal ionic environment (Morris, 1989; Sayer *et al*., 1993, Katoh *et al*., 2000; Ishimatsu *et al.*, 2004). However, there are higly specialized ionregulatory chlorid cells in the yolk sac membrane and body skin of larvae, which can change in abundance, distribution and size in order to cope with ionic stress (Hiroi *et al*., 1999; Kikkawa *et al.,* 2002). Bodenstein (2012) was able to detect chlorid cells in Baltic herring embryos, but could not show change in distribution or size under elevated pCO_2 (1000µatm - 4000µatm), assuming natural adaption responses to acidified conditions.

Results from the Kristineberg 2013 experiment showed further, that the larvae from the warmer CO_2 treatment were smaller than the larvae from the cold CO_2 treatment. In the warm and cold ambient treatments was no larval size difference at all. This stands in strong contrast to larval growth rates, which normaly increase with higher temperatures, due to faster metabolism and food consumption (Houde, 1989). One reason for that could be that the larvae in the warm treatment had to increase their mitochondrial density, which ultimately causes a rise in oxygen demand and reduces the energy availability for growth owing to elevated costs of mitochondrial maintenance (see also Pörtner *et al*., 2001). Especially, as the limited prey density (131 - 600 prey L^{-1}) does not allow for excessive food consumption.

5.3.2 Cleithrum and Maxilla lengths:

In the cold treatment, 25 dph larvae had cleithra and maxillae of the same size under ambient and high CO_2 conditions. Differences in size have been observed with higher temperatures. In the warm treatment, cleithra and maxillae of larvae from the high CO_2 treatment were smaller than of those from larvae of the ambient treatment. However, in the cold treatment the ratios of both bones related to SL showed that although larvae are bigger in the high CO_2 treatment, the cleithrum and maxilla were of the same size in both (ambient and CO_2) treatments, whereas under warm temperatures cleithrum and maxilla were smaller in larvae of the high CO_2 treatment than those from the ambient treatment, although the former had larger sizes. The ratio between cleithrum/SL and maxilla/SL in larvae of the ambient treatment for both temperatures showed a trend to be higher than in the larvae of the CO_2 treatment. This indicates an advanced bone growth relative to larval size in the ambient treatment compared to the high CO_2 treatment. In other words, these data indicate that larval growth and skeletal growth responded differently in environmental stressors. It can therefore be assumed that somatic growth and bone development is decoupled under OA. The ratio of ceithrum to maxilla was constant within the four treatment combinations, assuming same response to stress in both bones.

The only negative synergistic effect of elevated temperature and pCO_2 was shown for the cleithrum and the cleithrum to maxilla ratio. So far, only Pimentel *et al*. (2014) found these synergistic negative effects of warming and ocean acidification on the skeletegonesis in S. *senegalensis*. They detected faster growth, which was accompanied by an increase of skeletal deformities. Georgakopolous *et al.* (2010) examined a significant effect of increased water temperature (16°C, 19°C, 22°C) on the development of skeletal deformities in early life stages of Gilthead seabream (*Sparus aurata*, Linneaus 1758) due to higher growth.

The cleithrum related to larval size ratio and the respective maxilla ratio were smaller in the cold treatment than in the warm treatment, regardless of the pCO_2 level. This is due to bigger larvae in the cold treatment, as more energy can be invested in growth than in metabolism.

5.4 Comparative examination of all experiments:

All herring larvae of the different experiments originated from the Norwegian spring-spawning herring population, hence similar reactions to ocean acidification can be expected, depending on the similar environment they experienced. Larval stages (25, 39, 42 dph) from the different experiment were distingtively smaller than 20mm, a length that marks the energy demanding transition from one acid base regulatory site (skin, yolk sac) to the other (gill) (de Silva, 1974; Melzner *et al*., 2009). Therefore, it can be assumed that all herring larvae used in this study depended on cutaneous gas exchange, which leaves them less tolerant and most susceptible to ocean acidification (Raven *et al*., 2005).

However, the three experiments differed strongly according to set up, prey densities, and abiotic conditions such as temperature and pCO_2 levels. As food availability is the most crucial factor for growth, different prey densities in the different studies are the main reason for different growth.

The 2010 experiment established *ad libitum* food conditions (2000 prey L^{-1}) in land-based mesocosms and the 39 dph larvae showed highest and fastest growth in coldest temperatures. In the Kristineberg 2013 experiment, the 25 dph larvae were reared in the laboratory under food limitation (131 - 600 prey L^{-1}) and displayed medium high growth. As the 2015 experiment had the lowest prey densities (~5 prey L^{-1}) in a near natural environment (mesocosms), the 42 dph larvae showed the smallest growth. The low prey densities in this experiment could not be compensated by extra additions of prey organisms and caused high mortality. The resulting small sample size was the main problem for not allowing testing for significant results in this experiment.

Regardless of different elevated pCO_2 levels, 2010 and 2015 experiments showed a distinct treatment effect, with smaller larvae in the elevated CO_2 treatment than in the ambient treatment. This confirms the first hypotheses of this thesis. However, the 2013 experiment demonstrated the complete opposite result, with larger larvae in the high CO_2 treatment than in the ambient treatment, assuming that the pCO_2 of 1000 µatm was not high enough to trigger a negative response in larval size. Another possible explanation could be an adaption to natural high pCO_2 levels as it was demonstrated in Baltic herring under acidified conditions (Franke and Clemmesen, 2011). In the same study, they also revealed negative effects on the RNA/DNA ratio, which served as an indicator for condition and development in herring larvae (Clemmesen, 1994).

Although the larval growth was different between the experiments, they showed surprisingly similar sized bones. Indicating already different responses of somatic growth and skeletal

growth on food availability. This kind of decoupling was very well shown by smaller cleithrum/SL and maxilla/SL ratios in the 2010 and 2013 experiments, which showed the largest and most developed larvae, but with relatively small cleithrum and maxilla. Additionaly, in all experiments, a trend for developmental delay in ossification and decoupling of somatic growth and bone development could be revealed under elevated pCO_2. The ratios are overall smaller in the manipulated treatment than in the ambient one, which shows severe negative impacts on the development of the cleithrum and the maxilla. This could have negative consequences for foraging and feeding at the critical first-feeding stages. Furthermore, it hints to a clear disadvantage in acidified conditions and approves the second hypothesis of this study.

This study showed, although not significant and based on small sample size, that Atlantic herring larvae are expected to suffer under ocean acidification by diminished growth and skeletal development. Furthermore, growth as a condition factor has to be reconsidered, due to different stress responses of somatic growth and bone development.

6. Conclusion and Outlook

In the KOSMOS 2015, Espegrend 2010 and Kristineberg 2013 experiments, herring larvae were exposed to distinctivly different levels of pCO_2 (2200, 1830 and 1000 µatm) and prey densities (5, 2000 and 131 - 600 prey L^{-1}). The results showed, that positive growth of herring larvae depended largely on sufficient food availability and the level of pCO_2 values. As elevated pCO_2 of 1830 µatm and 2200 µatm tended to result in diminished growth, the lowest level of 1000 µatm had no negative effect on larval growth, assuming that they were already adapted to similar natural pCO_2 levels.

However, all levels of pCO_2 indicated to negatively impact the skeletal development of the shoulder girdle and the upper jaw of the larvae, which could have negative consequences on foraging and survival. Further, this study could show that somatic growth and bone growth differentiated in their responses to food and pCO_2 stress, assuming developmental decoupling. This leads to a reconsideration of somatic growth as a condition factor in larval development.

In conclusion, the results indicated, although not significant and possibly due to a low sample size, that ocean acidification could negatively affect the growth and skeletal development in herring larvae, which could lead to severe negative consequences of survival and recruitment. Further, they indicated that the Atlantic herring has the ability to adapt to relativly low elevated pCO_2 levels of 1000 µatm by showing no negative effects on somatic growth, although the internal growth of the skeleton shows the opposite response, also regarding food density.

Further ocean acidification studies are needed to validate these indications by analyzing the skeletal development in a sufficient amount of larvae and relate it to RNA/DNA ratios in order to give a better estimate of larvaes' condition. Additionaly, a baseline study of skeletal development and growth in herring under natural conditions would be a great advantage in order to accomplish certain comparability.

7. Eidesstattliche Erklärung:

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung anderer als der im Literaturverzeichnis angegebener Quellen angefertigt habe.

Die Arbeit wurde bei keiner anderen akademischen Institution zwecks Erlangung eines akademischen Grades eingereicht.

Mit der Aufnahme der Arbeit in die Bibliotheken des GEOMAR und der Christian-Albrechts-Universität Kiel bin ich einverstanden.

Kiel, den 19.02.2016

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Henrike Wunderow

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