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Effect of temperature and CO₂ concentration on the morphogenesis of sagittal otoliths in Atlantic herring (*Clupea harengus*) larvae

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Abstract

Otoliths are very useful biomarkers especially for fish growth. Climate change with the associated global changes in warming and acidification could affect the calcification and the shape of otoliths during the crucial larval period in teleost fish. To evaluate this predicted combined effect of temperature and CO₂, Atlantic herring (*Clupea harengus*) embryos and larvae were reared from hatching to respectively 47 and 60 days post-hatching (dph), under present day conditions and a scenario predicted for the year 2100 (IPCC RCP8.5). Otolith morphogenesis was tracked by analyzing area and normalized Elliptical Fourier coefficients. We found that otolith area for fish of similar size increased under the predicted 2100 climate change scenario compared to the present day. Climate change does not, however, seem to directly affect the otolith shape. Finally, the onset of otolith morphogenesis is hardwired, but the relationship between otolith and fish size is environment-dependent.

Keywords

otolith shape, otolith area, temperature effect, ocean acidification, Elliptic Fourier descriptors, directional asymmetry

1. Introduction

Otoliths are calcified biomineralised structures overlying the sensory epithelia in the inner ear of fish. They are formed by calcium carbonate crystals embedded in a non-collagenous organic matrix composed of acidic proteins and polysaccharides (Degens et al., 1969; Popper et al., 2005). They are metabolically inert and do not resorb in periods of stress, but grow throughout an individual's life, in correlation with its ontogenic growth (Casselmann, 1987). Because of the positive correlation between otolith and fish growths, otoliths have been widely used in fisheries science to understand the fish growth and the potential effects which controlled it (i.e. environment, fishing pressure; Enberg et al., 2011 ; Marty et al., 2014; Carbonara et al., 2022). In Atlantic herring (*Clupea harengus*), otolith microstructure has been used to study environmental effects (temperature, salinity or feeding activity) on the growth of the larvae (Folkvord et al., 1997 ; 2004 ; Johannessen et al., 2000; Berg et al., 2017; Denis et al., 2017; Tonheim et al., 2020), and otolith shape used as a tool for stock identification (Turan, 2000; Burke et al., 2008; Libungan et al., 2015). Atlantic herring are of high commercial importance, with around 1,640,000 tons caught in 2016 (FAO FishStat data), but are vulnerable to the effects of climate change, particularly at the larval stage (Hufnagl and Peck, 2011).

Otolith growth, and ultimately global otolith shape are well-known to result from the combination of genetic heterogeneity, ontogeny (physiological processes) and the influence of environmental (biotic and abiotic) factors (Vignon and Morat, 2010; Mahé, 2019; Hüsey et al., 2020). Global climate change is projected to cause warming of the ocean surface by 3 to 5°C by 2100, and acidification of waters with a drop of 0.4 pH units in the worst-case scenario (IPCC 2019). The speed at which global change is occurring will not allow some species to adapt; in addition, early life history stages which do not yet have fully functional physiological homeostatic mechanisms could be particularly affected

(Melzner et al., 2009). Some ontogenic processes, such as otolith morphogenesis, could thus be particularly impacted by the alteration of environmental conditions.

A previous study on herring larvae testing four temperature conditions showed that the size and the width of the otolith daily increment were both temperature-dependent (Folkvord et al., 2004). Other studies on the effects of ocean acidification on fish otoliths have been mostly conducted on larval developmental stages. Several studies have shown that elevated levels of CO₂ caused an increase in otolith size or modification of the otolith shape for multiple species (Munday et al., 2011; Bignami et al., 2013; Maneja et al., 2013; Reveillac et al., 2015; Coll-Llado et al., 2021). Consequently, acidification can lead to decoupling of otolith and body growth for some species (Reveillac et al., 2015; Franke and Clemmesen 2011; Frommel et al., 2013; Perry et al., 2021).

Here, we aimed to investigate in Atlantic herring how otolith shape and size variability is affected by the warming and acidification conditions projected by 2100 under the worst-case IPCC scenario during the first early life stages. This otolith shape was investigated within individual (i.e. directional bilateral asymmetry) and within the population (i.e. between individuals).

2. Materials and methods

2.1 Experimental design

A experiment with temperature and CO₂ concentration (through the measure of pCO₂ as partial pressure of CO₂) as combined factors was conducted, one close to present day conditions of temperature and pH in the winter in the English Channel (Amb; 11°C and pH 8.0, pCO₂ ~560 µatm), and the other a global change scenario of ocean warming and acidification (IPCC 2019, “Changing ocean, marine ecosystems and dependent communities”) (OWA; 14°C and pH 7.6, pCO₂ ~1660 µatm) (Supplementary Table 1). There were 3 replicates (tanks) for each experimental condition (i.e.

temperature-CO₂). Most of the eggs hatched on the 9th day of incubation (starting point of the experiment: 1 dph) and larvae were kept until 69 days post hatch (dph). Three days after hatching (3 dph), all larvae were counted and distributed equally in six 38 L conical black tanks (1500 larvae by tank), constituting the replicates of the experiment. A continuous flow through system of 20 L h⁻¹ was used (i.e. open circulation system). To allow the acclimation of the larvae, the temperature in the OWA tanks was progressively increased from 11 to 14°C over 48 h. Each OWA tank was supplied by a 200 L header tank, where the heated water was enriched with CO₂ to achieve the target value of pH 7.6.

Temperature and CO₂ were checked twice per day (pH meter 330i, WTW, Germany; Table S1). Oxygen saturation (oximeter: WTW Oxi 340, Bioblock scientific) and salinity were measured once per week, along with total alkalinity (TA) of each tank following the protocol of Anderson and Robinson (1946) and Strickland and Parsons (1972). Oxygen concentration was always above 88%. pCO₂ was calculated using the excel macro CO₂sys (Lewis and Wallace 1998) with the constant from Mehrbach et al., (1973) refit by Dickson and Millero (1987). To prevent any food limitation, the daily food quantity was distributed four times during the day to maintain an ad libitum level (Strain 2002), ensuring that there were always prey in the tank during the day. To be sure to allow proper feeding through time we used an increasing range of living prey sizes from phytoplankton to 24 h old nauplii, before weaning with feed granules (as described in Joly et al., 2021). Throughout the study, mortality was monitored daily, and was comparable between all ponds with no influence of environmental conditions (for more details, Joly et al., 2021).

Experimental animal came from wild Atlantic herring from the Downs stock of the southern North Sea and English Channel (Joly et al., 2021). Herring larvae were reared until they reached the last larval development stage respectively from hatching to 47 days post-hatching in the OWA treatment (14°C, 1660 µatm) and 60 days post-hatching in the Amb treatment (11°C, 560 µatm) to obtain the

same Growing Degree Days (GDD) for two temperature conditions. To follow the otolith growth and shape variation through time, 351 Atlantic herring from 32 to ~662°C.day (GDD, Figure 1) were sampled five times over the course of the experiment. At 32°C.day, 15 individuals were sampled, and then 42 fish for each experimental condition were sampled at each of the next four samplings. To assess the potential effect of temperature on the growth and shape of otoliths, we used the GDD approach which quantifies the thermal opportunity for growth by aggregating temperatures relevant to growth (McMaster and Wilhelm, 1997), and is thus more precise than the calendar time approach when describing growth (Neuheimer and Taggart, 2007; Mahé et al., 2019). The first sampling occurred at three days post-hatching (GDD=32°C.day). The four other samplings were then carried out after different time periods depending on the rearing temperature (Figure 1).

2.2 Otolith shape analysis

After measuring the total length ($TL \pm 0.1$ cm) of fish, their sagittal otoliths (left and right) were extracted from the cranial cavity and cleaned. The outline of each otolith was digitized using an image analysis system. To compare the shapes of the left and right otoliths, mirror images of the right otoliths were used. Otolith shape was assessed by analyzing first the otolith area (Oarea, μm^2) and Elliptic Fourier Descriptors (EFDs; e.g. Lestrel, 2008). Oarea seems to be a better univariate descriptor than longest length. For each otolith, the first 99 elliptical Fourier harmonics were extracted and normalized with respect to the first harmonic so as to be invariant to otolith size, rotation and starting point of contour description (Kuhl and Giardina, 1982). To determine the number of harmonics required to reconstruct the otolith outline, the cumulated Fourier power (F) was used. Only the first 6 harmonics were necessary to ensure reconstruction of each otolith shape with a precision of $F=99.99\%$ (Lestrel, 2008) and were thus used for further analyses.

The resulting matrix containing EFDs (as columns) for each otolith (as rows) was subjected to Principal Components Analysis (PCA) (Rohlf and Archie, 1984). and the 3 first principal

components (PCs) were selected as otolith shape descriptors or shape matrix according to the broken stick model (Legendre and Legendre, 1998), which, in this case, corresponded to a threshold of 3.4% of the total variance explained (Borcard et al., 2011). In total, these 3 PCs explained 82.4% of the total variance in the EFDs. This procedure allowed us to decrease the number of variables used to describe otolith shape variability through EFDs while ensuring that the main sources of shape variation were kept, and to avoid co-linearity between shape descriptors (Rohlf and Archie, 1984).

2.3 Statistical analyses

Each environmental condition was the combination of temperature and CO₂ concentration values. Otolith area and larvae size differences were analysed using a post-hoc Tukey-HSD test among several values of GDD. The relationship between larval total length (TL) and otolith area (Oarea) in response to the environmental conditions (temperature and CO₂ concentration) was tested using Analysis of Covariance after verifying the normality of residuals. Using the Fourier harmonics to describe the otolith shape, pRDA were used to test the explanatory variables of interest (i.e., side, combination of temperature and CO₂ concentration, GDD value) using total length to correct for fish size. This pRDA was combined with permutation tests on the selected PC matrix and the explanatory matrix consisted. To analyse the potential anatomical differences described by the directional bilateral asymmetry between left and right otolith shape, partial redundancy analysis (pRDA) was modelled on the selected principal components (PCs) matrix using otolith side (left/right) as the potentially influential variable and the individual as the conditioned variable. To test the potential effect of climate change, the pRDA was applied with the environmental explanatory matrix (Temperature and CO₂ concentration). To visualise shape differences, average shapes were rebuilt based on EFDs averaged for each group of individuals. Directional asymmetry and environmental effect amplitude were then computed as the percentage of non-overlapping surface between the reconstructed otolith average shapes relative to the total area they covered after superposition for 2

groups left side/right side or 2 environmental conditions as used for other otolith shape studies (Mahé et al., 2018 ; Mahé et al., 2019 ; Mahé, 2019).

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

3. Results

3.1 Otolith growth

There is a positive relationship between otolith and fish larvae sizes. Otolith and larvae sizes increased stepwise from 32 to 670°C.day (Figure 2). Over the course of the experiment, the shape was modified leading to the loss of the initial circular shape of the otolith. Otolith morphogenesis was positively correlated with GDD.. Herring larvae were long and thin at hatching, then progressively developed dorsal and caudal fins. Body height increased around 436°C.day, and the pelvic fins were the last fins to differentiate at around 662°C.day. Otolith area and larvae size differences were analysed among several values of GDD. Larvae size as otolith area grow during the larvae life (i.e. with GDD value; Figure 3A). Larvae size increased significantly between 32°C.day and 87°C.day, while the first changes in otolith area appeared only at 436°C.day. However, after this GDD level, otolith area (i.e. otolith 2-dimensional measure) increased faster than larval size measure (i.e. fish 1-dimensional measure). Neither larvae size nor otolith area were significantly different between the two environmental conditions (temperature/CO₂) at any value of GDD, with the exception of larvae size at 436°C.day ($P < 0.05$). The relationship between Oarea and TL was always significant ($P < 0.05$), but the slope was significantly higher in the OWA than in the Amb treatment (slopes difference; $P < 0.05$; Figure 3B). For the same fish length, the area of otolith was bigger for the 2100 scenario (14°C/CO₂ 1660 μ atm) than under present day conditions (11°C/CO₂ 560 μ atm) (Figure 3B); this difference increased with fish size. Our experimental study showed that this

significant correlation between otolith and fish growth was still observed in present day environmental conditions as well as for the 2100 scenario.

3.2 Otolith shape

No significant difference in shape was observed between left and right otoliths (Supplementary Table 2). The average percentage of non-overlapping surface between the two sides never exceeded 1% (Figure 4). Similarly, the results showed no significant environmental effect on otolith shape in herring larvae, with the average percentage of non-overlapping surface ranging from 0.73% to 1.48% (Supplementary Table 2; Figure 4).

4. Discussion

The otolith shape is regulated by a complex combination of endogenous and exogenous factors, including both abiotic environmental parameters (such as temperature and CO₂), and biotic parameters (such as food availability). The influence of these factors is dependent on the ontogenetic stage (i.e. the development stage of the individual). In addition, the otoliths can be different between right and left inner ears as a consequence of potential developmental lateralization (e.g. side effect). Environmental factors, especially temperature, have a greater influence than genetic differences for Atlantic cod (Cardinale et al., 2004; Hüsey, 2008; Irgens, 2018). For seabass (*Dicentrarchus labrax*), increased temperature speeds up otolith morphogenesis and modifies the developmental pattern of the otolith shape (Mahé et al., 2019). Ocean acidification can also alter otolith shape (Holmberg et al., 2019). For several species, individuals exposed to high CO₂ had a larger otolith area and maximum length compared with controls; the increases were larger than could be explained by an increase in CaCO₃ precipitation in the otoliths driven by the modification of the pH regulation in the endolymph (Checkley et al., 2009; Munday et al., 2011; Réveillac et al., 2015; Coll-Llado et al., 2018). Changes in extracellular concentrations of carbonate and bicarbonate caused by acid-base regulation in a high CO₂ environment could increase the precipitation of CaCO₃ in the otolith (Payan

et al., 1998). In studies on other species including herring, high CO₂ had no effect on the larval sagittal otolith (Franke and Clemmesen, 2011; Munday et al., 2011; Frommel et al., 2013; Perry et al., 2015). Our experimental study showed that there is the relationship between otolith area and fish length according to the environmental conditions and validated the results observed *in situ* (Folkvord et al., 1997; Johannessen et al., 2000; Berg et al., 2017; Denis et al., 2017; Tonheim et al., 2020). A previous study on herring larvae testing four temperature conditions (4°C, 12°C and two others shifted twice with 4/8/4°C and 12/8/12°C) showed that the size and the width of the otolith daily increment were both temperature-dependent (Folkvord et al., 2004). The otolith area per fish size in our study increased with higher temperature and CO₂ (i.e. lower pH). It should be noted that the feeding protocol chosen for this study provides *ad-libitum* feed, in order to avoid under-feeding situations that could have interfered with the otolith study. Nevertheless, it cannot be excluded that larvae under the 2100 scenario ingest more food than control larvae, which could also accelerate the increase of the otolith area in the 2100 scenario group. While growth mechanisms of otoliths and their morphogenesis during the early life stages of fish are poorly understood, shifts in otolith shape are linked to physiological modifications due to environmental disturbance (Geffen, 1987; Vignon, 2018). Early life stages are more vulnerable to environmental challenges because they have higher surface area to volume ratios and have not yet fully developed the homeostatic regulatory mechanisms which are found in adult fish (Hurst et al., 2013). For Downs herring, a shift affecting larval condition has previously been observed *in situ* for individuals at 13 mm, and was driven by environmental conditions (temperature and prey concentration) (Dennis et al., 2017). Our experimental study on Downs herring covered this length range but the otoliths did not show any ontogenetic differences. Several previous studies showed that the increase in otolith area by fish size could mainly result from water acidification (Checkley et al., 2009; Munday et al., 2011; Réveillac et al., 2015; Coll-Llado et al., 2018). Moreover, the elevated seawater CO₂ can cause directional asymmetry (Holmberg et al., 2019). For herring, the environmental factors we studied

(temperature/CO₂) did not affect the otolith shape between sides at the observed larval stage. Consequently, it is likely that the level of response to CO₂ increase might be a species-specific phenomenon. This capacity to maintain otolith shape in response to environmental changes is probably due to efficient intracellular ionic-regulation mechanisms in this species (Ishimatsu et al., 2008; Melzner et al., 2009). This result may suggest that herring larvae are well equipped to cope with the environmental changes projected for 2100, as long as their energy and nutritional needs are well covered, which would not necessarily be the case in the natural environment. The faster growth of the larvae under the 2100 scenario may indeed lead to a greater prey requirement, and it cannot be ruled out that the increase in the area of the otoliths may also have affected their hearing ability and behaviour. Nevertheless, it should be noted that this species certainly has a good intracellular ionic regulation because it is confronted with different environments (Ishimatsu et al., 2008; Melzner et al., 2009). Indeed, herring larvae typically hatch in littoral regions where pH and temperature can fluctuate because of freshwater inflow, so they may often experience such fluctuations and therefore be quite resistant to them. For more extreme values, however, the homeostatic mechanisms might not be sufficient to compensate for environmental stress and this could induce changes in otolith morphological development (Coll-Llado et al., 2018).

Ontogeny and environment are the factors that control most of the otolith development during the early stages of life. The otolith shape evolves from the basic round form to elongated shape due to faster growth along the anterior-posterior axis than along the dorsal-ventral axis (Galley et al., 2006; Mille et al., 2015; Bounket et al., 2019; Mahé et al., 2019). Our study showed that the otolith shape of herring grew in this way during the larval period. At the adult stage, significant directional asymmetry has been observed in herring (Bird et al., 1986), while no lateralization has been observed at the larval stage. This bilateral effect increases over the life of the fish when considering a different trajectory of otolith morphogenesis between left and right sides. However, the asymmetry between

left and right otoliths especially the fluctuating asymmetry may be underestimated in experiments due to the small number of tested individuals which are experiencing stressful conditions and present experimental conditions that do not require significant development of acoustic functions as in the natural environment (Grønkjær and Sand, 2003; Diaz-Gil et al., 2015). Consequently, no lateralization has been observed in the experimental condition for the herring larval stage does not mean that this result will be the same in the natural environment. Although otolith shape is influenced by both abiotic and biotic environmental parameters and depends on individuals' genotype, environmental effects can also be perceived more with ontogeny, consequently, this developmental lateralization could be a phenotypically plastic response to environmental drivers rather than the consequence of the individual genotype, as previously suggested in *Boops boops* (Mahé et al., 2018). Sagittal otolith size and shape for each species could therefore be adaptive traits to different habitats and ecological niches (Lychakov and Rebane, 2000; Lombarte et al., 2010).

Conclusions

The growth and shape of the otoliths studied here did not appear to be affected by the environmental conditions predicted for 2100. However, this experimental approach should be confirmed *in situ*. Only the relationship between otolith and fish sizes seems to be environment-dependent. This information is important to understand the otolith morphogenesis and consequently when the otolith shape was used as a tool in fisheries science. In the future, to complete this approach, a crossed-effect experiment (four different settings: control, temperature increase, acidification, temperature increase and acidification) could be applied to quantify the effect of each environmental factor. In the same way, it could be important to understand the link between the environment modifications and the food consumption by fish to separate the direct and indirect effects of the climate change on the otolith and fish growth.

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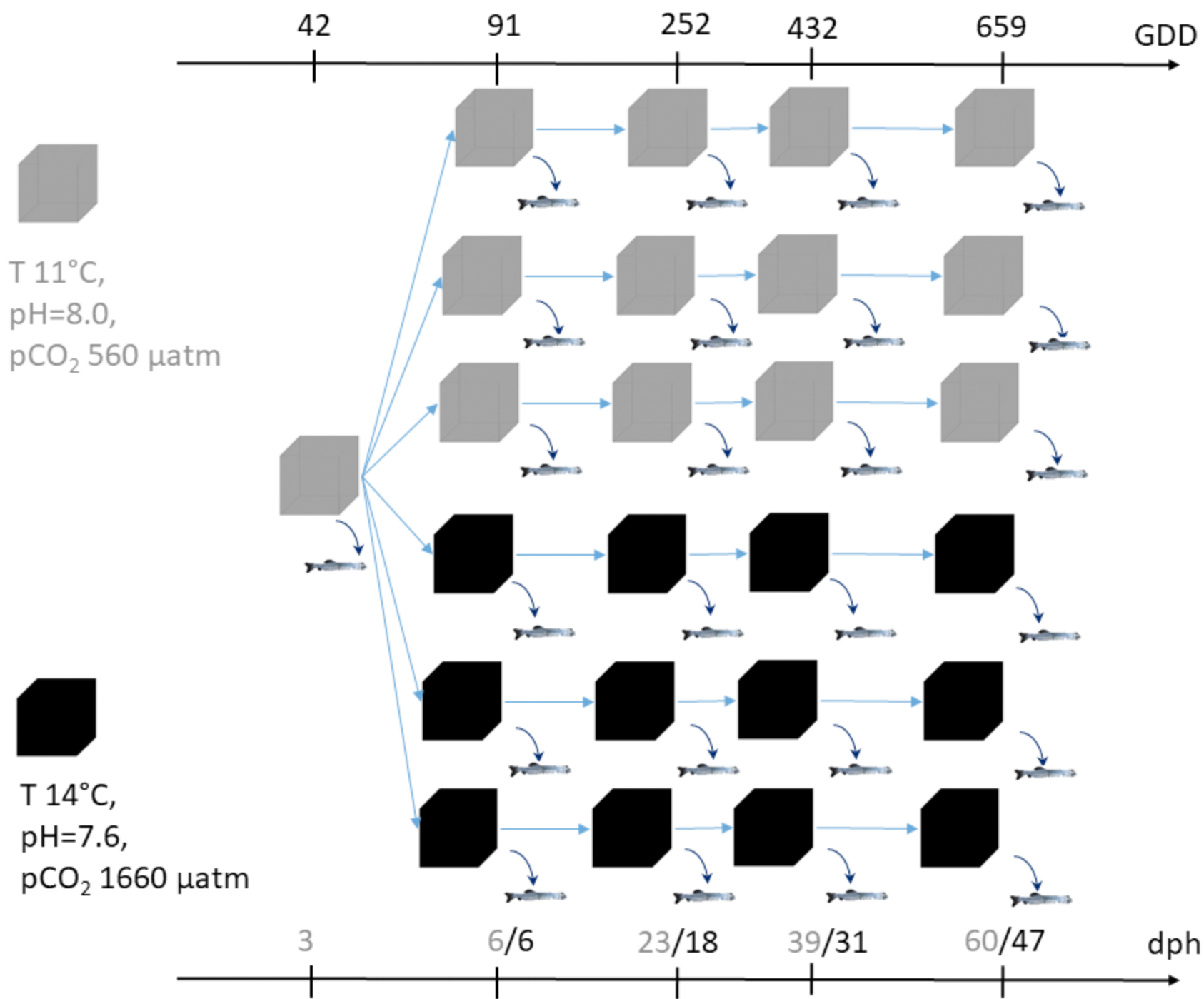
Figure 1: Main steps of the experimental design for herring with five samplings by the level of GDD (Growing Degree Days) and by the number of dph (Days Post Hatching; grey value for 11°C; black value for 14°C). Each condition of temperature and CO₂ concentration had three replicates. To test the effect of global change, present day conditions (11°C and pH 8.0, pCO₂ 560 µatm) versus the ocean warming and acidification conditions predicted for 2100 (14°C and pH 7.6, pCO₂ 1660 µatm) were used.

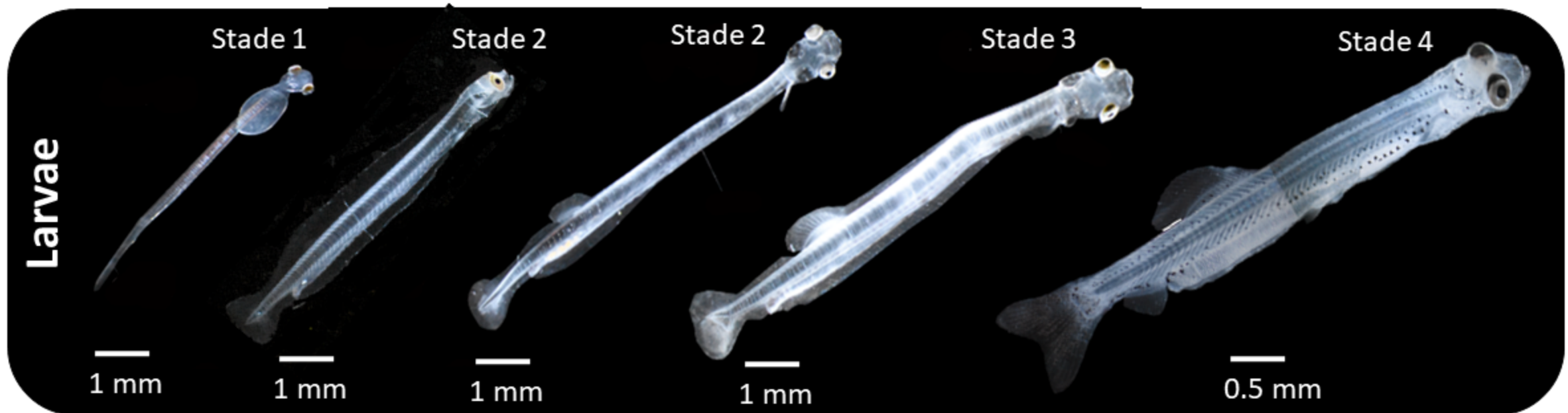
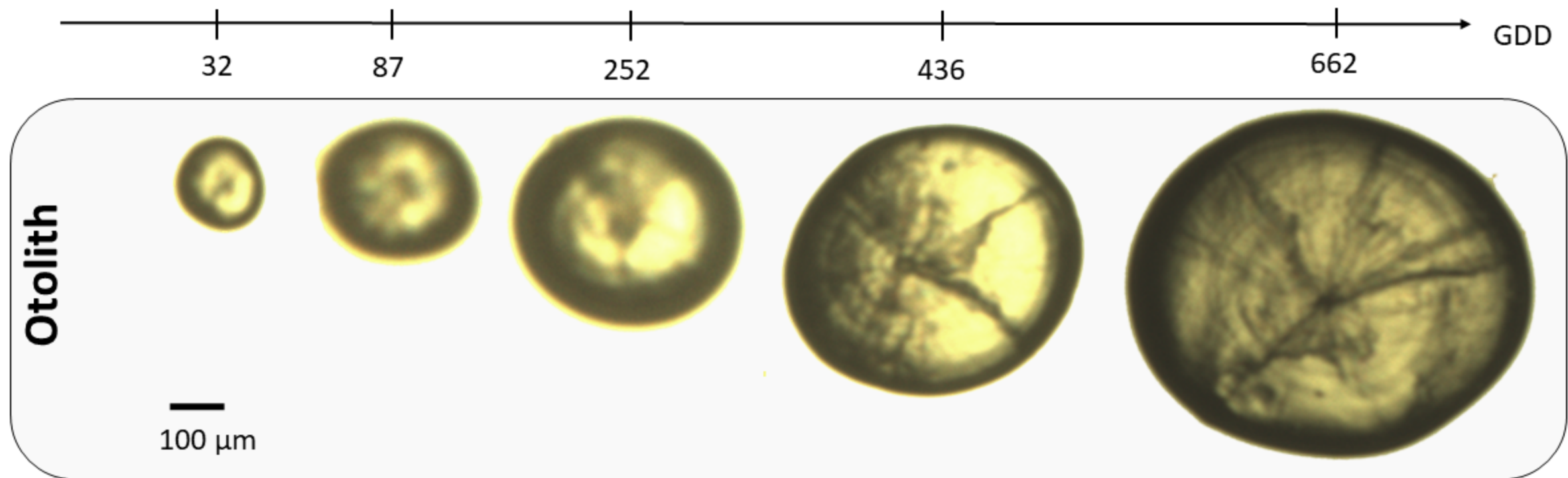
Figure 2: Growth of otolith shape and larval fish by GDD value (°C.day).

Figure 3: (A) Box-plot of otolith area (Oarea, µm²) and total length of fish (TL, cm) values by GDD value (°C.day) and environmental conditions (red for 14°C and 1660 µatm, and blue for 11°C and

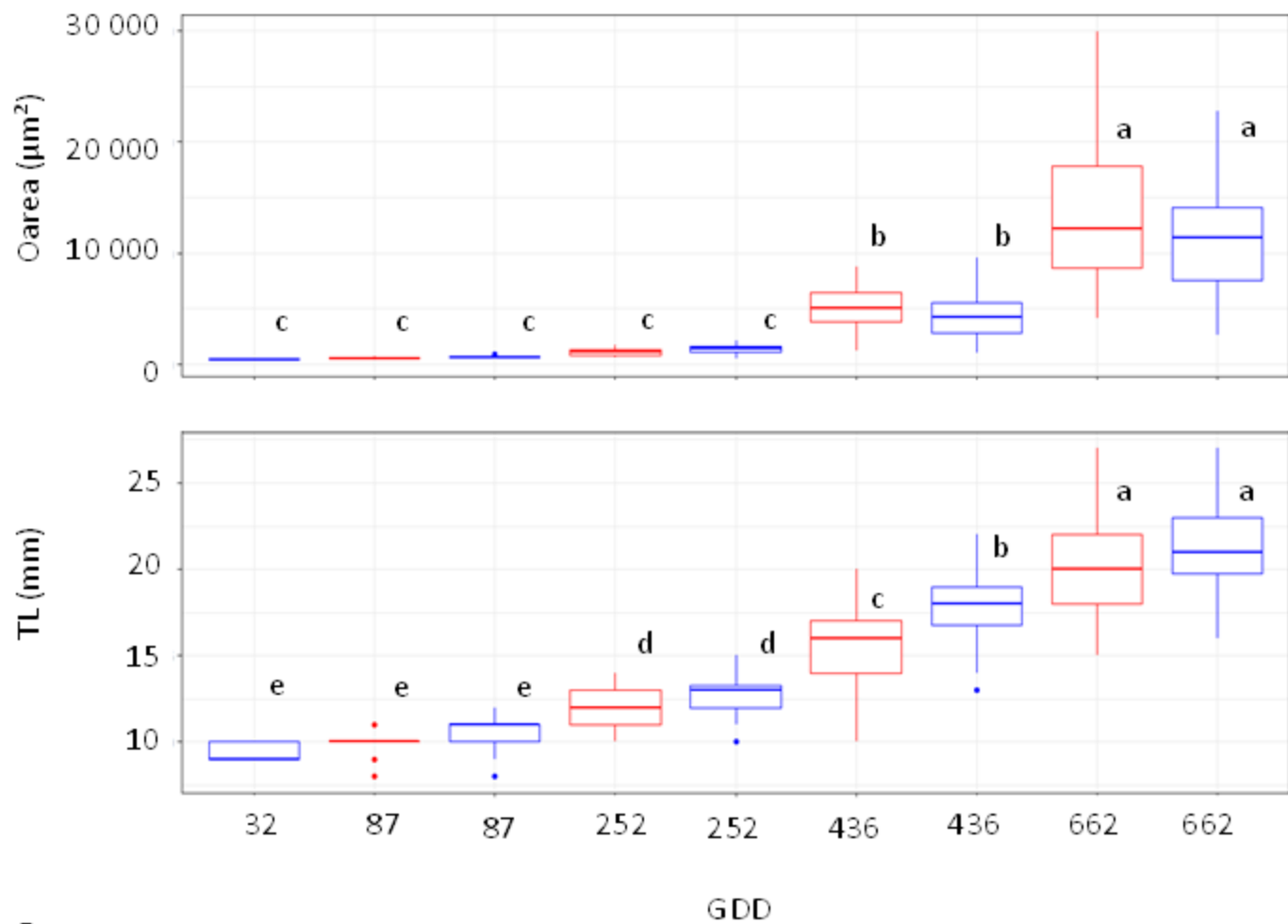
560 μatm). A different letter within each sampling time denotes a significant difference between the two groups. (B) Relationship between otolith average area (Oarea) and larval body length (TL) in response to the environmental conditions (red for 14°C and 1700 μatm , and blue for 11°C and 560 μatm) (smoothing method using level of confidence interval of 0.95).

Figure 4: Percentage of non-overlapping surface between left and right otolith shape and for two environmental conditions by GDD value ($^{\circ}\text{C}\cdot\text{day}$) (arrows identify the main areas of difference between both otoliths).

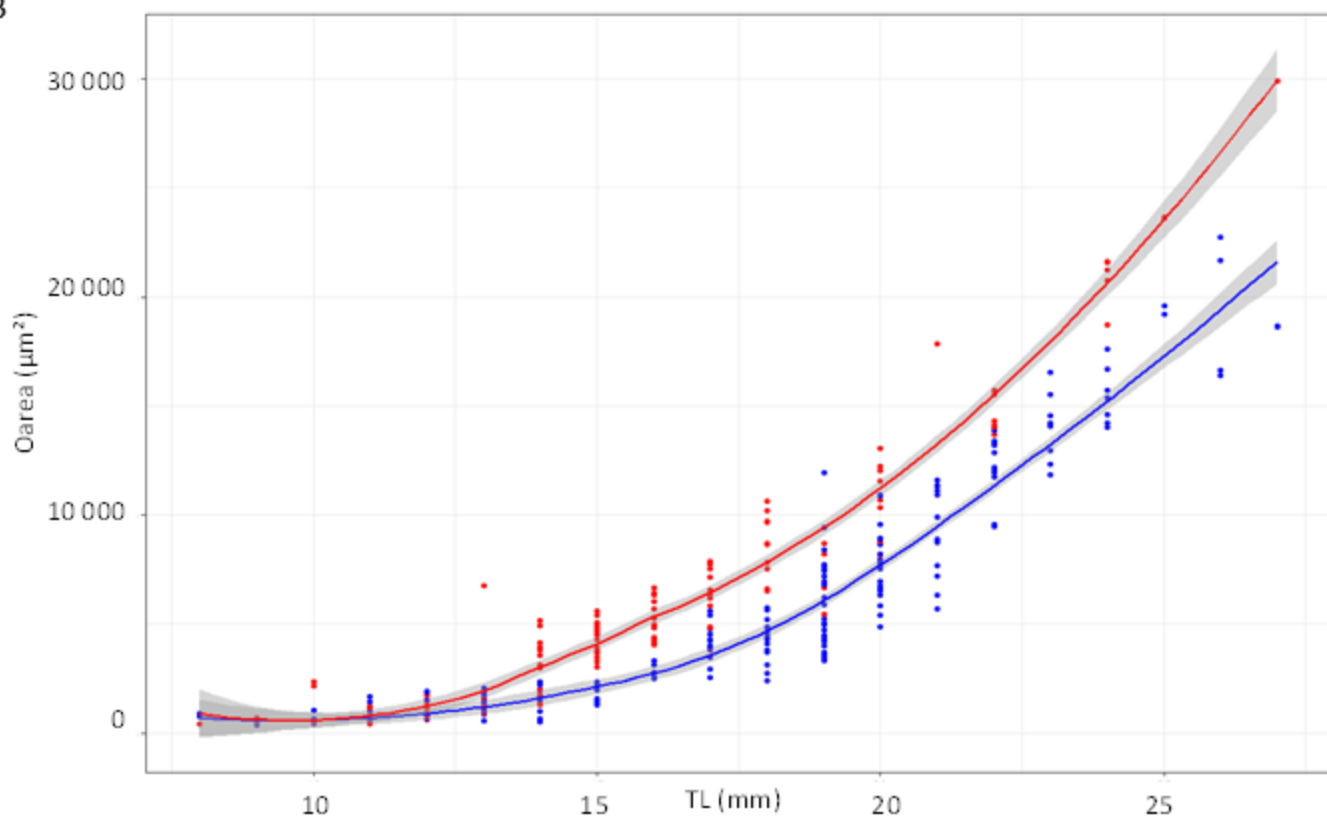




A



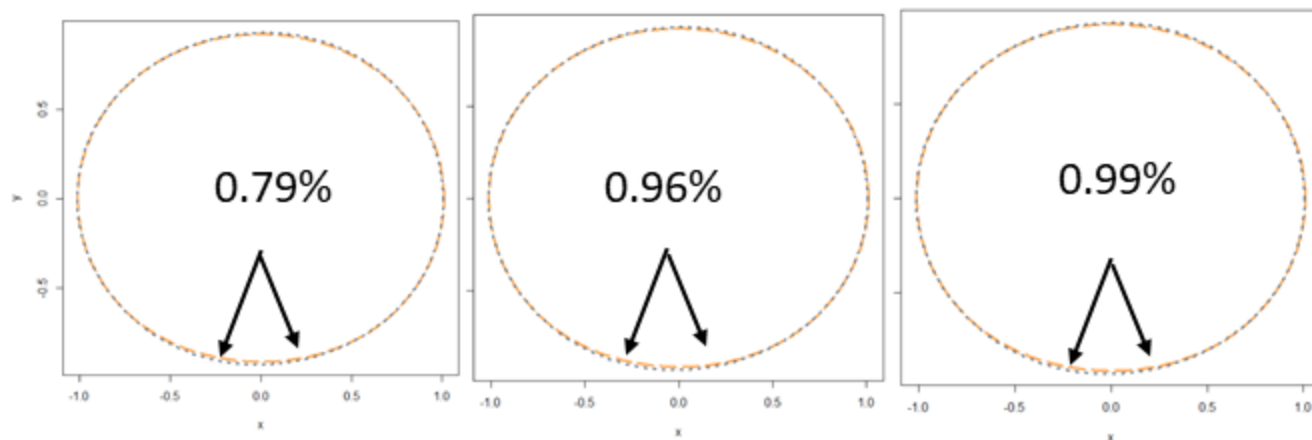
B



A) Side effect

Left

Right



B) Environmental effect

T=11°C

pH=8

CO₂ 560 μatm

T=14°C

pH=7.6

CO₂ 1660 μatm

