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DYNAMICS IN BROOD CHAMBER PH OF THE EUROPEAN FLAT OYSTER (*OSTREA EDULIS*) IN RESPONSE TO OCEAN ACIDIFICATION



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Content

Abstract	2
Introduction	3
Ocean acidification risks	3
Geographical distribution	3
Carbonate chemistry and seasonality	3
Reproduction strategies	4
Resilience in brooding oyster?	5
Questions for future scenarios	6
Aims and hypothesis	6
Materials and methods	7
Sampling and preparations	7
Design and measurements	7
Algae cultivation.....	9
Video and Statistical analysis	9
Results	9
Brood chamber pH analysis.....	9
Shell gape analysis.....	11
Discussion	13
Brood chamber pH fluctuations.....	13
Effect of shell gape on brood chamber pH	14
Future research	15
Study limitations.....	15
Conclusion	16
Acknowledgements	16
References	17
Appendix I: Popular science summary	19
Appendix II: Brood chamber pH fluctuations per treatment	21
Control treatment: Ambient pH – 18°C.....	21
Elevated temperature treatment: Ambient pH – 23°C.....	21
Decreased pH treatment: pH 7.5 – 18°C	22
Combined treatment: pH 7.5 – 23°C	22

Abstract

Ocean acidification is posing a threat to marine bivalve species who struggle to deposit calcium carbonate in order to grow their shell. Some oyster species have developed a brooding reproductive strategy which might help them cope with this acidification stress. Brooding oysters have shown to be more resilient against ocean acidification than broadcast spawning oyster species. It is suspected that because the brood chamber is on top of the maternal gills, the mothers add carbon dioxide into the chamber from her respiration. This suggests larvae evolved to develop in a more acidic environment than the surrounding water column. Through exaptation the larvae may have coopted traits needed for development in the brood chamber which now enable them to be more resilient to ocean acidification. In this study, we measured the pH inside the brood chamber of *Ostrea edulis* under current and future predicted ocean conditions (i.e., elevated temperature and decreased seawater pH) to get a better understanding of the ambient-maternal relationship on brood chamber pH fluctuations under ocean acidification scenarios. The results suggested that maternal respiration indeed makes the brood chamber always a more acidified environment than the surrounding water. Elevated temperatures in the surrounding water slightly lower the pH as a result of increased maternal metabolism. Yet lowering the ambient pH causes a much larger and significant reduction of internal pH levels since the oyster is constantly filtering the overlaying water while the valves open. Additionally, there seems to be a positive relationship between shell gape and internal pH changes suggesting that the mothers behaviour may also influence how fast and to which level pH values can drop inside the brood chamber. These results give an indication of what conditions brooding oysters larvae will have to face in the future and helps determine possible winners and beneficial strategies in an acidified ocean.

Introduction

Ocean acidification risks

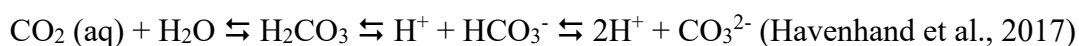
Global warming causes two major changes in our oceans. Increasing atmospheric concentrations of carbon dioxide (CO₂) causes sea water temperature to rise and pH levels to drop (Tan & Zheng, 2020). This ocean acidification (OA) process is expected to cause many marine species to live in stressful conditions (Kroeker et al., 2013). While some species tend to be more robust against these changes than others, calcifying bivalves are considered among the most vulnerable species when it comes to coping with reduced environmental pH values (Kroeker et al., 2013; Waldbusser et al., 2016). Surface seawaters show a reduction of approximately 16% of their carbonate ion (CO₃²⁻) levels since pre-industrial values (Sabine et al., 2004). The carbonate ions (CO₃²⁻) form a vital building block for bivalves to form their calciferous shells. The reduced availability of suitable habitats exposes many calcifying species to these stressors which causes shell deformations, increased risk of disease outbreaks and negative effects on the survival of bivalve early life stages (Tan & Zheng, 2020). Oysters often act as key species in their habitat and their disappearance can lead to changes in ecosystem community structures and loss of important ecosystem functions (Smith et al., 2023). Oysters are ecologically important species that provide ecosystem functions such as creating structural reefs and providing shelter and food for other community species (Thurstan et al., 2024). Additionally, they are of great economical value and create a source of food and income for many people (Thorngren et al., 2019).

Geographical distribution

The *Ostrea edulis* has been abundantly present and therefore commercially fished for hundreds of years. Yet, overexploitation, a decline in water quality in combination with the introduction of diseases caused by *Bonamia ostreae* caused a decline in suitable habitat. This led to a general decline in oyster reefs and the collapse of *O. edulis* populations (Thurstan et al., 2024). Today *Ostrea edulis* is present in the Mediterranean and north sea ranging from the Peloponnese into the Adriatic sea, all around the coastline of the Iberian peninsula and the UK and Ireland, up until the southwestern parts of Sweden and Norway. The species has been introduced as alien species along the East coast of the USA (LifeWatch Belgium & VLIZ, 2024).

Carbonate chemistry and seasonality

The amount of emitted carbon dioxide (CO₂) dissolved in the atmosphere is measured as the partial pressure caused by CO₂ (pCO₂). Since the industrial revolution atmospheric pCO₂ significantly increased which causes an increased influx of CO₂ in our oceans. The CO₂ that dissolves in seawater reacts with water molecules forming hydrogen ions and carbonate ions (Maes, 2023):



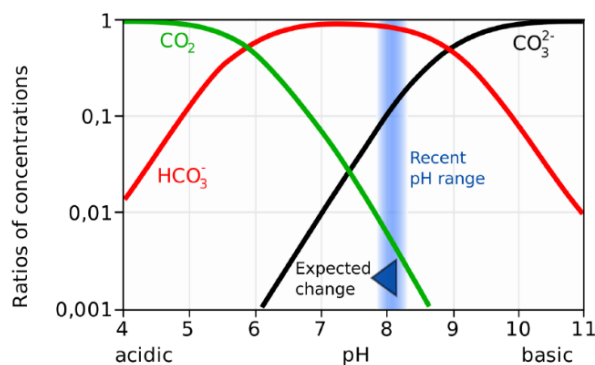


Figure 1. Dynamics of oceanic dissolved carbon molecule concentrations in varying pH conditions (Maes, 2023)

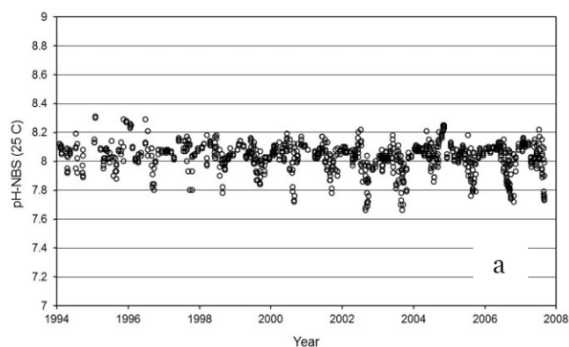


Figure 2. Time series of pH in the top 20 m of the Kattegat region (Havenhand et al., 2017)

Marine calcifying organisms are in need of these carbonate ions (CO_3^{2-}) and calcium ions (Ca^{2+}) to mineralize calcium carbonate (CaCO_3) as the main component of their shell or skeletal structure. Higher pCO_2 in the water results in higher concentrations of hydrogen ions (H^+) which in turn react with the carbonate ions (CO_3^{2-}) to form more stable HCO_3^- , thereby reducing the availability of vital carbonate building blocks for the marine calcifiers (**Figure 1**) (Maes, 2023).

Although the concentration of available carbonate ions might be reduced in the future, the main cause for larval sensitivity might be energy limitation. Waldbusser et al. found that in *M. gigas*, fast shell growing rates require a lot of energy and that larvae in early life stages have the highest calcification rates. Therefore they are more vulnerable to acidified environments as a result of a kinetic constraint. Older larvae develop organs for feeding and shell formation (eg. gills and mantle tissue) (**Figure 3**) providing help to reduce the energetic stress under OA (Waldbusser et al., 2013).

The amount of pCO_2 in the ocean is not constant but undergoes seasonal fluctuations. In spring and summer, the high rate of primary production consumes CO_2 and H^+ thereby reducing the pCO_2 and increasing the pH in the seawater. In winter, the opposite happens; breakdown of organic matter consumes oxygen (O_2) and produces CO_2 , decreasing the seawater pH (Havenhand et al., 2017). The actual observed pH fluctuations in a specific region are also influenced by the buffer capacity of the oceanic waters where salinity plays an important role. Higher salinity usually provides a stronger buffer capacity. In the Kattegat region between the Baltic sea and the North sea the average observed seasonal pH fluctuations range between 8.2 in summer and 7.8 in winter (**Figure 2**) (Havenhand et al., 2017).

Reproduction strategies

Based on reproductive strategies, there are 2 types of oysters: brooding species and broadcast spawning species. Brooding species brood their larvae in a brood chamber for a species specific length of time while broadcast spawning species release their larvae into ambient waters immediately after fertilization. *Ostrea edulis* is a brooding species and a protandric hermaphrodite meaning that they are born male and periodically switch to female when conditions are suitable during the reproductive season (Chaparro et al., 2019; Jacobs et al., 2020). Spawning oysters release eggs into the brood chamber (**Figure 3**) and fertilise them with sperm present in ambient water. In European populations, this event usually happens when temperatures reach above 15°C on average, although this threshold varies depending on the

geographical location of a population. It has been observed that at higher latitudes, some oyster populations need temperature up to 25°C to trigger spawning while in some southern populations 12°C can be sufficient (Jacobs et al., 2020). Previous research on the well-studied *Ostrea chilensis* has shown larval movement patterns in the brood chamber. Depending on the developmental state of the larvae, their circulation patterns can vary but usually include transportation to the anterior side over grooves in the maternal gills (Figure 3) and return to the posterior side through maternal counter currents (Chaparro et al., 2019). Inside the brood chamber, the larvae undergo organogenesis of their first shell which is called the prodissoconch I (PDI). During this time, the larvae are at their most vulnerable stage when it comes to acidified environments (Waldbusser et al., 2016). The eggs are incubated for 8-10 days on top of the maternal gills before the mother releases the larvae out of the pallial cavity into the water column (Jacobs et al., 2020). In the *O. chilensis*, larval ejection happens through physical contraction of the maternal shell (Chaparro et al., 2019). During a pelagic period of 6-10 days, the veliger larvae swim around until they find suitable substrate (small shell fragments, adult oyster shells, etc.) (Didderen & Sas, 2019). They will develop an eyespot and a foot making them competent to settle down and are now called pediveliger larvae (Jacobs et al., 2020). Once settled the larvae undergo metamorphosis taking on adult morphology and keep growing until reaching their adult size after 3-4 years. At this point the oysters are matured and can start reproducing (Didderen & Sas, 2019).

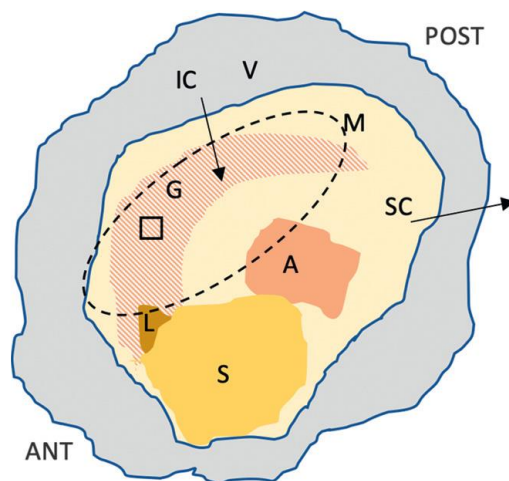


Figure 3. *Ostrea edulis* morphology: anterior region (ANT), posterior region (POST), adductor muscle (A), gills (G), labial palps (L), mantle tissue (M), stomach and gonad (S), left valve (V), brood chamber (outlined with dashed line), infrabranial cavity (IC) with inhalant flow direction (arrow) and suprabranial cavity (SC) with exhalant flow direction (arrow) and probe location within brood chamber above gills (open square). IC and SC together form the pallial cavity. (Gray et al., 2019).

Resilience in brooding oyster?

In comparison with other present broadcast spawning oyster species, such as *Magallana gigas*, all oysters of the family Ostreidae use the brooding strategy (Ó Foighil & Taylor, 2000). Brooding oysters have previously been shown to be more resilient against ocean acidification than broadcast spawning oysters (Waldbusser et al., 2016). Vulnerability to ocean acidification stress is often shown in early shell-building life stages. However, Waldbusser et al. (2016) found that extracted fertilized eggs from the brooding *Ostrea lurida* show little response to different ocean acidification conditions in the PDI stage. Compared to the broadcast spawning *M. gigas*, the rate of calcification and shell growth is much slower in *O. lurida*. The slow shell formation, associated with brooding oysters, may reduce the energetic stress that comes with rapid shell development in an acidified environment (Waldbusser et al., 2016).

One possible mechanism for OA resilience extends from the exaptation of traits used in early life-stages to cope with the acidified environment inside the brood chamber (Gray et al., 2019). Exaptation is the mechanism where a trait increases fitness in a new situation yet the evolution of this trait was caused by a different selective agent that occurred prior to this new situation (Gould & Vrba, 1982). In this case resistance against ocean acidification is a trait that evolved under the selective pressure of brooding conditions but also serves very well as a protection against growing up in acidified ambient waters. The selective agent is not ocean acidification but brooding conditions selected for a trait that can be coopted to increase fitness in future OA scenarios. Through exaptation, the development of OA resistance can bypass natural selection, since this trait has already been present in brooding oysters for a long time. As previously discussed, slow shell building might be a larval strategy to cope with acidified conditions in the brood chamber and through exaptation might serve as a strategy against ocean acidification.

Because of its location right above the maternal gills (**Figure 3**), maternal respiration might add additional CO₂ and thus cause a reduced pH inside the brood chamber. Previous research has shown that pH in the brood chamber is positively correlated with ambient water over a small pH range, suggesting that environmental conditions do influence the internal state of the oysters, possibly through maternal respiration (Gray et al., 2019). Additionally, some environmental conditions may elevate metabolism and maternal respiration (i.e. temperature), indirectly mediating brood chamber pH. Contrarily, Gray et al. (2019) found that oysters with fully closed valves actively slow down their respiration rate. This also slows down acidification and deoxygenation rates in the brood chamber and might serve as a mechanism to prevent the pallial cavity from reaching levels that could harm larvae survival (Gray et al., 2022).

Questions for future scenarios

This raises interesting questions on what will happen once ocean acidification scenarios become reality. If ambient waters become more acidified and increased temperatures raise metabolism and therefore maternal respiration, will brood chamber conditions reach values that larvae can no longer cope with? Will strategies such as slow shell building and slowed respiration upon valve closure, remain sufficiently helpful if pH in the pallial cavity plummets? However, more research is needed to understand the individual influence of environmental factors such as temperature, pH, pCO₂, Ω_{arg} , etc. on the internal state of the pallial cavity. For future conservation purposes of this species and its ecosystem services it is important to understand the drivers behind the apparent resistance of brooding oyster species.

Aims and hypothesis

In this study, we seek to explore the environmental influence on the internal state of the European flat oyster, *Ostrea edulis*. Although previous work has demonstrated strong connectivity between overlying water and the brood chamber conditions, the question remains, how does the relation between brood chamber and ambient water pH change under ocean acidification scenarios?

This research will look further into the individual and combined effects of elevated temperature and reduced pH levels as predicted for 2100. The aim of this research is to get a better

understanding of the ambient-maternal relationship when it comes to brood chamber pH fluctuations under ocean acidification conditions. Therefore, oysters will be reared under different environmental conditions (pH and temperature) while microsensors will be used to measure pH inside the brood chamber.

Hypothesis 1: pH inside brood chambers will always be lower than pH in ambient water caused by additional carbon from maternal respiration.

Hypothesis 2: Since oysters respire the overlaying water, a decrease in ambient pH should result in a decrease in brood chamber pH.

Hypothesis 3: Increasing the ambient water temperature will increase the oyster's metabolism, increasing maternal respiration and therefore decrease brood chamber pH .

Materials and methods

Sampling and preparations

On September 6th 2024, 30 European flat oysters (*Ostrea edulis*) were sampled from wild populations in the waters around Tjärnö, Sweden. Additionally, some oysters were previously sampled and stored in baskets hanging from the docks for later use in any experiment. Since these oysters were stored in seawater in the bay outside Tjärnö, they experienced the same feeding and weather conditions as they would in non-captured, wild populations. About 90 of these oysters were collected from the docks outside the Tjärnö marine laboratory. Collected oysters, were placed in tanks supplied with unfiltered, constant flowing surface seawater.

Of these, a total sample size of 20 oysters ($n_t = 20$) with a size range of 64 - 93 mm were prepared for the brood chamber experiments. First the oysters were cleaned to make sure no living organisms attached to the oyster shell would disturb any measurements or calculation regarding oyster mobility, food supply calculations, etc. When cleaned and dry, all 20 oysters were marked with an individual ID number and their size in mm was recorded. Next a hole of 16 mm in width, was drilled into their shell above the maternal gills to access the brood chamber (**Figure 3**). The holes were closed off using parafilm to avoid excessive turnover with ambient water. Lastly, 2 small white plastic markers, each featuring a black dot, were glued onto the left and right valve of each oyster, positioned opposite to each other. This allowed for later video analysis and measurement of the shell gape of the oysters. When the oyster is actively pumping, the distance between the 2 black dots could be calculated and converted to shell gape as a measure for maternal respiration. After these preparations, all 20 oysters were placed in a resting tank of 30.359 l supplied with flowing unfiltered seawater and left to rest for 48h to ensure minimal impact of the drilling procedure on the experimental measurements. The temperature of this resting tank was set at the same level as the ongoing treatment (18°C or 23°C) in order to condition the oysters to this environmental value before starting measurements.

Design and measurements

To examine the environmental influence on brood chamber conditions in the *Ostrea edulis*, 4 treatments were designed based on varying temperatures and pH values. The current

temperature value during the reproductive season was based on a database from Tjärnö Marine Laboratory averaged over the past 5 years. From 2019-2023, the average surface sea water temperature in the reproductive season, lasting July-September, was 18.3°C (Tjärnö Marine Laboratory, 2024). Given the seasonal sea water pH fluctuations in the Kattegat region (**Figure 2**) combined with observed sea water pH in the oyster sampling area, the pH value to simulate the average reproductive season over last years would be approximately pH 7.8. Following the SSP5-8.5 climate change model, the sea water temperature is predicted to increase 3-5°C by 2100, (IPCC, 2015). This model assumes a business as usual scenario, with minimal mitigation efforts to reduce the impact of carbon emissions. This same model predicts a decrease in seawater pH of 0.3-0.32 by the end of the century (IPCC, 2015). These values made up the conditions for the 4 treatments. Treatment 1 was carried out in a seawater filled heated bath at 23°C (predictions for 2100). The pH remained as in the Oyster’s natural habitat as unfiltered seawater from the Tjärnö bay was used as treatment water. Treatment 2 consisted of a seawater bath at 18°C with a current pH values. This treatment mimicked the natural conditions during the reproductive season of the past 5 years and therefore acted as a control treatment. During treatment 3, the oysters were held in a seawater bath at 23°C while the pH was lowered to 7.5. This treatment allowed us to measure the response of the oysters to the predicted environmental conditions for the year 2100. In the 4th treatment, a pH of 7.5 was maintained combined with a temperature of 18°C. By testing all combinations of current and predicted temperature and pH values, it is possible to distinguish between the individual and combined effects of these variables (**Table 1**).

Experimental conditions	Temperature	pH
Average current reproductive season	18°C	7.8
Prediction 2100	23°C	7.5 – 7.48

Table 1. Experimental conditions including temperature and pCO₂ averaged over the reproductive seasons of the last 5 years and predicted for the year 2100 following the SSP5-8.5 climate change model. Each combination of temperature and pCO₂ will be tested.

During each treatment, one oyster at a time was transferred to the experimental tank. The parafilm plug was removed and the oyster was left to accommodate for some minutes. The interests of this experiments are focused on the data when the oysters are open or actively pumping. Therefore measurements were started when the oyster in the experimental tank was visibly seen opening. To measure the pH inside the brood chamber a micro pH probe (Microelectrodes, MI-414 Series Micro-Combination pH Probe, 15 gauge) was used connected to a pH logger (REED R3000SD Data Logging pH/ORP Meter). The pH probe was mounted to a micromanipulator for a stable and accurate use and calibrated daily before the start of any measurements. The temperature in the experimental tank was controlled using a temperature regulator (Inkbird, Temperature Controller ITC-308S) connected to the heating system in the floor of the tank. Ambient water temperature and pH were logged using a Bluetooth connected pH probe (Hanna Edge Blu pH Meter, HALO HI-11102 Glass Body Gel Filled Bluetooth pH Electrode). Video data of the oyster’s valve gape were recorded using a GoPro HERO8 Black. Once the oyster started pumping, the micro pH probe was inserted into the brood chamber and video and pH recordings were started for a period of 2 hours. After 2 hours all recordings were stopped, the micro pH probe returned to its buffer solution and the oyster returned to the resting tank. Some oysters might only open for a few minutes and close for the remaining time of data measurement. Although these data are relevant for investigating the extent of pH reduction within the brood chamber, the focus of this study is on data collected from oysters that are open

and actively pumping. Accordingly, the following procedure was repeated until 10 datasets of actively pumping oysters, lasting a duration of 2 hours, were obtained (n = 10).

Algae cultivation

The oysters in the resting tank were supplied with unfiltered deep sea water. This water did not contain sufficient food supply for the oysters, who were sampled in very shallow waters of 2-3 meters depth. Therefore, they were supplemented with a combination of live algae and an algae paste. 5 species of live micro algae were grown as a food supplement: *Isochrysis galbana*, *Rodomonas spp.*, *Chaetoceros spp.* (diatom), *Dunaliella spp.* and *Tetraselmis spp.*. Each species was cultivated in a 4l plastic container filled with filtered, autoclaved seawater and F2 medium and was aerated through gentle bubbling of filtered air.

Video and Statistical analysis

The video data were analysed using the image processing software ImageJ and FIJI. The TrackMate plugin for FIJI was employed to track the movement of the black dots on the white markers glued to the oyster's valves as it opens and closes. The measured distances between the dots were converted to mm and incorporated into the dataset as a new variable, representing the shell gape.

To study the influence of ambient water pH and temperature on the brood chamber pH, a linear mixed effects model was fitted on the data using R. Both main effect and interaction models were tested for significant influences. ANOVA's were used to look at differences between treatments. The relation between shell gape and brood chamber pH was analysed by regressing the slope of pH per minute (change in pH) over the slope in shell gape per minute (change in valve position) and comparison with similar previous studies.

Results

Brood chamber pH analysis

After monitoring 10 oysters per treatment for 2 hours, the following results were obtained. A linear mixed effects model (lmer) was used, fitting brood chamber pH in function of the treatments with individual oysters as a random variable to account for individual variation. Normality of residuals was confirmed using Shapiro-Wilks normality test (W=0.978). One outlier yet no influential observations were found. Homogeneity of variances was tested by fitting a model that allowed for varying variances between treatments which had a higher AICc value. The realized pH in the overlaying treatment water in the first two treatments, which resulted from unaltered seawater during autumn 2024, was measured at pH 8.0. In the last two treatments the realized treatment water pH was kept at pH 7.5 (**Table 2**).

Treatment	Estimate	SE	95% confidence interval		Realized ambient pH	SD	Realized temperature	SD	Δ pH	p
			Lower bound	Upper bound						
Ambient pH - 18°C	7.651091	0.032523	7.585067	7.717116	7.976752	0.03222162	17.94777	0.2216178	0.4114033	<.0001
Ambient pH - 23°C	7.548212	0.032185	7.482873	7.61355	7.962026	0.04223295	22.13626	0.2280825	0.3369892	<.0001
pH 7.5 - 18°C	7.269177	0.032203	7.203802	7.334552	7.491522	0.03210598	18.21077	0.4167728	0.2195438	<.0001
pH 7.5 - 23°C	7.216497	0.032169	7.15119	7.281805	7.508296	0.02703653	22.9007	0.1537077	0.2912721	<.0001

Table 2. Average brood chamber pH of *O. edulis* per treatment (Estimate) with its standard error (SE) and 95% confidence intervals; the actual obtained pH and temperature in the treatment water the oysters were held in during measurements with their respective standard deviations (SD) and the difference between brood chamber pH and ambient pH (Δ pH) with their p-values for significance.

Post hoc comparison on the previously mentioned lmer was conducted to show which treatments significantly differed from one another (**Table 3**). The brood chamber pH averaged over 2 hours and 10 replicates, was measured at pH 7.65 (95% CI:7.59-7.72) for the control treatment. The elevated temperature treatment showed a borderline significantly ($p=0.099$) decreased value of pH 7.55 (95% CI:7.48-7.61). The decreased pH treatment showed a significantly ($p<0.0001$) lower value of pH 7.27 (95% CI:7.20-7.33). The last treatment combining elevated temperature and decreased pH resulted in an average of pH 7.22 (95% CI:7.15-7.28). This is a significant lower value compared to the control ($p<0.0001$) and the elevated temperature ($p<0.0001$) treatments, yet there is no difference with the decreased pH treatment ($p=0.59$). In every treatment a significant difference between the overlaying water pH and the brood chamber pH was found ($p<0.0001$)(**Table 2**).

Compared treatments	Difference of means	SE	df	t	p
Ambient pH, 18°C					
Ambient pH, 23°C	0.1029	0.0428	27.7	2.406	0.0993
pH 7.5, 18°C	0.3819	0.0433	28.3	8.812	<.0001
pH 7.5, 23°C	0.4346	0.0417	24.7	10.434	<.0001
Ambient pH, 23°C					
pH 7.5, 18°C	0.279	0.041	24.7	6.806	<.0001
pH 7.5, 23°C	0.3317	0.0424	26.9	7.829	<.0001
pH 7.5, 18°C					
pH 7.5, 23°C	0.0527	0.0415	24.9	1.27	0.5898

Table 3. Post hoc analysis comparing means of the brood chamber pH in each treatment and calculating significance levels (p).

An additive negative trend of temperature and ambient pH was seen when compared to the control treatment. The increased temperature treatment was nearing significance compared to the control treatment with an effect 0.10 pH reduction (**Table 3, Figure 4**). The reduced pH and combined treatments both showed a significant reduction of 0.38 and 0.43 pH respectively compared to the control treatment (**Table 3, Figure 4**).

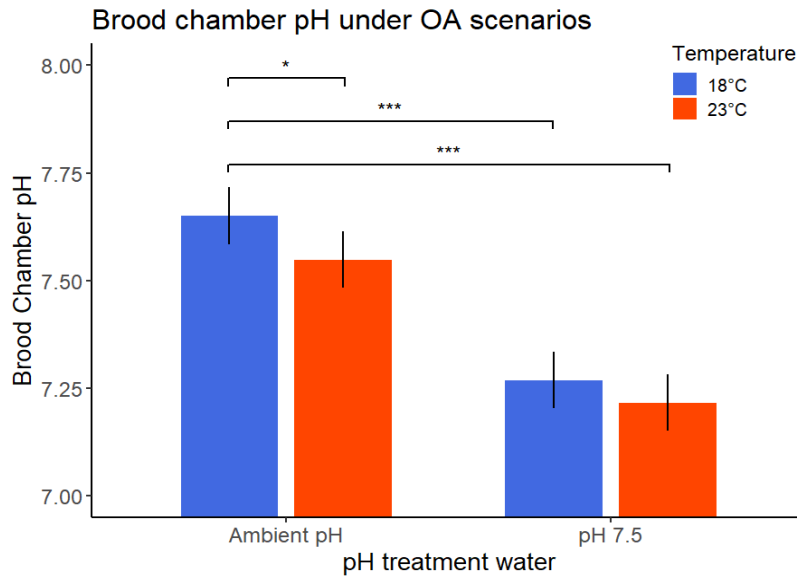


Figure 4. Brood chamber pH in the 4 treatments: Ambient pH – 18°C, Ambient pH – 23°C, pH 7.5 – 18°C and pH 7.5 – 23°C. Values for ambient pH correspond with pH values of the seawater in October-November 2024 (see Table 3). Significance levels between treatments are displayed using asterisks: * = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$

A second linear mixed effects model was fitted to check for the overall influence of temperature, ambient pH and oyster shell size on brood chamber pH. Normality of residuals was again checked using Shapiro-Wilks normality test ($W=0.960$). One outlier yet no influential observations were found. A model relaxing the assumption for homogeneity of variances had a lower AICc value and was therefore used for the values in **Table 4**. There was no significant interaction term between temperature and ambient pH. The temperature of the surrounding treatment water the oysters were held in had a small, yet significant ($p=0.0087$), negative effect on the brood chamber pH. On the other hand, the pH of the surrounding treatment water had a significant ($p=0.000$) positive effect of 0.699 meaning that if the pH in the surrounding water drops by 1 unit, the pH in the brood chamber would drop by 0.699 units. The brood chamber pH is not influenced by the size of the oysters ($p=0.96$).

	Estimate	SE	df	t	p	
Temperature	-0.014054	0.00483	20	-2.91001	0.0087	Table 4. Overall effect of temperature, ambient pH and oyster size on the brood chamber pH of <i>O. edulis</i> . Effect sizes (Estimate) are significant when their respective p-values < 0.05.
Ambient pH	0.699309	0.016277	20	42.96423	0.0000	
Size	0.000197	0.004153	16	0.04742	0.9628	

The lowest pH values obtained in each treatment were 6.93 in the control treatment, 6.51 in the elevated temperature treatment, 6.67 in the lowered ambient pH treatment and 6.78 in the combined (elevated temperature and lowered ambient pH) treatment. These values were recorded after the oysters had naturally closed their shells for some time.

Shell gape analysis

The relationship between the shell gape and the brood chamber pH was analysed to understand how maternal behaviour can influence brood chamber conditions. TrackMate proved to be a highly valuable method for analysing hundreds of thousands of data points (**Figure 5**). These data provide an additional, highly-resolved layer of data that blend the environmental and maternal respiration effects over the brood chamber.



Figure 5. A FIJI plugin called Trackmate was used to measure shell gape over time by tracking the position of a dot on a marker glued to the top valve of the oyster.

The results of the time lapse analysis are displayed by colouring the brood chamber pH graphs of each oyster by their shell gape over time. The maximum opening of the valves can differ between oysters based on the placement of the markers and the size of the oysters. Therefore, the relative shell gape (percentage of maximum opening) was adopted for the analysis. **Figure 6. A.** shows the combined results of brood chamber pH and shell gape fluctuations in one oyster of the control treatment (ambient pH – 18°C).

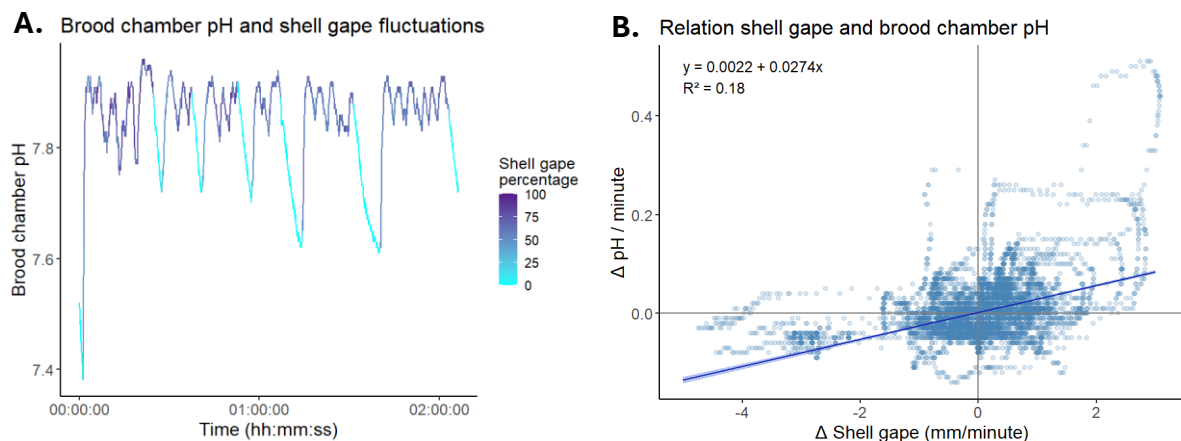


Figure 6. A. Visualisation of brood chamber pH fluctuations in one oyster of the control treatment over the duration of the 2h measurement period. The line is colored by the shell gape, standardized to the percentage of the maximum opening for this oyster. **B.** Effect plot of the relationship between the rate of change in brood chamber pH and the rate of change in shell gape on the same oyster as in A. Negative Δ shell gape values represent the oyster closing, positive values represent the oyster opening. Negative Δ pH values mean the pH is dropping, positive values mean the pH is increasing.

Figure 6. B. shows the relation between the rate of change in the brood chamber pH and the rate of shell gape opening or closing. This linear model was performed on data from the same oyster in the control treatment as in **Figure 6. A.** Residuals were normally distributed. Some outliers yet no influential observations were found. Based on the spread level plot, no obvious deviation from heteroscedasticity was observed. The graph shows a significant positive correlation (estimate = 0.027, $p < 2e-16$) between both variables meaning the faster the oyster's valves open, the faster the pH inside the brood chamber decreases. The faster the valves close, the faster the internal pH decreases. This same analysis performed on all oyster data in a treatment combined, shows no correlation.

Discussion

Brood chamber pH fluctuations

The results show a consistent significant difference between the pH in the overlaying water and the pH inside the brood chamber across present day and all simulated future ocean scenarios. This study provides strong evidence for supporting the hypotheses that maternal respiration of the brood chamber fluid acidifies the chamber but also that these chamber conditions are influenced by the prevailing environmental conditions. The mother adds additional carbon, emitted through respiration, into the brood chamber at all times making it a more acidified environment than the surrounding seawater. The data suggests these conditions applied evolutionary pressure on larvae to evolve traits to develop in acidified conditions and successfully perform organogenesis of their first shell, before being released into the surrounding water column. Even in current ocean conditions (control treatment) the pH in the brood chamber reaches values between 7.59-7.72 (95% CI), or 0.41 pH unit reduction from ambient seawater. These measurements already resemble ocean conditions predicted to occur in 2100 (e.g. pH reduction of 0.30-0.32 units; IPCC, 2015). These data help explain why some brooding *Ostrea* species display larvae to be resistant to OA (Waldbusser et al., 2016). Since they evolved to develop in a pH range of 7.59-7.72, OA conditions do not seem to pose a threat to them.

When comparing the 4 treatments, the individual effects of ambient pH and temperature on brood chamber pH are shown. Where temperature had an overall small yet significant negative effect on the brood chamber pH, after accounting for the change in ambient pH this effect is no longer significant. If only the temperature of the sea water would increase to 23°C during the reproductive season in the future (control treatment vs increased temperature), the analysis shows a nearly significant reduction of 0.10 pH units in the brood chamber (**Table 3, Figure 4**). In an already acidified ocean, there is no additional effect of increased temperature ($p=0.59$) (**Table 3**). The third hypothesis predicted that an increased water temperature would increase metabolism and, therefore, increase maternal respiration, which would further acidify the brood chamber by adding greater amounts of respired CO₂. The results cannot confirm this hypothesis but do provide evidence for this mechanism. Possibly, a temperature change of 5°C is not severe enough to cause a significant change in the brood chamber pH. The effect caused by this temperature change might be altered by the mother's shell gape behaviour or by actively slowing down her respiration when the valves are closed (Gray et al., 2019) in order to protect the larvae in her brood chamber

On the other hand, the individual effect of lowering the ambient pH has a much bigger impact on the brood chamber pH. If only the pH of the seawater would decrease to 7.5 in the future (control treatment vs decreased pH) the analysis shows a significant reduction of 0.38 pH in the brood chamber (**Table 3, Figure 4**). Even in an already heated ocean the additional effect of decreasing the pH in the surrounding water (increased temperature treatment vs combined treatment) causes a significant reduction of 0.33 pH in the brood chamber (**Table 3**). These results show that the pH inside the brood chamber is strongly influenced by the pH of the overlying water. Of course, the connection between the brood chamber and the overlying water requires mothers to be open and pumping this water into the chamber.

Following the SSP5-8.5 global change model and observations around the Kattegat region, it is predicted that both the temperature of the seawater will increase by 5°C and the seawater pH will drop to ~7.5 in the Kattegat region by 2100 (Havenhand et al., 2017; IPCC, 2015). If this scenario were to become reality, the brood chamber pH is expected to significantly drop by 0.43 pH (**Table 3, Figure 4**). Given the natural pH fluctuations inside the brood chamber, this reduction doesn't significantly differ from the scenario where a future ocean would be acidified yet at the same temperatures as today.

Among closed oysters that seal themselves off from the overlying water, we observed brood chambers to become truly acidic (pH < 7.0). We assume that there are likely times that brooding oysters close up periodically, such as when coming into contact with a predator (Clements & Comeau, 2019) and expose their young to these extreme carbonate chemistry conditions. However, it is unclear if there is a limit to the ability of brooded larvae to cope with acidified conditions that will be reached in the near future. To examine larval carbonate chemistry limits, the data collected here can now be used to challenge brooded or extracted larvae (see Gray et al., 2019 and Waldbusser et al., 2016 for information on larvae extraction studies) to predicted seawater conditions and future brood chamber conditions. Although the individual effect of temperature is barely significant, a reduction of 0.10 pH units would be a considerable change given pH is expressed on a logarithmic scale. Yet, natural fluctuations measured inside the brood chamber (**Table 2**) have shown to exceed this value suggesting that larval development and survival of brooding oysters likely won't be disrupted by increased temperatures in the future.

Effect of shell gape on brood chamber pH

A clear and straightforward relationship between the valve gape and the brood chamber response in one oyster of the control treatment (ambient pH – 18°C) can be seen (**Figure 6**). When the oyster is closed (shell gape = 0 % open), the pH inside the brood chamber declines rapidly. Quickly after the oyster reopens its valves, the internal pH increases again. When the valves of the oyster are completely closed, there is no exchange with the surrounding seawater. At the same time, the maternal respiration is still emitting CO₂, causing acidification inside the brood chamber. Upon reopening the shell gape, the exchange with the surrounding seawater, which now has a higher pH compared to the acidified brood chamber, causes the pH to increase again. This relation is not as obvious in all oyster, while there are many individuals where the shell gape data does not line up with the measured pH data.

Fluctuations in shell gape cannot be used as a complete measure for maternal respiration since it has been shown that valve gape does not always strongly correlate with the rate of water exchange or pumping rate of the oyster (Frank et al., 2007). Therefore, it cannot entirely explain the fluctuations in the brood chamber pH while the valve gape is open. Clearly, while the animal is opened there is still variation in either respiration activity or pumping rate by the gills that we cannot account for that is driving small brood chamber pH variations. Nevertheless, valve gape data can certainly account for some of the greatest observed pH variation. If brooding oysters can regulate the duration and frequency of opening and closing events and combine this with actively regulating the rate of maternal respiration, they can strongly determine the conditions inside the brood chamber where larvae will develop. These mechanisms could provide a buffer for future changes in oceanic waters and protect the larvae from energetic stress caused by ocean acidification.

The shell gape data was analysed following a similar approach Gray et al. (2022) used on the *O. chilensis*. Noticeably, the regression equation Gray et al. (2022) found was $Y = 0.00027 + 0.022x$, which is very similar to the regression equation calculated from *O. edulis* measurements in this study: $Y = 0.0022 + 0.027x$. Since the regression closely passes through the origin, it suggests that the brood chamber pH stays relatively stable in case the shell gape does not change. Regularly opening and closing the valves (pumping) could explain the small yet constant fluctuations in the measured brood chamber pH. The approach used for analysing valve gape effects on brood chamber pH fluctuations inherently incorporates noise. The strongest signal of valve gape effects on brood chamber pH are received when there is a sudden and large change in valve gape that is accompanied by a dramatic shift in brood chamber pH (Gray et al., 2019). When slope estimates were created that overlap stable periods and fluctuating periods within one minute, noise in the valve gape-brood chamber pH relationship was created. Further refinement can be achieved with greater post-processing of these data that focus on periods of transition.

Future research

Further analysis on this shell gape data can be performed to see if not only the rate of shell gape change but perhaps also the periodicity or the amount of opening and closing events has an effect on its internal state. Whether this maternal behaviour changes when exposed to heated and acidified conditions could show if it might serve as a strategy to protect the larvae from developing in hostile environments.

Now that the internal fluctuations of the brood chamber pH are known, a valuable next study could look at larvae extracted from the brood chamber and grown in these conditions to see if larval development and survival are effected. This could help determine whether there is a threshold for brood chamber conditions past which larvae can no longer perform organogenesis of their first shell. Not only pH values in the brood chamber can be altered by ocean acidification. This same method and treatments can be used to measure fluctuations of pCO₂ and aragonite saturation states, giving a more complete insight into the carbon chemistry of the brood chamber.

The question remains, Will tactics such as actively regulating respiration and shell gape behaviour by mothers and slow shell development by larvae serve as mechanism to counter the energetic stress created by OA in the future? Therefore larval survival studies in these measured conditions are needed. Additionally, genetic studies would be beneficial to get an idea of the genetic diversity of these resistance traits and the potential of further evolution of these traits.

Study limitations

When inserting the pH probe into the brood chamber of the oysters, it is not always clear whether the tip of the probe is located at the right position. As the name suggests the European flat oysters have a limited volume in their pallial cavity. Lowering the probe too much would result in measuring pH values in between the gills, whereas not lowering the probe enough could result in measuring the pH of the overlaying water instead.

Oysters kept in the resting tank were supplementary fed with excess algae cultures and algae paste mixture from additional experiments. The feeding regime was not equal for every day.

Although the main feeding source is the flow through unfiltered seawater supply, following experiments are best to keep the feeding regime even throughout the entire experiment.

Oysters may have been influenced by the duration of the experiments. The oysters used in the experiment were sampled in September and held in lab conditions for several months. It is uncertain if their time away from their natural habitat may have caused changes in their pumping behaviour.

Conclusion

Larvae of the *O. edulis* develop in an environment that is always more acidified than the surrounding seawater because of the added CO₂ emitted by the maternal respiration and/or reduced exchange with the overlying water. It seems like the main threat to this brood chamber becoming too acidified is ocean acidification, while increased temperatures won't influence larval survival in the future. Maternal behaviour of the shell gape seems to have a small impact on the brood chamber pH as well. If our oceans keep warming and acidifying, by the end of the century brooding oyster larvae will have to cope with brood chamber pH levels as low as 7.2. Further research will have to point out if these low pH conditions are harmful for larvae survival and development.

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Appendix I: Popular science summary

European flat oysters in the future: Do they have what it takes to overcome ocean acidification stress?

Oysters are both economically and biologically important species as they provide food, shelter and substrate for many other organisms living in the same habitat. They act as key species in many environments yet in the face of current global climate change scenarios, it is not guaranteed that the oyster reefs and their services will continue to survive.



1. Sampling of European flat oysters (*Ostrea edulis*) at Tjärnö, Sweden. Source: Lore Van Acker

The threat of ocean acidification

Climate change is causing two major changes in our ocean: Do you know which two? Exactly, the temperature is rising and the seawater is becoming more acidic. Right now the acidity of our oceans is measured at an average pH of 8 but this level is predicted to drop to 7.7 by the year 2100. This can cause serious problems for many bivalve species such as oysters, mussels, scallops, clams, To build their shell they need carbonate ions (CO_3^{2-}) present in the water yet when the pH decreases this important mineral becomes less available and causes the animals to have smaller and deformed shells. Aside from the limited carbonate ions, ocean acidification also limits the energy supply that especially growing larvae are in need of.

Are they perhaps resistant?

Luckily, there are some signs that many shell building animals in the ocean have found a way to cope with this ocean acidification stress. One of those is the European flat oyster. This oyster developed a special way of taking care of its offspring. Instead of immediately releasing all the tiny larvae into the ocean (like many other oysters do), this oyster broods its larvae in a cavity inside the mothers shell, called the brood chamber. The larvae stay there up to 10 days, while they slowly start forming their first shell. This seems to be a very effective strategy, since these brooding oysters perform much better than their non-brooding cousins when they are faced with ocean acidification.

A brood chamber as a sanctuary

Just like we humans do, when an oyster breathes, it takes up oxygen and releases CO₂. A mother oyster will therefore add CO₂ into the brood chamber, located right on top of her gills, and in doing so she makes the brood chamber more acidic. This would mean that the larvae grow up in an environment that might already be more acidic than what is predicted to happen in our oceans in about a 100 years. Wouldn't it make sense that if you learned to deal with a stressful environment ever since you were born, you become an expert in handling that stress later on in life as well?

That's why in this study we try to get a better understanding of what exactly is going on inside that brood chamber. By raising the temperature and the acidity in the surrounding water, we can mimic ocean acidification and create an environment that the oysters will have to face in the future. If we know how the pH inside the brood chamber is changing under ocean acidification scenarios, we can start to understand if this species will thrive or barely survive when these scenarios become reality.

What did we find?

The collected data shows that the average brood chamber pH is always lower than in the surrounding water suggesting that the mother's respiration is indeed contributing to an acidified environment for the larvae to grow up in. Additionally, lowering the pH in the surrounding water lowers the pH inside the brood chamber as well which makes sense since these two water bodies are connected. Raising the temperature seems to make the brood chamber even more acidic although there is no significant difference when oceans would already be acidified. Under ocean acidification scenarios, the pH inside the brood chamber can drop to 7.2 with fluctuations even going below 7. Now more research is needed to see if the larvae will be able to survive these conditions in the future.

Appendix II: Brood chamber pH fluctuations per treatment

Control treatment: Ambient pH – 18°C

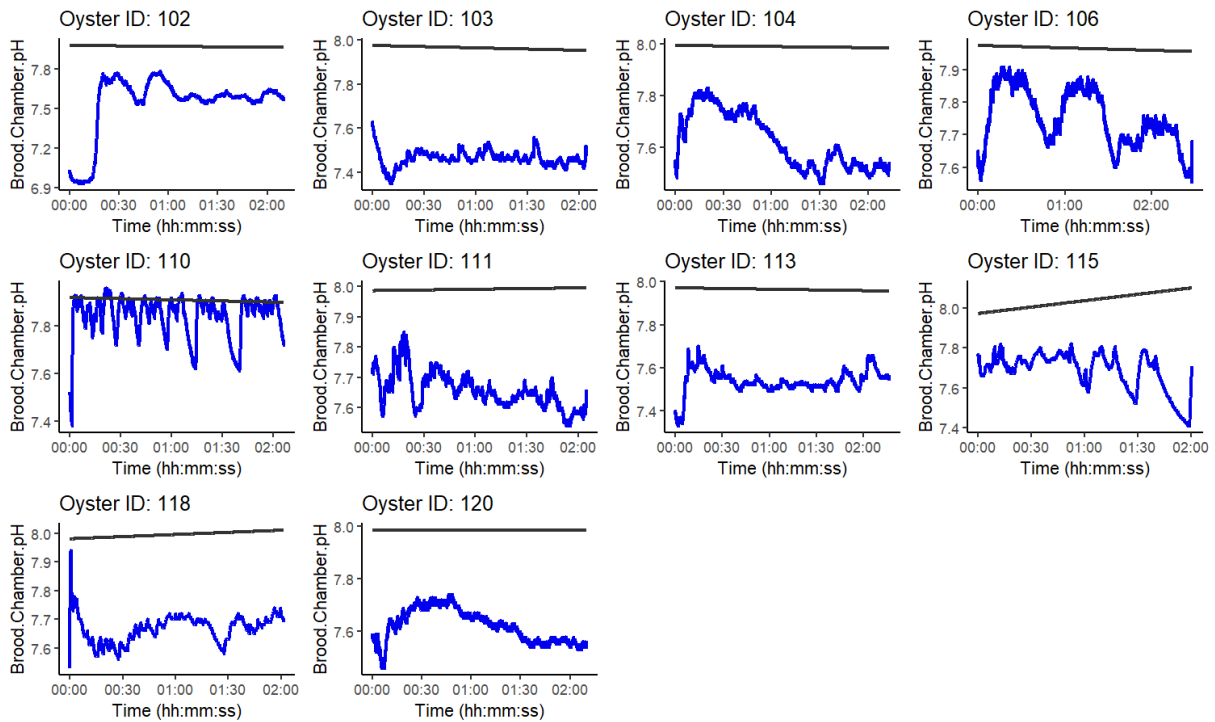


Figure 7. Brood chamber pH fluctuations data from the control treatment (Ambient pH – 18°C) after filtering out 10 datasets to use for analysis. Black lines represent pH values in the overlying water. Blue lines represent brood chamber pH values.

Elevated temperature treatment: Ambient pH – 23°C

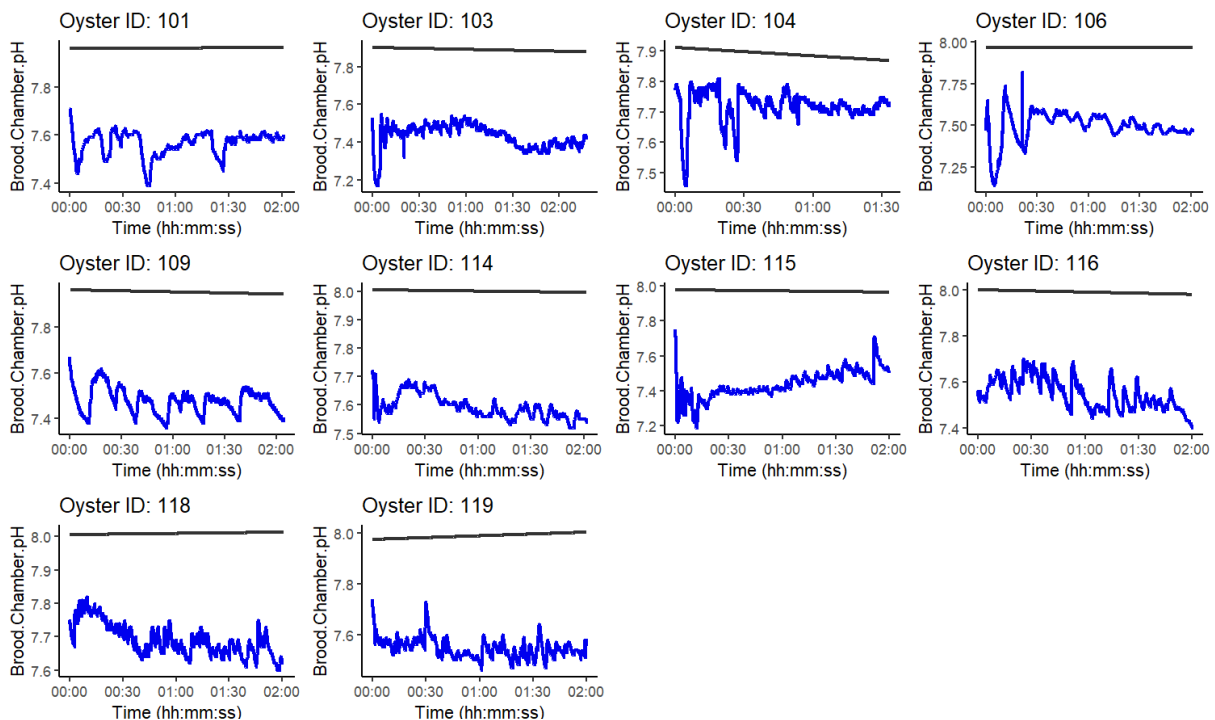


Figure 8. Brood chamber pH fluctuations data from the elevated temperature treatment (Ambient pH – 23°C) after filtering out 10 datasets to use for analysis. Black lines represent pH values in the overlying water. Blue lines represent brood chamber pH values.

Decreased pH treatment: pH 7.5 – 18°C

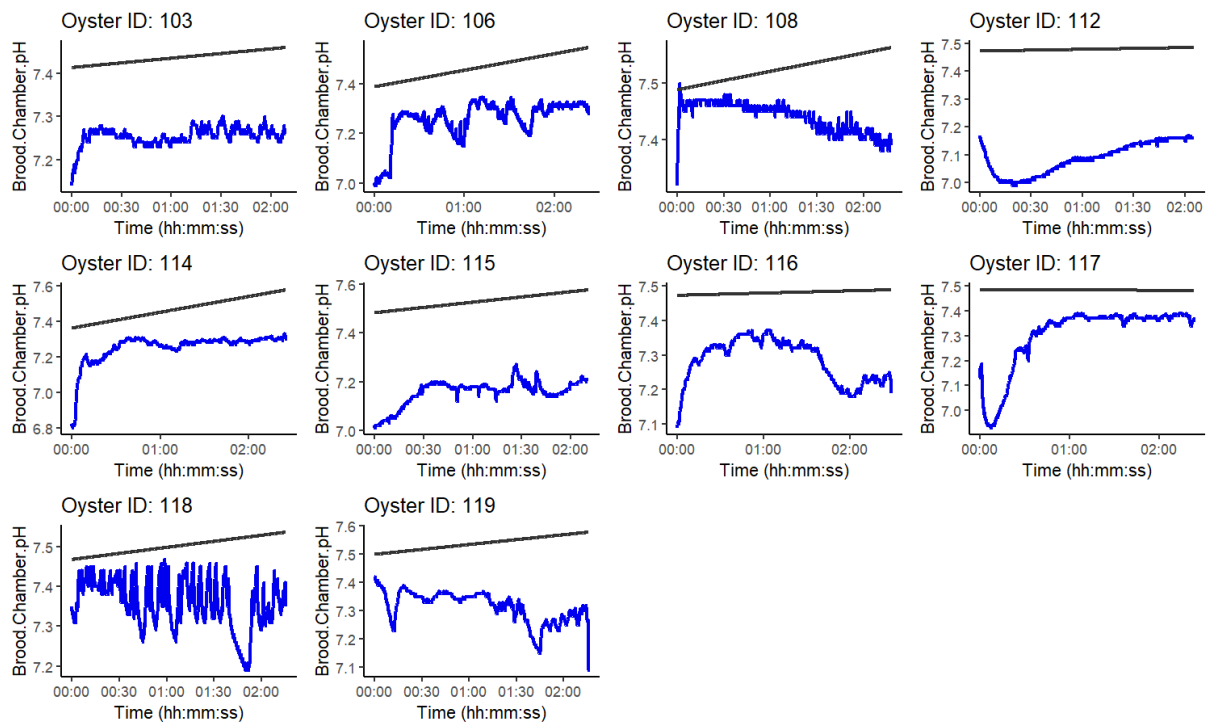


Figure 9. Brood chamber pH fluctuations data from the decreased pH treatment (pH 7.5 – 18°C) after filtering out 10 datasets to use for analysis. Black lines represent pH values in the overlying water. Blue lines represent brood chamber pH values.

Combined treatment: pH 7.5 – 23°C

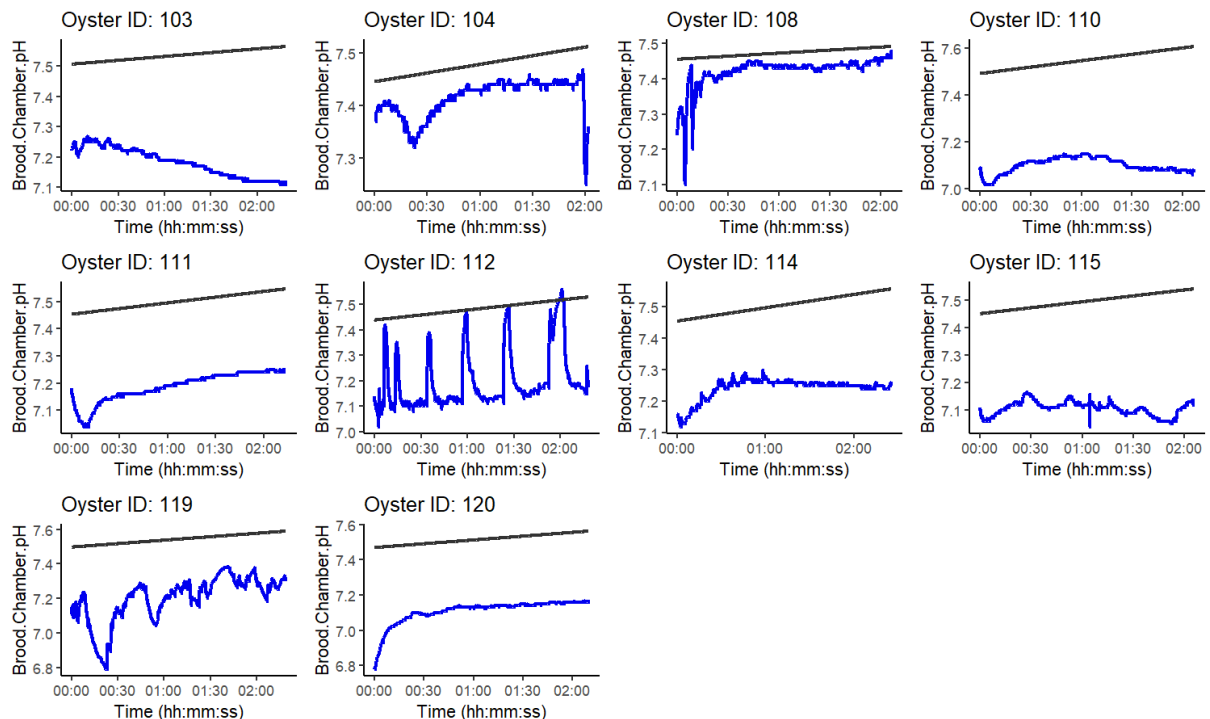


Figure 10. Brood chamber pH fluctuations data from the combined treatment (pH 7.5 – 23°C) after filtering out 10 datasets to use for analysis. Black lines represent pH values in the overlying water. Blue lines represent brood chamber pH values.