

The modulating role of natural variability in the biological response to ocean acidification

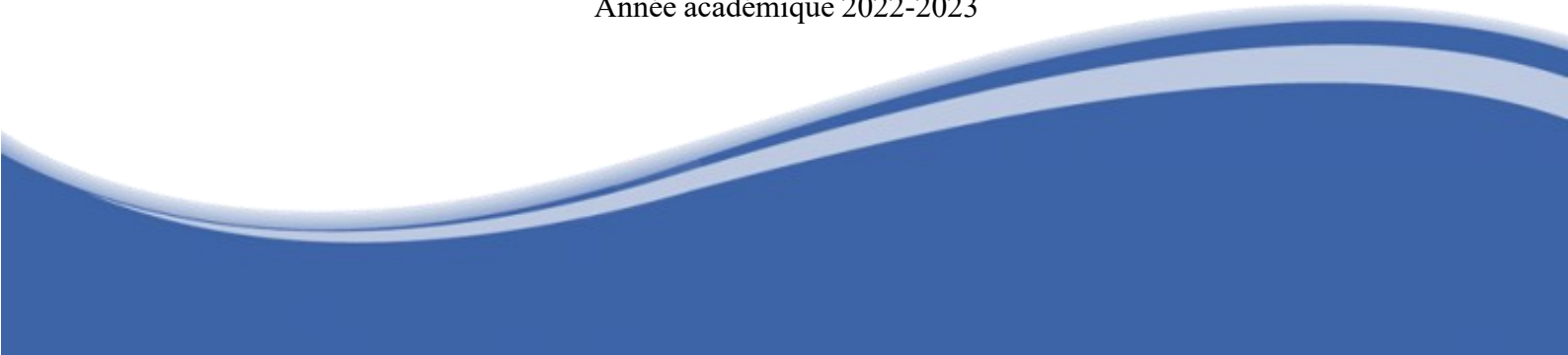
De Neyer Louise

Mémoire présenté en vue de
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Ocean acidification (OA) is the consequence of the uptake of excess carbon dioxide from the atmosphere. Along the coastal zone, ocean acidification is influenced by other processes such as biology and currents, leading to high levels of natural variability in pH. While the impact of pH on marine organisms is better resolved, the modulating role of this natural variability is poorly understood. This master's thesis aimed at evaluating diel pH fluctuations using the larval stages of the brittle star *Amphiura filiformis*. Results revealed the importance of acknowledging pH variations with individuals exhibiting higher fitness. Diel analyses also underscored the existence of an intrinsic circadian cycle where individuals would grow more during the daytime than nighttime, possibly explained by better conditions encountered during the day. In addition, observations revealed a carryover effect that could also be associated with a stage sensitivity. We suggest that future studies should integrate natural variations and delve into the different species' adaptations as they have an important role in the biological responses to upcoming OA.

Introduction

With global industrialization and population growth, the atmospheric concentration of CO₂ has increased by about 38 percent since the pre-industrial era, reaching around 415ppm today (NOAA - Ocean Acidification Program). The ocean serves as a major sink by absorbing half of the surplus CO_{2(atm)}. When CO_{2(atm)} dissolves in seawater, it forms carbonic acid which dissociates to create an equilibrium with carbonate, bicarbonate and hydrogen

ions. This interplay of chemical reactions ultimately leads to a reduction in the ocean's pH, giving rise to the phenomenon known as ocean acidification (OA). With the increase uptake of CO_{2(atm)} by seawater, the average surface ocean's pH could fall around 7.8 by 2050, with a current value of ± 8.0 (Gattuso & Hansson, 2011). Additionally, under future scenarios, it is estimated that CO₃²⁻ concentration will decrease by $\pm 50\%$ in the year 2100 (Feely et al., 2004; Orr et al., 2005).

Consequently, multiple studies have delved into evaluating OA as a potential stressor for marine species and ecosystems. Globally, there has been a growing body of evidence that calcification was a primary sensitive parameter when exposed to high-CO₂ level for different development stages of marines species (Dupont et al., 2010). However, available results have sometimes shown contrasting results. The bloom-forming coccolithophore *Emiliania huxleyi* serves as a compelling example. Several studies have indicated a decrease in its calcification when subjected to high *p*CO₂ levels (e.g Riebesell et al., 2000), while another study found that under similar conditions, the coccolithophore exhibited twice the usual calcification rates (Iglesias-Rodriguez et al., 2008). Similarly, a study carried out on *Amphiura filiformis* revealed an unexpected positive effect of pH reduction on

calcification (Wood et al., 2008), contradicting the prevailing scientific assumptions. While this study has been subjected to many criticisms (e.g. Hu et al., 2014; Dubois, 2014), it remains intriguing to acknowledge that although calcification may increase, there is a probable underlying trade-off, such as metabolic depression, as indicated by Hu et al. (2014). In addition, other important biological traits such as morphology (symmetry and allometry), growth or survival have also been proven to be sensitive to OA. It has notably been demonstrated that shape changes under decreased pH could have significant biomechanical impacts on larval movement (Chan et al., 2015) and stability in flow (Clay & Grünbaum, 2011) changing larvae's ability to carry out ecological functions. A recent meta-analysis also revealed that far-future ocean acidification scenario have negative effects on echinoderms on larval growth, fertilization success and coelomic fluid pH (Leung et al., 2022). Furthermore, the heightened susceptibility to low pH levels may result in increased mortality rates (e.g. Shirayama & Thornton, 2005) and in some extreme cases, can lead to the total extinction of the studied population. A notable example was observed with the brittlestar *Ophiothrix fragilis*, where all individuals perished with deformities after being exposed to a pH of 7.7 for a duration of 8 days (Dupont et al.,

2008). Globally, this involves that OA can act upon multiple biological processes of larvae development by creating novel environment conditions. This indicates that it's of utmost importance to factor in a range of different biological responses and their interrelationships when assessing scenarios of near- and far-ocean acidification.

In addition, coastal ecosystems are known to experience variations in seawater chemistry through time and between ecosystems (Hofmann et al., 2011). pH variations are also observed at small temporal scale. A study conducted in Ischia in Italy revealed minute-variation between studied coastal stations. They also showed variations among the different benthic stations with some having high and stable benthic pH fluctuation with diel periodicity, presumably driven by community photosynthesis and respiration, and others with very variable benthic mean pH including large pH fluctuations (Kerrison et al., 2011). Other physical, chemical, and biological processes can lead to coastal variations such as upwelling (Dabuleviciene et al., 2018), seasons and interannual differences (Carstensen & Duarte, 2019) and interactions between land, open ocean and atmosphere with coastal environment (Aufdenkampe et al., 2011). Many studies have focused on examining the biological responses of

coastal species under future constant $p\text{CO}_2$ levels projected for the open ocean by the Intergovernmental Panel on Climate Change (Vargas et al., 2017). However, by doing so, they have disregarded natural variability, a vital natural factor, that may play a substantial role in the acclimation and adaptability of coastal species to ocean acidification. For instance, contrasting physiological performance have been observed between coastal and open ocean diatoms indicating that they differ in their ability to deal with dynamic $p\text{CO}_2$ that could be explained through local acclimation to habitat and diurnal changes interacting with OA (Li et al., 2016). This suggests that only when natural pH variations are considered, can we evaluate the real impacts of OA and align them with the future scenarios we may face in marine coastal ecosystems and species as notably suggested by Leung et al. (2022).

Amphiura filiformis is a brittlestar belonging to the family Amphiuridae which is living buried in soft-bottoms sandy substrate and is found in north-east Atlantic at depth between 15 meters and 100 meters (or more). Its disc is buried from 4 up to 8cm below the surface. The population densities of *A. filiformis* are known to reach approximately up to 3,000 individuals m^2 , making it one of the dominant macrobenthic species on soft bottoms (Rosenberg, 1976).

Through its biological activities, the brittlestar is influencing generative and regenerative microbial-mediated processes, including nutrient transformation and decomposition which are vital condition for the good maintenance of marine coastal ecosystems (Solan & Kennedy, 2002; Vopel et al., 2003). When present, *A. filiformis* can account for 80 percent of the total flux of O_2 into the soft bottom (Vopel et al., 2003). This implies that this species is particularly interesting to study, for its ecological importance as a keystone species as well as being an established model species, notably for regeneration (e.g Dupont & Thorndyke, 2006) and evolutionary developmental studies (e.g Dylus et al., 2016).

In this research article, our objective was to assess the effect of pH variability in contrast to constant pH on different pertinent morphometrics and survival responses. We mimicked natural fluctuations occurring between day and night at the Swedish coasts and also evaluated whether reversed fluctuations could influence responses. Additionally, we investigate whether local adaptability to fluctuations could be associated with a plasticity or canalization cost. Species residing in fluctuating habitats, where behavioral adjustment to changes are constrained by limited mobility, are likely to exhibit wide norms of reaction

(Ebert, 1996). Nevertheless, it appears that calcification could be a highly conserved trait through geological time (Gold & Vermeij, 2023), meaning some biological processes might be resilient through favorable selections. Understanding what adaptation has been selected and its associated cost is important to estimate and predict the ecological and evolutionary consequences expected from *A. filiformis* facing future OA scenario in a fluctuant environment.

We focused on larval developmental stage because it is a critical dispersal phase and is considered one of the most vulnerable to environmental perturbations (Dupont et al., 2010)

Method and materials

Animal collection and fertilization

The brittlestar *Amphiura filiformis* was collected using a box corer to collect sediment at the mouth of the Gullmarsfjord, west Sweden at ~40 meters depth (58° 14.72' N, 11° 25.80' E). A total of 116 individuals were extracted from the sediment by gentle sifting on board and sorted between male and female based on distinct physical features. Each individual was then placed separately in a Falcon tube of 50 ml filled with deep water sea (15°C). In the lab, individuals were kept in brightness for 12h until spawning.

Individuals were then transferred to darkness to initiate the release of gametes. Every 15 minutes after transfer, gametes from spawning individuals were placed together in a 2.5L Erlenmeyer flask (corresponding to 1 batch). Percent of fertilization and total number of eggs in each batch was estimated based on a 10-20 µl sample where we counted the total number of eggs and the number of fertilized eggs. When not enough males were spawning, we collected sperms directly from the gonads of one male and spread it in the different Erlenmeyer flasks. When transfer to darkness was not sufficient to drive spawning, we used a heat shock procedure to induce more spawning. Individuals were transferred in 20°C water for 5 to 8 minutes and then transferred again in 15°C water in the dark. These combined procedures allowed to reach a fertilization rate of $\sim 90 \pm 5\%$. Once fertilization had occurred, we transferred about 10 000 fertilized eggs per 1L bottle replicate (21 in total- 3 replicates per treatment).

Experimental design

Seven different treatments with three replicates each were established and maintained for 14 days. The experiment was divided into 2 phases of 7 days as *A. filiformis* larvae follow a logarithmic growth with the first 7 days corresponding to a linear growth followed by a

stabilization around the 7th/8th day. The seven different treatments were used in different combinations between the two phases. These included 3 different constant pH regimes (respectively pH 8.0, 7.75, 7.5 – corresponding notably to current, near-future and far-future scenario pH predictions), and 2 fluctuating treatments following natural pH fluctuation encountered in coastal environment between night (pH 7.5) and day (pH 8.0). One was following the natural pH fluctuating following the diurnal cycle (high pH during the day, low pH during the night) and one inverted light and pH cycle (low pH during the day, high pH during the night).

The 7 combinations are presented in the Figure 1 and allowed to test 3 hypotheses. By comparing the 3 constant treatments and the fluctuating treatments following the natural diurnal cycle throughout the 2 phases (treatments 1, 2, 3 and 6), we could test the hypothesis that the fluctuating environment led to better fitness than constant treatments (H1) as well as confirming that low pH had a negative effect on fitness. Comparing the two fluctuating treatments throughout the two phases (treatments 6 and 7) but following different diurnal cycles, allowed to test the hypothesis that reversing the natural cycle is detrimental for fitness (H2). Testing for

canalization and plasticity cost (H3) were evaluated through the comparison of a constant treatment on one phase and fluctuant on the second phase (treatment 4) and a fluctuant treatment on phase one and constant on phase two (treatment 5). If plasticity reveals to be costly, we expect to see lower fitness values once individuals in fluctuant treatments are transferred in constant treatment. On the other hand, we expect canalization cost if individuals first raised in constant conditions and then switched to fluctuations demonstrate a lower fitness.

Maintenance of cultures, sampling, and carbonate chemistry

For each treatment, water was changed twice a day, at 7 a.m. and at 7 p.m. During the procedure, a 10 ml sample was collected using a pipette from which the number of larvae were counted (larval density; ind. ml⁻¹) and measured with Zeiss software (Leica digital microscope ; Axiocam camera)) after being fixed with a drop of paraformaldehyde solution (4% PFA in FSW, buffered at pH 8.3). Evening samples were stored at 4°C in 4% PFA solution until the measurements. When less than 15 individuals were found, 20 ml sample were taken instead. In addition, as individuals number decreased through time, we decided to concentrate the 1L bottles into 500 ml bottles at day 7 to increase the density.

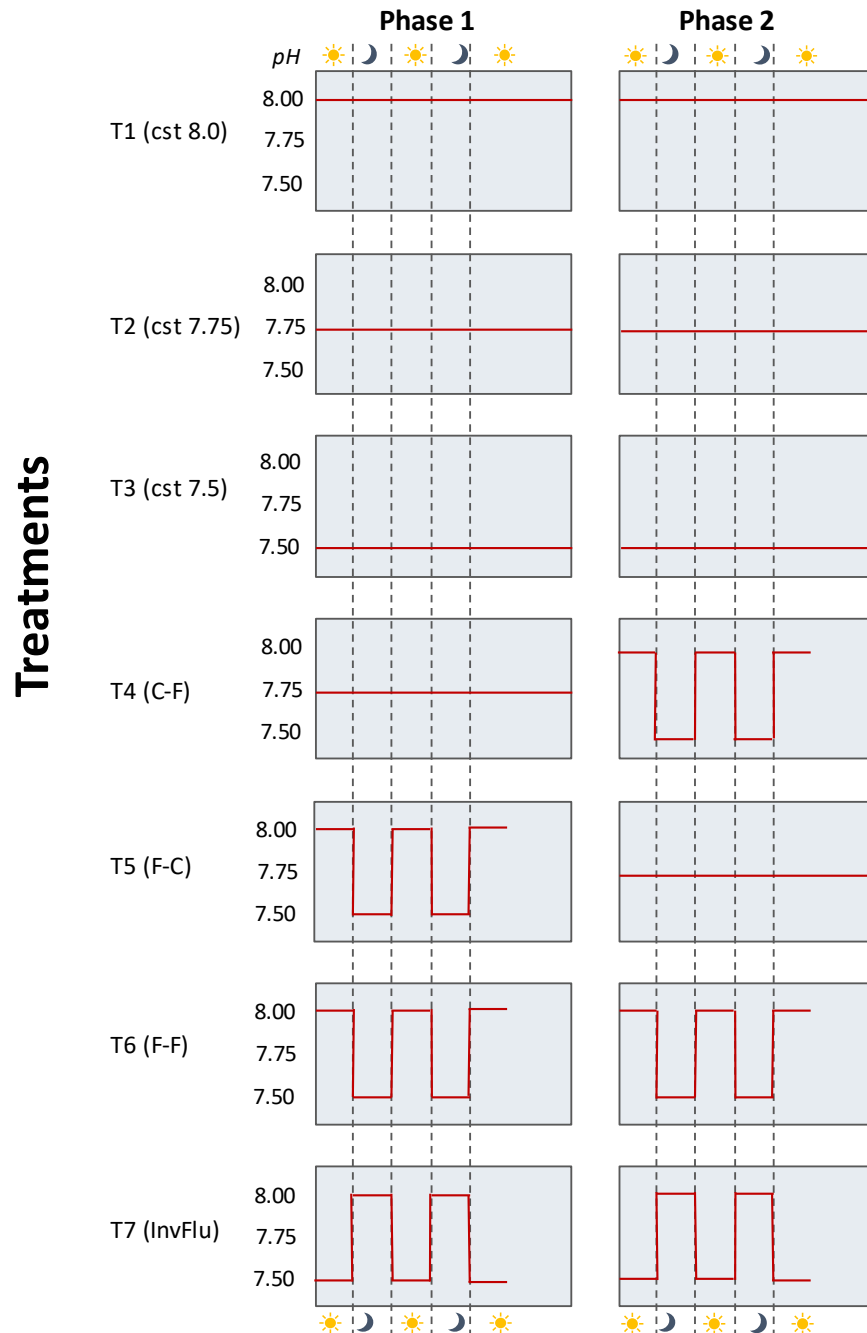


Fig. 1: Experimental design showing the application of the different pH treatment. **H1** corresponds to the comparison of treatments 1,2,3 and 6. **H2** corresponds to the comparison of treatments 6 and 7. **H3** corresponds to the comparison of treatments 2, 4, 5 and 6. Only 2 complete days were represented as an example.

We used deep filtered water (3 filters: 10 μm , 3 μm , 0.5 μm). The water pH was maintained in 3 tanks with a pH computerized feedback system (AquaMedic) adjusting the desired pH through CO_2 gaseous flux (± 0.02 pH units; for more details see Annex 2 and 3). Larvae

were reared at 14°C and salinity 32 and were fed *Rhodomonas sp* at $150 \mu\text{gC l}^{-1}$ ($\sim 4000 - 5000 \text{ cells ml}^{-1}$) starting from 5 d post-fertilization.

Seawater chemistry parameters were measured through the experiment on day

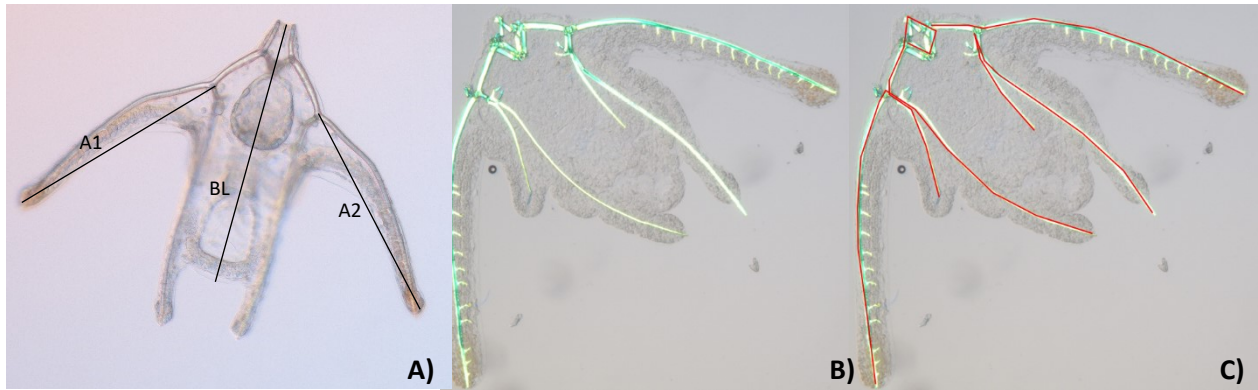


Figure 2: Example of measurements. **A)** Different body parts length (BL = Body Length, A1 = postoral arm one, A2= postoral arm two); **B)** Skeleton picture. **C)** Skeleton picture with the calcification measurement in red.

2.5, 3, 7, 7.5, 8, 10.5, 11, 14 (0.5 digit is equivalent to day shift, while no digits corresponds to night shift). pH was measured with a Metrohm 827 pH lab electrode adjusted for pH measurements on the total scale (pHT) using TRIS (Tris – HCl) and AMP (2-aminopyridine/HCl) buffer solutions with a salinity of 32 (provided by Unité d’Océanographie Chimique, Université de Liège, Belgium). Alkalinity was measured by titration. Carbonate system speciation ($p\text{CO}_2$, ΩCa , and ΩAr) was calculated from pH_T , alkalinity, temperature and salinity using CO2SYS (Pierrot et al., 2006). Seawater chemistry can be found in Table 2.

Picture analysis

Pictures were analyzed with ImageJ. The body length, the two postoral arms and the total skeleton length were measured for 10 individuals per replicate and per sampling time (Figure 2). Note that from day 1 to 2.5, only the body length was measured, as no arms and skeleton had appeared yet. The

measurements were transformed from pixel to μm .

Endpoints

Description and calculations of the different studied responses can be found in Table 1.

Mortality rate (D/N shift^{-1})

A cumulative correction factor was applied on the individual density to take into account the different sample size (10 vs 20 ml) and the replicate volume (1L vs 0.5L). Relative density was calculated by the normalization of the density relative to the initial density. Mortality rate corresponds to the difference of relative density between consecutive day – night shift, ranging between 0 (no mortality) and 1 (100% mortality).

Calcification rate ($\mu\text{m} \times \text{D/N shift}^{-1}$), growth rate (D/N shift^{-1}) symmetry index (D/N shift^{-1})

Calcification rate was calculated as the mean difference between consecutive night and day shift. Growth rate corresponds to relative growth rate per 12 hours. Symmetry

Table 1, List of variables used in this experiment. *RD* = Relative density, *BL* = Body length, *D/N* = Day/Night shift, *Cal* = Calcification, *i* = specific observations (*I* = 10), *j* = specific D/N shift (*J* = 29)

Abbreviation	Name	Description	Unit
<i>MR</i>	Mortality rate	$\forall i \in J: G_{ij} ; G_{ij} = \sqrt{(RD_{i,j_0} - RD_{j_{-1}})}$	D/N shift ⁻¹
<i>GR</i>	Growth rate	$\forall i \in J: F_{ij} ; F_{ij} = \frac{BL_{(i,j_0)} - \overline{BL}_{(j_{-1})}}{\overline{BL}_{(j_{-1})}}$	D/N shift ⁻¹
<i>CR</i>	Calcification rate	$\overline{Cal}_{(i,j_0)} - \overline{Cal}_{(j_{-1})}$	$\mu\text{m} \times \text{D/N shift}^{-1}$
<i>AL</i>	Allometry	Average per day or night shift of the linear regression model slope of individuals' longer limb over the body length.	D/N shift ⁻¹
<i>SI</i>	Symmetry index	Average per day or night shift of the ratio between the two postural arms	D/N shift ⁻¹

index was calculated as the ratio between the smallest and longest post-oral arms.

Allometry (D/N shift⁻¹)

In the context of this study, we decided to evaluate allometry as the differential growth between the total body length and the longer post-oral arm. This information will give an overall idea of the growth pattern of *A. filiformis* under different pH conditions.

Statistics analysis:

All statistics were realized using RStudio. Tukey's method was used to delete any outliers. Normality was checked with Shapiro-Wilk test and homoscedasticity was tested using Bartlett method. Linearity was visually checked and approved for all treatments. For all hypothesis, all parameters revealed to be appropriate for the conduction of 3-ways ANOVA (detailed results in Annex A1- A.5 and Table A2) apart from the mortality rate which showed

heteroscedasticity (Bartlett-test; p-value = <0.000). For the latest, a squared root transformation was applied, reaching a p-value of 0.192 on the Bartlett-test, solving heteroscedasticity. Other transformations (logarithmic, squared and cubic) revealed to be non-efficient. In addition, not all treatments revealed normality on both phase for each parameter (Annex Table A1). While this would usually mean that 3-ways ANOVA could not be conducted as the chance of false positive increases, multiple simulation studies have revealed that ANOVA was not very sensitive to moderate deviations from normality, which was the case in this experiment, with a false positive rate not affected by the violation of the assumption (Glass et al., 1972; Harwell et al., 1992; Lix et al., 1996). When needed, Tukey test was realized after parametric test.

Table 2: Seawater carbonate chemistry parameters for the seven treatments presented as the mean $\pm$ SD

	Measured pH _T										Calculated													
						pCO ₂					Temperature (°C)					Wca					War			
	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5	8	7.75	7.5
1	8.00±0.06	—	—	—	470.78±70.62	—	—	—	—	14.50±0.28	—	—	—	3.27±0.41	—	—	2.09±0.26	—	—	—	—	—	—	—
2	—	7.80±0.06	—	—	—	905.4±145.75	—	—	—	14.30±0.34	—	—	—	1.96±0.23	—	—	1.25±0.15	—	—	—	—	—	—	—
3	—	—	7.50±0.16	—	—	—	1814.07±644.88	—	—	—	—	—	14.20±0.42	—	—	1.23±0.43	—	—	—	—	—	—	—	0.78±0.16
4	8.09±0.00	7.75±0.05	7.43±0.09	364.19±9.45	885.98±100.95	1965.77±432.58	14.65±0.35	14.01±0.326	14.13±0.23	3.96±0.11	1.97±0.23	1.00±0.2	2.53±0.07	1.25±0.14	0.64±0.61									
5	7.94±0.11	7.76±0.09	7.51±0.02	561.37±148.34	883.19±212.46	1580.29±33.76	14.07±0.29	14.33±0.42	13.70±0.46	2.94±0.65	2.03±0.34	1.15±0.12	1.87±0.41	1.29±0.22	0.73±0.08									
6	8.04±0.03	—	7.48±0.17	426.14±31.72	—	2637.55±2973.71	14.18±0.34	—	14.14±0.26	3.55±0.17	—	1.34±0.50	2.26±0.11	—	0.86±0.32									
7	8.04±0.07	—	7.67±0.16	430.00±75.81	—	1144.58±374.26	14.12±0.28	—	14.21±0.38	3.50±0.43	—	1.73±0.69	2.23±0.27	—	1.10±0.29									

In addition, an ACP analysis was realized on phase 2 to verify the underlying structure of the dataset that could explain the most variance, notably to understand the relationship between explanatory variables.

Results

Mean $\pm$ SD for seawater carbonate chemistry parameters measured and calculated for all treatments are reported in Table 2. Mean pH_T were 8.00 $\pm$ 0.06, 8.09 $\pm$ 0.00, 7.94 $\pm$ 0.11, 8.05 $\pm$ 0.03 and 8.04 $\pm$ 0.07 for nominal pH 8.0. For nominal pH 7.75, we reached mean pH_T value of 7.80 $\pm$ 0.06, 7.75 $\pm$ 0.05 and 7.76 $\pm$ 0.09. For nominal pH 7.5, we reached mean pH_T value of 7.50 $\pm$ 0.16, 7.43 $\pm$ 0.09, 7.51 $\pm$ 0.02, 7.48 $\pm$ 0.17 and 7.67 $\pm$ 0.16. Though the latest treatment revealed higher values than expected, we decided to keep it as its pH_T was still lower than the other nominal pH conditions. pCO₂ was the lowest for pH 7.5 treatments and seawater was under-saturated concerning calcite and aragonite in the lowest pH treatments. Temperature revealed values around 14 degrees for all treatments.

H1: Constant versus fluctuant pH regime

Each of the studied responses revealed to be significantly different between both phases apart from symmetry index. On one hand, calcification rate and growth rate exhibited mean positive values on phase one and

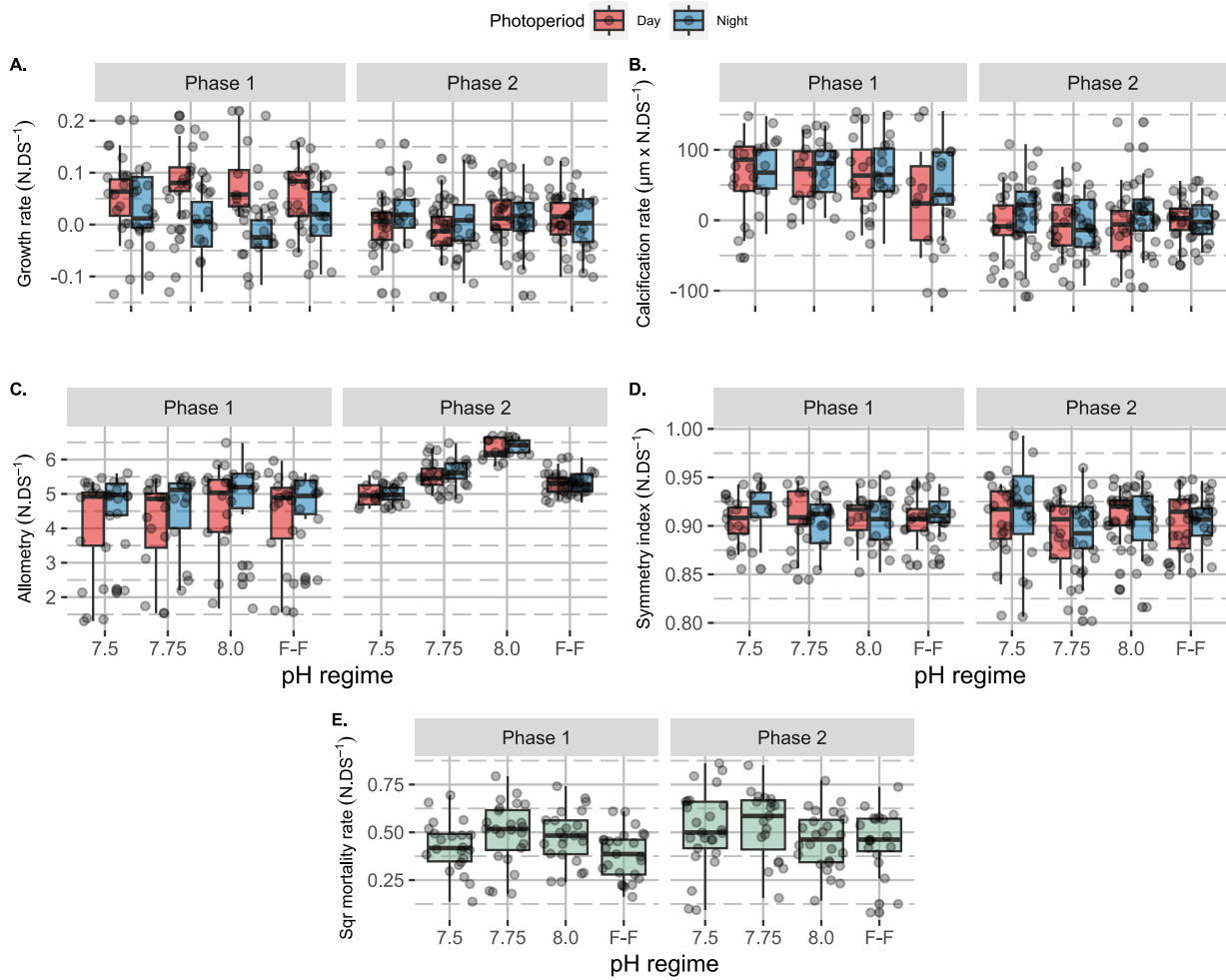


Fig. 3: Test of the H1: results over treatments 7.5, 7.75, 8.1 and F-F. **A.** Growth rate. **B.** Calcification rate. **C.** Allometry. **D.** Symmetry index. **E.** Squared root mortality rate. N.DS = Night and day shift.

values close to zero on phase two. On the other hand, on average allometry was 1.25 times higher and mortality 1.11 times higher on phase two compare to phase one (ANOVA III, Table A2, Fig.3, GR: $F_{3,277}$ -value = 12.677; p-value = <0.000, CR: $F_{3,252}$ -value = 102.1; p-value = <0.000, AL: $F_{3,222}$ -value = 38.990; p-value = <0.000, SI: $F_{3,252}$ -value = 0.100; p-value = 0.752, MR: $F_{1,175}$ -value = 3.978; p-value = 0.048). Phase and pH regime showed significant interaction on allometry values (ANOVA III, Table A2, $F_{3,222}$ -value = 9.892; p-value = <0.000).

When looking at each phase separately, results exhibited significant allometry

differences between pH treatments on phase two (ANOVA II, Table A4, $F_{3,114}$ -value = 31.710; p-value = <0.000), with constant 8.0 treatment revealing the highest allometry values, followed respectively by treatments 7.75, F-F and 7.5 (*Post hoc* Tukey's test, Table A12, p-value < 0.05).

There was also a significant difference of mortality rate between pH regimes on phase one, with treatment 7.75 revealing significant higher mortality rate value compared to F-F treatment (ANOVA II, Table A3, $F_{3,90}$ -value = 3.095; p-value = 0.031, *Post hoc* Tukey's test, Table A12, p-value < 0.05).

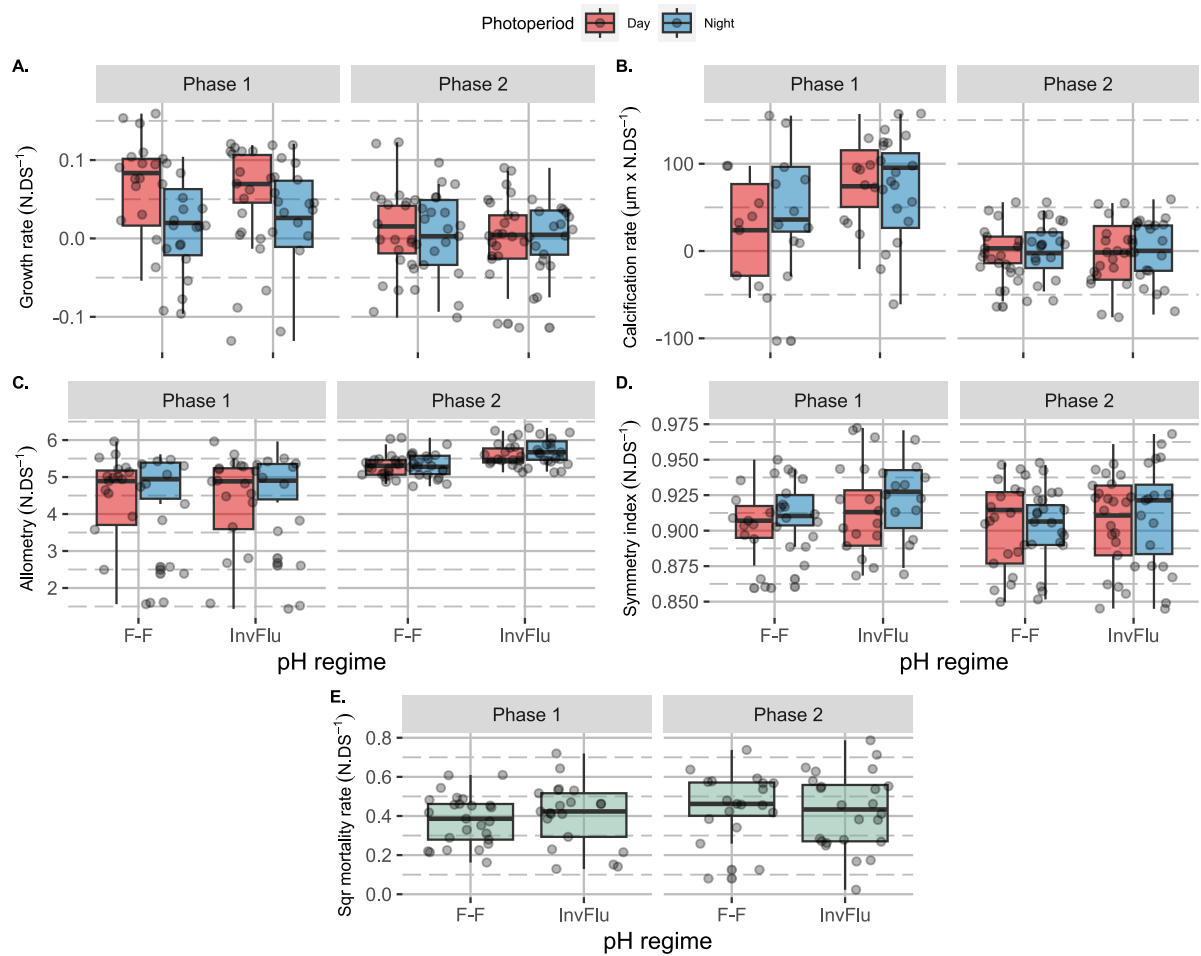


Fig. 4: Test of the H2: results over treatments F-F and InvFlu. **A.** Growth rate. **B.** Calcification rate. **C.** Allometry. **D.** Symmetry index. **E.** Squared root mortality rate. N.DS = Night and day shift.

In addition, ANOVA III revealed a significant interaction between photoperiod and phase regarding growth rate (ANOVA III, Table A2, $F_{1,277}$ -value = 25.483; p -value = <0.000). When evaluating phase one, results exhibited a significant difference of growth rate between day and night, with daytime showing higher growth rate values compared to nighttime (ANOVA II, Table A3, $F_{1,277}$ -value = 18.435; p -value = <0.000).

H2: Natural and reversed fluctuations

Each response revealed to be significantly different between both phases apart from

symmetry index and mortality rate. Precisely, phase one revealed positive average growth rate and calcification rate values compared to phase two, exhibiting close to zero values for both of those responses. In addition, similarly to hypothesis one, allometry revealed 1.25 times higher values on phase two compared to phase one (ANOVA III, Table A5, Fig.4, GR: $F_{1,144}$ -value = 14.240; p -value = <0.000, CR: $F_{1,126}$ -value = 48.820; p -value = <0.000, AL: $F_{1,132}$ -value = 26.000; p -value = <0.000, SI: $F_{1,131}$ -value = 1.854; p -value = 0.176, MR: $F_{1,85}$ -value = 1.846; p -value = 0.178). Phase parameter also exhibited a significant interaction with pH regime

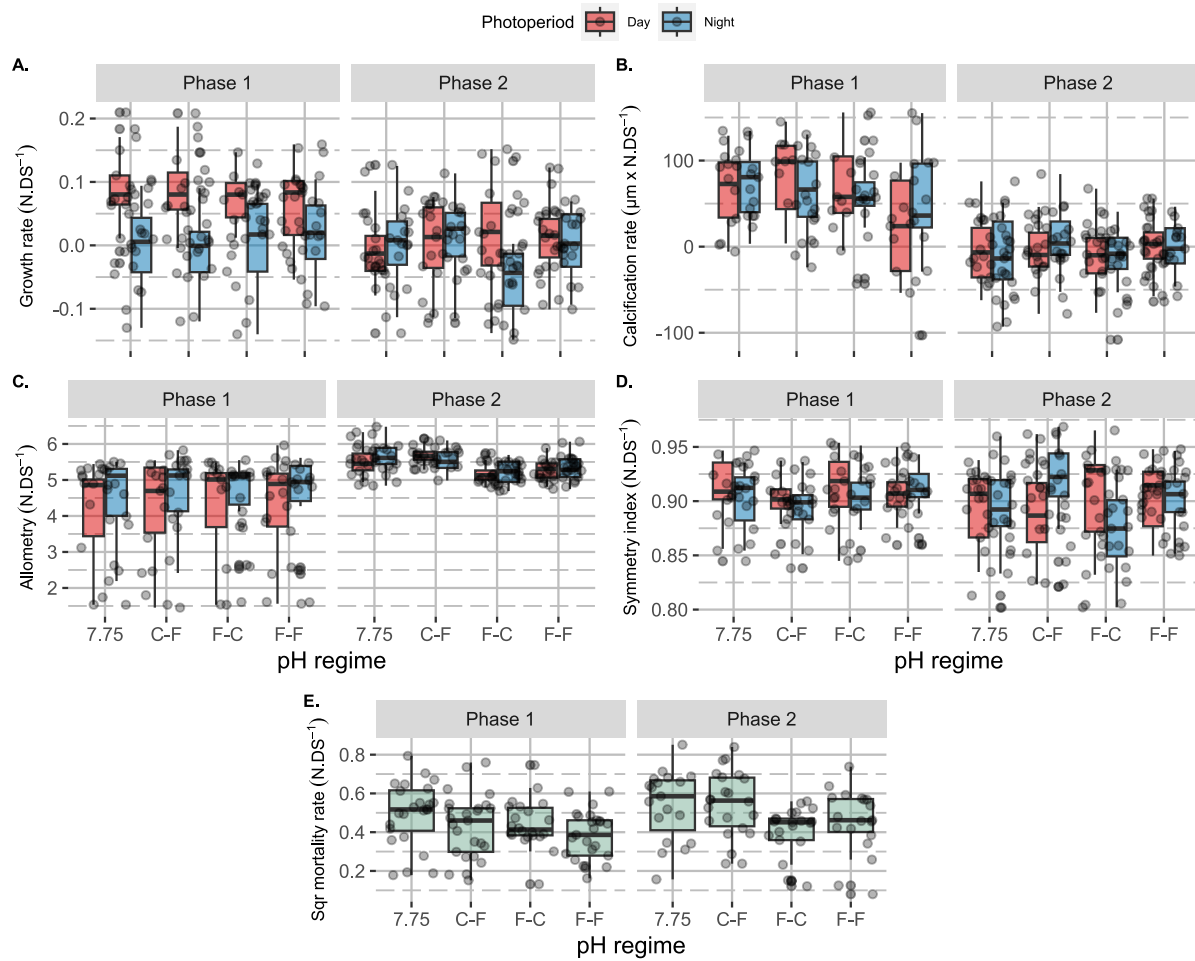


Fig. 5: Test of the H3: results over treatments 7.75, C-F, F-C and F-F. **A.** Growth rate. **B.** Calcification rate. **C.** Allometry. **D.** Symmetry index. **E.** Squared root mortality rate. N.DS = Night and day shift.

parameter regarding allometry and calcification rate variables (ANOVA III, Table A5, $P \times pH$ on AL: $F_{1,132}$ -value = 4.962; p-value = 0.028, $P \times pH$ on CR: $F_{1,126}$ -value = 6.354; p-value = 0.013).

When studying phase one, it appeared that there was a significant difference between pH treatments on calcification rate, with a reversed fluctuant treatment revealing 38.66 calcification rate higher than natural fluctuant treatment (ANOVA II, Table A6, $F_{1,46}$ -value = 5.108; p-value = 0.029).

Allometry was also different between the two pH treatments but on phase two, with

reversed fluctuant treatment revealing 0.334 higher allometry than fluctuant treatment (ANOVA II, Table A7, $F_{1,76}$ -value = 20.360; p-value = <0.000). Similarly to H1, night and day shift was significant for growth rate on phase one, with daytime revealing higher value than nighttime (ANOVA II, Table A6, $F_{1,67}$ -value = 12.140; p-value = 0.001).

H3: Plasticity versus canalization cost

All responses revealed significant differences between both phases apart from symmetry index. Phase one exhibited mean

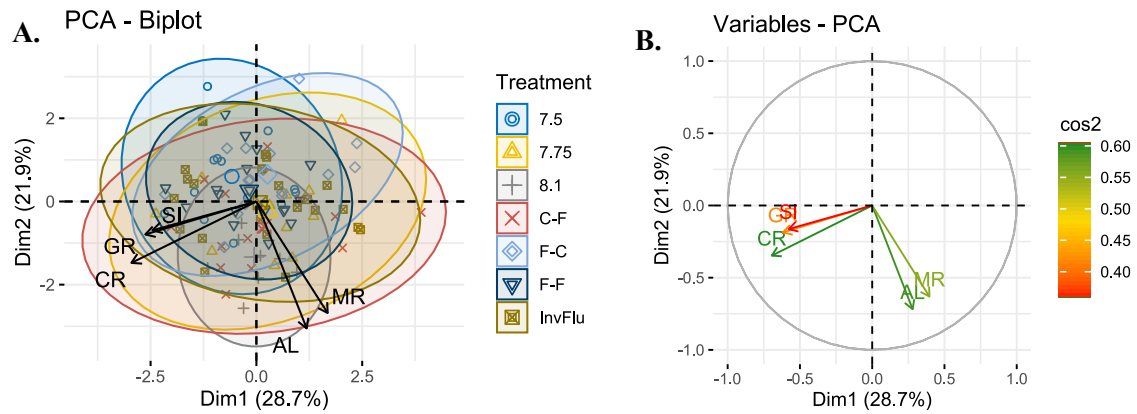


Fig. 6: ACP results over phase two according to dimensions 1 and 2, explaining respectively 28.7% and 21.9% of the total variance. **A.** Individual representation according to their respective treatment and studied responses. **B.** Cercle of correlations between the studied responses. CR = Calcification rate, GR = Growth rate, SI = Symmetry index, MR

positive growth rate and calcification values while phase two exhibited values close to zero. Phase two also revealed 1.25 times higher allometry values and 1.12 times higher mortality values compared to phase one (ANOVA III, Table A8, Fig.5, GR: $F_{1,281}$ -value = 24.290; p-value = <0.000, CR: $F_{1,249}$ -value = 143.800; p-value = <0.000, AL: $F_{1,247}$ -value = 32.550; p-value = <0.000, SI: $F_{1,254}$ -value = 0.035; p-value = 0.852, MR: $F_{1,169}$ -value = 4.512; p-value = 0.035). Analyses also revealed that Phase interacting with pH regime had a significant impact on allometry, as well as on growth rate when interacting with night and day shift (ANOVA III, Table A8, $P \times pH$ on AL: $F_{1,247}$ -value = 4.024; p-value = 0.008, $P \times N.DS$ on GR: $F_{1,281}$ -value = 16.520; p-value = <0.000). *Post hoc* test revealed that treatment C-F had 0.374 higher allometric values on phase two compared to phase one while treatment C-F exhibited no allometric differences between phases (*Post hoc* Tukey's test, Table A14, p-value < 0.05).

Results over phase one demonstrated that mortality rate was close to be significantly influenced by pH regime (ANOVA II, Table A9, MR: $F_{3,92}$ -value = 2.401; p-value = 0.073), with F-F treatment demonstrating a 0.112 lower value compared to constant 7.75 treatment (*Post hoc* Tukey's test, Table A13, p-value < 0.05).

Additionally, there were also significant differences between pH regimes on mortality and allometry on phase two (ANOVA II, Table A10, AL: $F_{3,137}$ -value = 14.460; p-value = <0.000, MR: $F_{3,73}$ -value = 3.740; p-value = 0.014). Precisely, treatments C-F and 7.75 revealed significant higher allometry values compared to F-F and F-C treatments - an increase of 0.325 and 0.446 respectively for treatment C-F and 0.215 and 0.386 units for treatment 7.75. (*Post hoc* Tukey's test, Table A13, p-value < 0.05). Furthermore, treatment F-C revealed a significant 0.139 lower mortality rate value compared to 7.75 treatment and treatment C-F revealed a 0.143 significant increase in mortality rate

compared to F-C treatment on phase two (*Post hoc* Tukey's test, Table A13, $p\text{-value} < 0.05$).

Additionally, growth rate and calcification rate revealed to be significantly influenced by night and day shift on phase one with higher values during the daytime compared to nighttime (ANOVA II, Table A9, GR: $F_{1,136}\text{-value} = 52.820$; $p\text{-value} = <0.000$, CR: $F_{1,109}\text{-value} = 4.118$; $p\text{-value} = 0.045$).

PCA analysis

PCA on the two first dimensions revealed an inertia percentage value of 50.6%. Growth rate, calcification rate and symmetry revealed to be related as the angles between each variable is small. Allometry has similar patterns of variation with mortality rate but revealed globally no interaction with calcification rate, growth rate and symmetry index as the angle between allometry and those variables are close to 90° (Fig 6A). Those results must be considered with caution since variables variations are explained at 60% maximum through $\cos^2$ parameter with symmetry index and calcification rate revealing poor quality of representation (Fig.6B). Other dimensions were also explored but revealed to be less pertinent and explained a poorer amount of the variable's variations (Annexes A6 and A7).

Discussions

Assessing allometry as an indicator of stress highlights the significance of natural fluctuations.

Allometry revealed to be significantly influenced by pH regime on all hypotheses on the second phase. This suggests that allometry is a sensitive and fast responding indicator. It's not surprising that the notable outcomes related to allometry emerged after seven days, as early on, individuals primarily rely on the resources supplied by their eggs. It's only once this initial phase passes that they begin to depend on the resources present in their environment. They then use their arms to catch organic particles in water on which they feed themselves, similarly to the larvae of the brittlestar *Ophiopholis aculeata* (Hart, 1991).

In addition, allometry was also correlated with mortality rate through PCA, meaning that allometry is a potential stress indicator. This last result must be taken with caution since PCA only represents two dimensions of the total points distribution, meaning that a 0° angle in two dimensions could become a 90° angle in three dimensions. However those results are consistent with the body of research demonstrating a significant effect of pH on echinoderms growth pattern (e.g Dorey et al., 2013; O'Donnell et al., 2010), though no pH effects have manifested on

growth rate in this study. Those observations highlight the necessity to take into account not just the growth rate but also the growth pattern. This broader perspective is essential for capturing the multifaceted OA issue on larval development.

Likewise, higher allometry, equivalent to longer arms compare to the total body length, usually means that individuals need more food and energy to cope with the stress their metabolism is facing (e.g. Podolsky & McAlister, 2005). Results on H1 revealed longer arms compared to body length for pH 8.0 followed by 7.75, fluctuant and then 7.5 treatment. Since *A. filiformis* larvae are used to face fluctuations in coastal environment, being exposed to a constant extreme high pH value could be associated with a higher level of stress, resulting in a higher allometry response. On the other hand, pH 7.5 resulted in what could have been first thought as counter-intuitive with low allometry values, though being an extreme low pH. However, this observation is consistent with a metabolic depression response, also associated with a stress. Metabolic depression involves a reduction in the larval metabolic rate, allowing for energy conservation in order to survive for an extended period of time under a stress (Padilla-Gamiño et al., 2013; Yuan et al., 2007). This provides individuals better chances to find suitable settling site or

favorable conditions. Metabolic depression has already been documented on *A. filiformis* adults where low constant 7.0 pH was associated with an 80% reduction in arms regeneration rate (Hu et al., 2014). Both those explanations are coherent with the intermediate allometry values obtained on pH 7.75 corresponding to the average pH value larvae are naturally facing in coastal environment and thus more able to cope with.

Additionally, fluctuant treatment reveals allometry values between treatment 7.5 and 7.75, and lower mortality rate value compared to constant 7.75 pH treatment (H1). This indicates that individuals raised in fluctuations are facing less stress during their development. Consequently, it might be more suitable to consider fluctuations as a more effective control. H2 also revealed lower allometry values for natural fluctuations compared to reversed cycle. These findings emphasize the importance of natural fluctuations over reverse fluctuations, demonstrating that the variations occurring in natural context should be taken into consideration when studying OA. A 2009 study, evaluating temperature fluctuations on the sea cucumber *Apostichopus japonicus*, demonstrated higher growth rate under temperature variations (An et al., 2009). Similarly, shortage or extend photoperiod of

light revealed gametogenesis delays on the sea urchin *Eucidaris tribuloides* (McClintock & Watts, 1990). All those observations highlight the importance to consider natural fluctuations in experiments. This involves that while constant pH has been used as control treatment through many different studies evaluating OA (Vargas et al., 2017), natural fluctuant treatment appears more appropriate for that purpose. This is particularly important if we want to understand the full extent of future OA effect on the biological responses of different species living on coastal environments, as indicated by Leung et al. (2022).

Intrinsic circadian cycle: pH sensitivity?

On all hypotheses, growth rate and calcification rate revealed a significant difference between the two phases. This is not surprising considering the logarithmic growth pattern of *A. filiformis* larvae, with the 7 first being an approximate linear growth followed by a stabilization around day 7 or 8. This implies that individuals do not experience significant growth beyond this time period.

In addition, all hypotheses revealed a clear tendency for a differential growth rate between night and day, even when individuals were raised in constant pH

treatment. This indicates the existence of an intrinsic circadian cycle. In particular, this circadian cycle is aligned with the most suitable conditions to grow. Algae photosynthesis during the day causes the reduction of $[\text{CO}_2]_{(\text{aq})}$, leading to the elevation of pH. Though it was been demonstrated that *A. filiformis* can adjust its metabolism to deal with bicarbonate levels up to 8 mmol L^{-1} when exposed to 1500 and 3200 $\mu\text{atm CO}_2$ (Stumpp & Hu, 2017), obtained results suggest that daytime is the most favorable period to activation metabolism. On the other hand, during the night, algae respiration takes place. This induces a $[\text{CO}_2]_{(\text{aq})}$ rise, resulting in the decrease of pH. This suggests that the pH conditions are less stressful during the day, providing individuals with more favorable conditions for growth. To our knowledge, this is the first time an experiment is conducted on pH variation.

Additionally, other parameters such as temperature and oxygen concentration (Baumann & Smith, 2018; Booth et al., 2021) show diel variations at coast environment and could also influence diel circadian cycle for the selection of most suitable conditions. Though not tested in this paper, within natural variation temperature range, daytime manifests higher temperatures, leading to a metabolic activity increase while nighttime presents

lower temperatures, associated with a decline in the metabolism activity. This involves that individuals can present a differential growth rate between night and day, as encountered with the sea cucumber *A. japonicus* (An et al., 2009). In order to test this hypothesis, we suggest a multi-stressor experimental design evaluating pH, temperature and other factors through fluctuations.

Furthermore, the absence of any effect on the calcification rate due to pH fluctuations between day and night can possibly be attributed to the distinct metabolic pathways employed during skeletogenesis and overall growth, leading to varying degrees of chemo-sensitivity. A study driven in 2014 on *A. filiformis* adults' arm regeneration revealed that, a total of 694 genes and 194 proteins were found undergoing differential expression during the initiation of regeneration process, divided into different processes such as cytoskeleton remodeling, cell adhesion integrin and translation initiation (Purushothaman et al., 2014). This involves that skeletogenesis and cell growth undergo different pathway, making them responsive to varying sets of factors.

Resilience and carryover effect

The fact that growth rate and symmetry index were not impacted reveals that *A. filiformis* might not be as sensitive as

previously thought. Calcification rate was only positively affected when exposed to reversed fluctuations. While this can be counter-intuitive, it is possible a stress indicator of hyper-calcification to compensate for the suboptimal conditions as reported in other studies (e.g Weerathunga et al., 2023).

In addition, multiple marine calcifiers have shown to be non-affected by pH (Leung et al., 2022). Fossil record also indicates that there has been a significant resilience in the durability of skeletons throughout geological history (Gold & Vermeij, 2023). This implies the presence of an inherent and enduring "deep resilience" in the stability of skeleton composition over the long term. Furthermore, there appears to be a positive correlation between skeletal mineral composition and a lower extinction risk over time (Gold & Vermeij, 2023; Murdock, 2020). It's important to highlight that each existing species has evolved from countless predecessors that successfully preserved their skeletons for hundreds of millions of years. This includes enduring through eras when the Earth experienced much higher temperatures than the present and the chemistry of the oceans was less favorable to the process of calcification (Scotese et al., 2021; Turchyn & DePaolo, 2019). Those observations align with our results, meaning calcification, symmetry and growth might

be highly conserved traits for *A. filiformis* development. However, a study driven on the same brittlestar larvae revealed a 100% decrease in growth rate between pH 8.0 and 7.7 (Chan et al., 2016). Those contrasting results might be the consequence of different experimental design and conditions such as sperm concentration and quality, pH stability, polyandry vs single male-female crosses, etc. The lack of impacts on symmetry has also been documented on Dupont & Thorndyke, (2008).

In addition, H3 revealed that individuals raised in fluctuant treatment after being exposed to constant conditions (C-F) had a significant higher allometry. On the other hand, F-C treatment exhibited no allometric differences between the two phases. Since allometry is considered a stress indicator, it is tempting to conclude on a canalization cost, as larvae did not manage to acclimate but suffered from the fluctuant conditions. pH regime C-F also exhibited no significant difference from pH 7.75 regarding mortality rate and allometry after the 14-day experiment. However, it did demonstrate higher values for these two responses compared to both the F-F and F-C treatments. This implies that it is difficult to conclude on either canalization cost or plasticity cost since none of the two tested treatments (C-F and F-C) were significantly

different from both 7.75 and F-F treatment at the end of the experiment.

Nonetheless, those results demonstrate a potential latent effect where larvae reared in constant conditions experienced the repercussions of this exposure after a specific duration, even when replaced in naturally fluctuant conditions. This phenomenon could be described as a time-dependent exposure effect where stressed individuals exposed to unfavorable conditions for a certain period of time will eventually suffer from irreversible damage as the maintenance of their metabolism is too expensive in term of energy (Dorey et al., 2013).

An alternative explanation would be that the stress experienced at embryos and early larval stage in constant conditions resulted in a carryover effect. This implies that, as individuals were raised in constant stressed conditions, they must face energetic constraints on the long term even when replaced in fluctuant treatment. In other words, impacts initiated in early life are exacerbated over time in the presence or absence of other stressors. It appears that carryover is particularly important when evaluating OA effects on echinoderms populations survival since its effects can arise within one generation, as revealed in this experiment, where individuals experiencing stress conditions at larval

stage can influence juvenile and adult success, or across a generation where environmental conditions experienced by one generation directly impact the success of the subsequent generation (Byrne, 2010, 2010; Munday, 2014). For instance, a study driven on *Ostrea lurida* from its planktonic larval form to metamorphosis into early juvenile life, revealed 41% decrease in larval shell growth rate when experiencing 7.8 pH values, even when replaced in control 8.0 pH (Hettinger et al., 2012).

It is worth noting that this phenomenon seems particularly associated with the stage at which the stress was applied since sensitivity differs between developmental phases (Dupont et al., 2010). This involves that it's difficult to dissociate the exact carryover effect from the influence of the development stage sensitivity.

We believe it would be interesting to conduct the same experiment on a longer time frame and at different larval stage over an entire lifespan to verify the existence of a time-exposure dependency, carryover effect and/or stage sensitivity. This would also allow to discriminate the real effect from the confounding factors linked to temporal affects -such as development and differential growth-, as pointing out by Dupont et al. (2010).

Conclusion

Ocean acidification driven by the emission of anthropogenic CO₂, has been a focal point of extensive research. While many early papers demonstrated drastic negative effects of decreased pH, there is a growing body of evidence that experiments need to integrate conditions met in natural environment, such as pH fluctuations to accurately evaluate future OA scenario. In this paper we evaluated the effect of diel pH fluctuations on the development of *Amphiura filiformis* larvae. Our results confirm the importance to consider natural fluctuations as it appeared that individuals raised in fluctuations demonstrated better allometric and survival responses. Diel analyses also underscored the existence of an intrinsic circadian cycle where individuals would grow more during daytime than nighttime, possibly explained by better conditions met during the day. Since pH did not affect the diel differences, we suggest that marine scientists should establish multi-stressor experiments with fluctuations on biological responses to evaluate which parameter might cause a defect on this circadian cycle.

Surprisingly, calcification rate, symmetry index and growth rate were not impacted by the lowering of pH. This could suggest a resilience to maintain those biological processes functioning though we could not

conclude on a potential canalization cost nor plasticity cost. Instead, it appears that there could be a carryover effect where individuals raised in stressed conditions at early stage suffered from this exposure even after being replaced in natural conditions. However, this effect is difficult to distinguish from a specific stage sensitivity or a time-exposure dependency. We suggest future experiments to evaluate biological responses through different time exposure and on different developmental stage to assess the different plausible adaptations to ocean acidification. This gap is a crucial point to investigate in order to understand the full extend of future OA scenarios and the evolutionary consequences it could have on different species.

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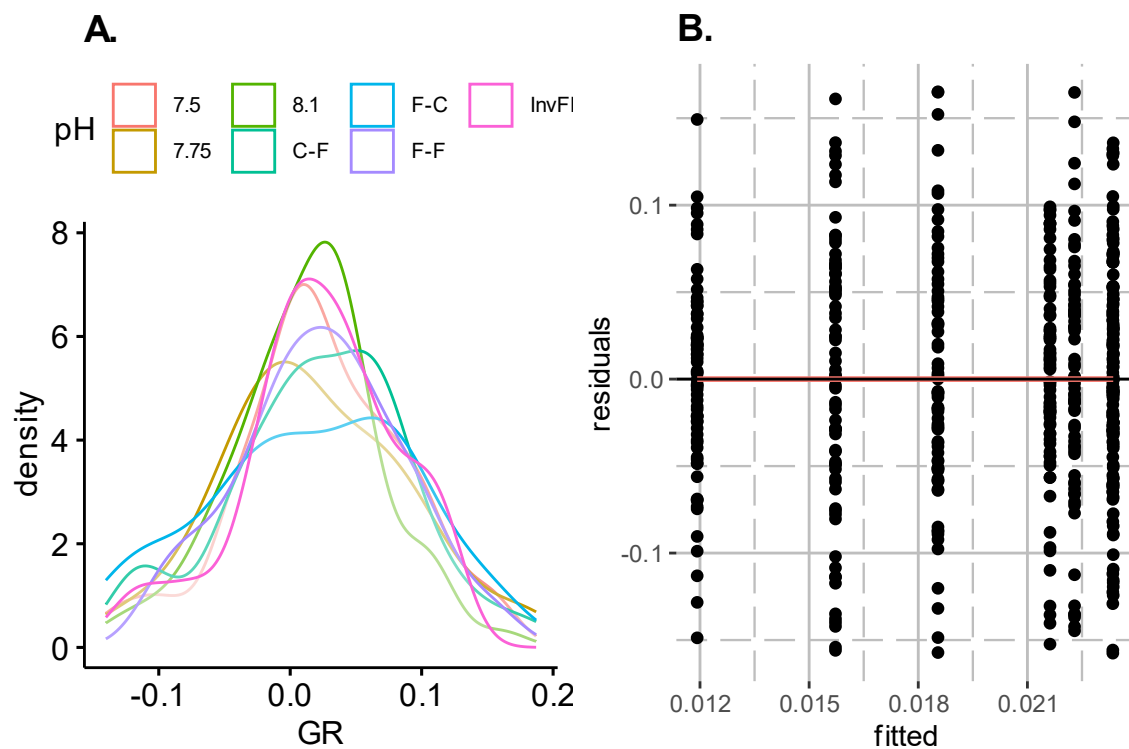
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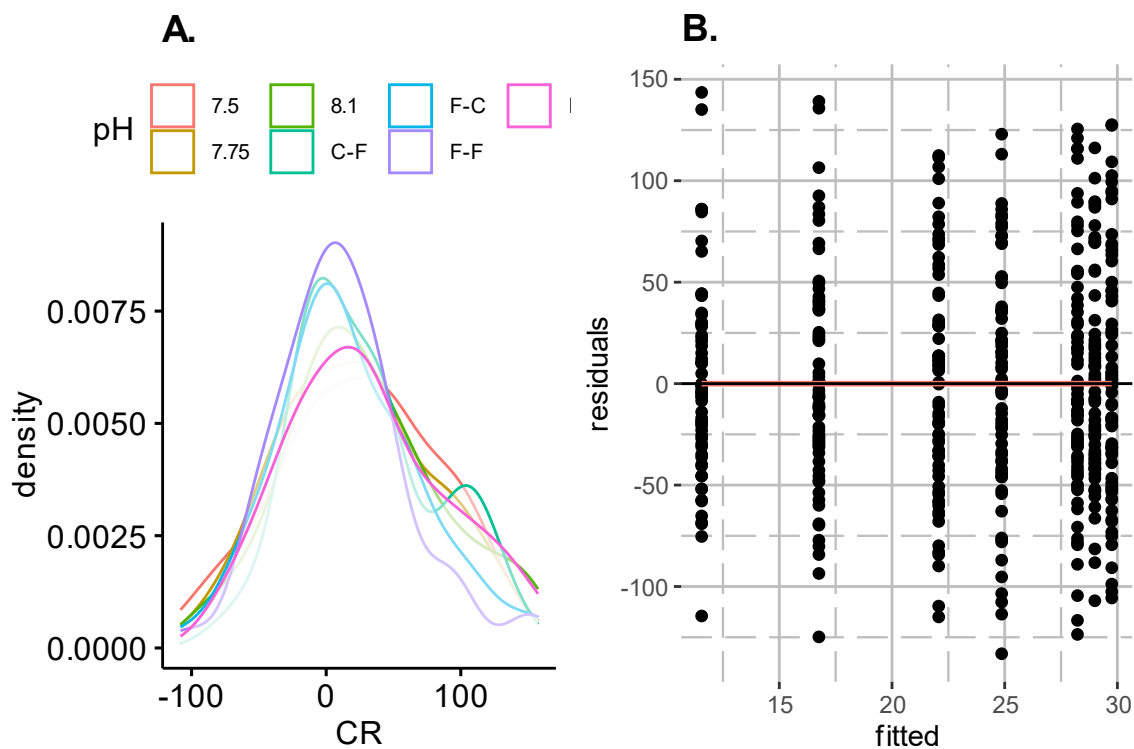
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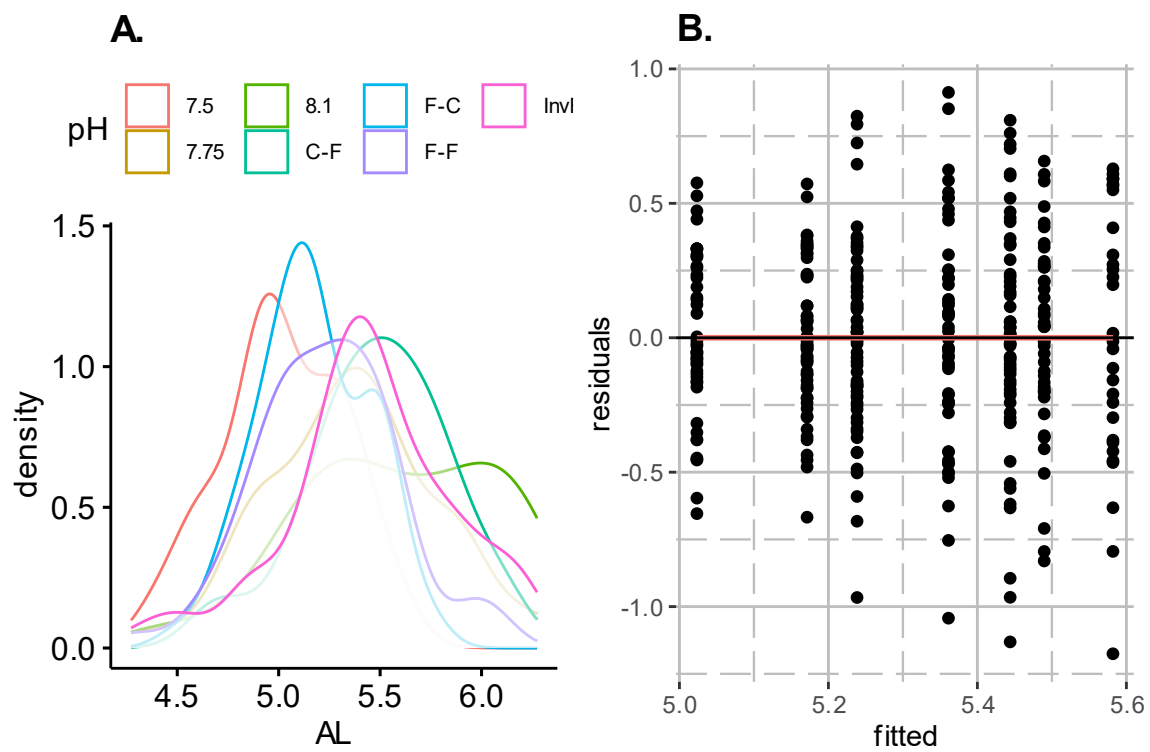
Annexes



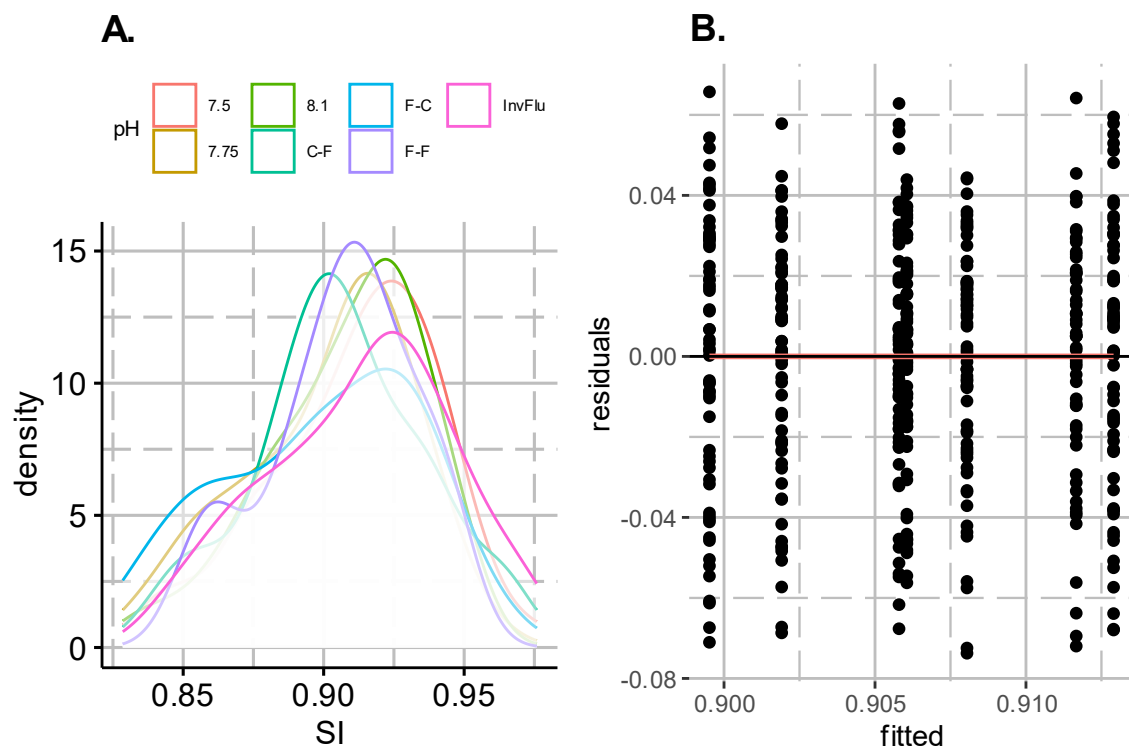
A1. A. Distribution of growth rate observations. **B.** Residuals versus fitted values distribution of growth rate.



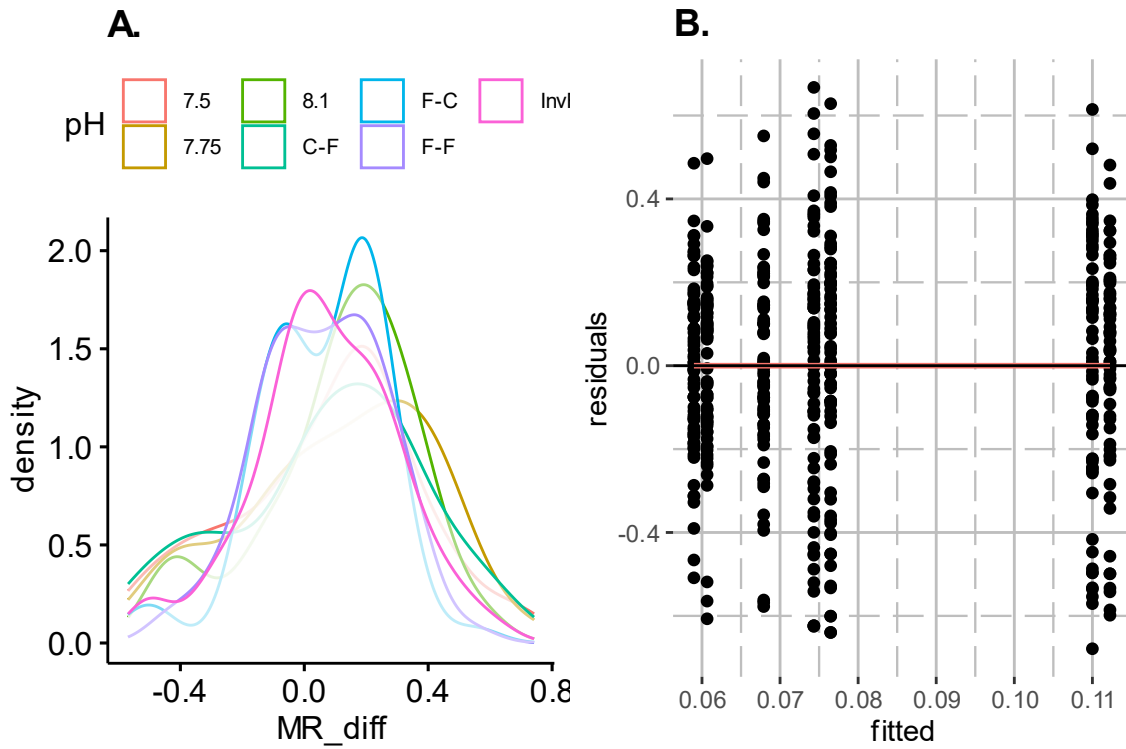
A2. A. Distribution of calcification rate observations. **B.** Residuals versus fitted values distribution of calcification rate.



A3. A. Distribution of allometry observations. B. Residuals versus fitted values distribution of allometry.



A4. A. Distribution of symmetry index observations. B. Residuals versus fitted values distribution of symmetry index.



A5. A. Distribution of mortality rate observations. **B.** Residuals versus fitted values distribution of mortality rate.

Table A1, Bartlett test results regarding homoscedasticity				
Parameter	Bartlett's K-squared	Df	Phase	p-value
GR	11.674	6	Both	0.0694
CR	4.0857	6	Both	0.6651
AL	20.733	6	Both	0.002049
AL	5.2264	6	P1	0.515123
AL	11.138	6	P2	0.08419884
SI	6.8988	6	Both	0.3303
MR_diff	28.597	6	Both	7.251e-05
MR_diff	15.955	6	P1	0.014
MR_diff	21.327	6	P2	0.001602
LMR_diff	19.68	6	Both	0.003157
QMR_diff	8.6811	6	Both	0.1923

<i>Table A2, H1 ANOVA III P1 & P2</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.353	277	3	0.353	0.787
	<i>Calcification rate</i>	10472	252	3	1.592	0.192
	<i>Allometry</i>	7.000	222	3	22.240	0.000
	<i>Symmetry index</i>	0.000	252	3	1.167	0.323
	<i>Mortality rate</i>	0.219	175	3	2.869	0.038
<i>Photoperiod</i>	<i>Growth rate</i>	0.063	277	1	18.435	0.000
	<i>Calcification rate</i>	3375	252	1	1.539	0.216
	<i>Allometry</i>	0.000	222	1	0.228	0.634
	<i>Symmetry index</i>	0.000	252	1	0.326	0.569
<i>Phase</i>	<i>Growth rate</i>	0.044	277	1	12.677	0.000
	<i>Calcification rate</i>	223861	252	1	102.1	0.000
	<i>Allometry</i>	4.000	222	1	38.990	0.000
	<i>Symmetry index</i>	0.000	252	1	0.100	0.752
	<i>Mortality rate</i>	0.101	175	1	3.978	0.048
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.020	277	3	1.985	0.116
	<i>Calcification rate</i>	1766	252	3	0.268	0.848
	<i>Allometry</i>	0.000	222	3	0.455	0.714
	<i>Symmetry index</i>	0.000	252	3	0.876	0.454
<i>pH:Phase</i>	<i>Growth rate</i>	0.009	277	3	0.857	0.464
	<i>Calcification rate</i>	13263	252	3	0. 2.02	0.112
	<i>Allometry</i>	3.000	222	3	9.892	0.000
	<i>Symmetry index</i>	0.000	252	3	0.500	0.683
	<i>Mortality rate</i>	0.083	175	3	1.087	0.356
<i>Photoperiod:Phase</i>	<i>Growth rate</i>	0.088	277	1	25.483	0.000
	<i>Calcification rate</i>	32.37	252	1	0.0147	0.903
	<i>Allometry</i>	0.000	222	1	0.514	0.474
	<i>Symmetry index</i>	0.000	252	1	0.455	0.501
<i>pH:Photoperiod:Phase</i>	<i>Growth rate</i>	0.007	277	3	0.650	0.583
	<i>Calcification rate</i>	6367	252	3	0. 0.968	0.408
	<i>Allometry</i>	0.000	222	3	0.221	0.882
	<i>Symmetry index</i>	0.000	252	3	0.244	0.866

<i>Table A3, H1 ANOVA II P1</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.006	129	3	0.367	0.777
	<i>Calcification rate</i>	15444	93	3	1.793	0.154
	<i>Allometry</i>	3.209	108	3	0.579	0.630
	<i>Symmetry index</i>	0.000	103	3	0.145	0.933
	<i>Mortality rate</i>	0.186	90	3	3.095	0.031
<i>Photoperiod</i>	<i>Growth rate</i>	0.182	129	1	44.919	0.000
	<i>Calcification rate</i>	833	93	1	0.290	0.591
	<i>Allometry</i>	4.135	108	1	2.307	0.132
	<i>Symmetry index</i>	0.000	103	1	0.018	0.894
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.024	129	3	2.003	0.117
	<i>Calcification rate</i>	1665	93	3	0.193	0.901
	<i>Allometry</i>	0.030	108	3	0.006	0.999
	<i>Symmetry index</i>	0.001	103	3	0.572	0.635

<i>Table A4, H1 ANOVA II P2</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.002	148	3	0.223	0.881
	<i>Calcification rate</i>	2892	159	3	0.537	0.658
	<i>Allometry</i>	22.380	114	3	31.710	0.000
	<i>Symmetry index</i>	0.005	149	3	1.440	0.234
	<i>Mortality rate</i>	0.115	85	3	1.237	0.302
<i>Photoperiod</i>	<i>Growth rate</i>	0.001	148	1	0.181	0.671
	<i>Calcification rate</i>	2679	159	1	1.491	0.224
	<i>Allometry</i>	0.111	114	1	0.768	0.383
	<i>Symmetry index</i>	0.000	149	1	0.624	0.431
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.011	148	3	1.110	0.347
	<i>Calcification rate</i>	7642	159	3	1.418	0.239
	<i>Allometry</i>	0.096	114	3	0.273	0.845
	<i>Symmetry index</i>	0.001	149	3	0.453	0.716

<i>Table A5, H2 ANOVA III P1 & P2</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.000	144	1	0.065	0.799
	<i>Calcification rate</i>	11920	126	1	5.912	0.016
	<i>Allometry</i>	0.777	132	1	6.573	0.012
	<i>Symmetry index</i>	0.000	131	1	2.120	0.148
	<i>Mortality rate</i>	0.000	85	1	0.002	0.965
<i>Photoperiod</i>	<i>Growth rate</i>	0.029	144	1	9.216	0.003
	<i>Calcification rate</i>	1091	126	1	0.541	0.463
	<i>Allometry</i>	0.005	132	1	0.043	0.836
	<i>Symmetry index</i>	0.000	131	1	0.783	0.378
				1		
<i>Phase</i>	<i>Growth rate</i>	0.045	144	1	14.240	0.000
	<i>Calcification rate</i>	98425	126	1	48.82	0.000
	<i>Allometry</i>	3.072	132	1	26.000	0.000
	<i>Symmetry index</i>	0.000	131	1	1.854	0.176
	<i>Mortality rate</i>	0.050	85	1	1.846	0.178
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.001	144	1	0.308	0.580
	<i>Calcification rate</i>	557.6	126	1	0.277	0.600
	<i>Allometry</i>	0.019	132	1	0.161	0.689
	<i>Symmetry index</i>	0.000	131	1	0.398	0.530
<i>pH:Phase</i>	<i>Growth rate</i>	0.001	144	1	0.235	0.629
	<i>Calcification rate</i>	12812	126	1	6.354	0.013
	<i>Allometry</i>	0.586	132	1	4.962	0.028
	<i>Symmetry index</i>	0.000	131	1	0.411	0.523
	<i>Mortality rate</i>	0.016	85	1	0.612	0.436
<i>Photoperiod:Phase</i>	<i>Growth rate</i>	0.021	144	1	6.591	0.011
	<i>Calcification rate</i>	170	126	1	0.084	0.772
	<i>Allometry</i>	0.179	132	1	1.517	0.221
	<i>Symmetry index</i>	0.000	131	1	0.420	0.518
<i>pH:Photoperiod:Phase</i>	<i>Growth rate</i>	0.000	144	1	0.124	0.725
	<i>Calcification rate</i>	2019	126	1	1.001	0.319
	<i>Allometry</i>	0.002	132	1	0.015	0.903
	<i>Symmetry index</i>	0.000	131	1	0.060	0.807

<i>Table A6, H2 ANOVA II P1</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.000	67	1	0.038	0.846
	<i>Calcification rate</i>	18199	46	1	5.108	0.029
	<i>Allometry</i>	0.007	56	1	0.004	0.951
	<i>Symmetry index</i>	0.002	54	1	2.173	0.146
	<i>Mortality rate</i>	0.007	44	1	0.342	0.562
<i>Photoperiod</i>	<i>Growth rate</i>	0.047	67	1	12.140	0.001
	<i>Calcification rate</i>	480.4	46	1	0.135	0.715
	<i>Allometry</i>	2.657	56	1	1.542	0.219
	<i>Symmetry index</i>	0.001	54	1	1.193	0.280
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.000	67	1	0.016	0.900
	<i>Calcification rate</i>	1848	46	1	0.519	0.475
	<i>Allometry</i>	0.067	56	1	0.039	0.845
	<i>Symmetry index</i>	0.000	54	1	0.389	0.536

<i>Table A7, H2 ANOVA II P2</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.001	77	1	0.343	0.560
	<i>Calcification rate</i>	11.02	80	1	0.010	0.922
	<i>Allometry</i>	1.961	76	1	20.360	0.000
	<i>Symmetry index</i>	0.000	77	1	0.344	0.559
	<i>Mortality rate</i>	0.009	41	1	0.282	0.599
<i>Photoperiod</i>	<i>Growth rate</i>	0.000	77	1	0.151	0.699
	<i>Calcification rate</i>	274.3	80	1	0.243	0.623
	<i>Allometry</i>	0.087	76	1	0.908	0.344
	<i>Symmetry index</i>	0.000	77	1	0.029	0.865
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.001	77	1	0.540	0.465
	<i>Calcification rate</i>	312	80	1	0.277	0.600
	<i>Allometry</i>	0.007	76	1	0.069	0.793
	<i>Symmetry index</i>	0.000	77	1	0.080	0.777

Table A8, H3 ANOVA III P1 & P2

Parameter	Response	Sum sq	Dfd	Dfn	F-value	p-value
<i>pH</i>	<i>Growth rate</i>	0.131	281	3	0.218	0.884
	<i>Calcification rate</i>	10194	249	3	1.958	0.121
	<i>Allometry</i>	2.000	247	3	7.088	0.000
	<i>Symmetry index</i>	0.000	254	3	0.597	0.618
	<i>Mortality rate</i>	0.309	169	3	4.318	0.006
<i>Photoperiod</i>	<i>Growth rate</i>	0.131	281	1	32.190	0.000
	<i>Calcification rate</i>	0.4018	249	1	0.000	0.988
	<i>Allometry</i>	0.000	247	1	0.386	0.535
	<i>Symmetry index</i>	0.000	254	1	0.352	0.554
<i>Phase</i>	<i>Growth rate</i>	0.131	281	1	24.290	0.000
	<i>Calcification rate</i>	249623	249	1	143.800	0.000
	<i>Allometry</i>	3.000	247	1	32.550	0.000
	<i>Symmetry index</i>	0.000	254	1	0.035	0.852
	<i>Mortality rate</i>	0.108	169	1	4.512	0.035
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.131	281	3	0.627	0.598
	<i>Calcification rate</i>	2924	249	3	0.562	0.641
	<i>Allometry</i>	0.000	247	3	0.411	0.745
	<i>Symmetry index</i>	0.000	254	3	1.582	0.194
<i>pH:Phase</i>	<i>Growth rate</i>	0.131	281	3	0.248	0.863
	<i>Calcification rate</i>	15261	249	3	2.931	0.034
	<i>Allometry</i>	1.000	247	3	4.024	0.008
	<i>Symmetry index</i>	0.000	254	3	1.992	0.116
	<i>Mortality rate</i>	0.145	254	3	2.017	0.114
<i>Photoperiod:Phase</i>	<i>Growth rate</i>	0.131	281	1	16.520	0.000
	<i>Calcification rate</i>	173.8	249	1	0.1001	0.752
	<i>Allometry</i>	0.000	247	1	0.008	0.927
	<i>Symmetry index</i>	0.000	254	1	1.344	0.248
<i>pH:Photoperiod:Phase</i>	<i>Growth rate</i>	0.131	281	3	1.214	0.305
	<i>Calcification rate</i>	7138	249	3	1.371	0.252
	<i>Allometry</i>	0.000	247	3	1.368	0.254
	<i>Symmetry index</i>	0.000	254	3	1.583	0.194

Table A9, H3 ANOVA II P1

Parameter	Response	Sum sq	Dfd	Dfn	F-value	p-value
<i>pH</i>	<i>Growth rate</i>	0.000	136	3	0.028	0.994
	<i>Calcification rate</i>	15702	93	3	2.013	0.118
	<i>Allometry</i>	0.194	110	3	0.037	0.990
	<i>Symmetry index</i>	0.002	106	3	1.011	0.391
	<i>Mortality rate</i>	0.156	92	3	2.401	0.073
<i>Photoperiod</i>	<i>Growth rate</i>	0.203	136	1	52.820	0.000
	<i>Calcification rate</i>	215.2	93	1	0.08276	0.774
	<i>Allometry</i>	4.642	110	1	2.667	0.105
	<i>Symmetry index</i>	0.001	106	1	1.022	0.314
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.000	136	3	0.028	0.994
	<i>Calcification rate</i>	6089	93	3	0.7806	0.508
	<i>Allometry</i>	0.026	110	3	0.005	1.000
	<i>Symmetry index</i>	0.001	106	3	0.347	0.792

Table A10, H3 ANOVA II P2

Parameter	Response	Sum sq	Dfd	Dfn	F-value	p-value
<i>pH</i>	<i>Growth rate</i>	0.005	153	3	0.392	0.759
	<i>Calcification rate</i>	3993	156	3	1.091	0.355
	<i>Allometry</i>	4.227	137	3	14.460	0.000
	<i>Symmetry index</i>	0.006	152	3	2.059	0.109
	<i>Mortality rate</i>	0.298	73	3	3.740	0.014
<i>Photoperiod</i>	<i>Growth rate</i>	0.006	153	1	1.347	0.248
	<i>Calcification rate</i>	66.34	156	1	0.054	0.816
	<i>Allometry</i>	0.030	137	1	0.309	0.579
	<i>Symmetry index</i>	0.000	152	1	0.141	0.707
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.018	153	3	1.400	0.245
	<i>Calcification rate</i>	2821	156	3	0.771	0.512
	<i>Allometry</i>	0.224	137	3	0.768	0.514
	<i>Symmetry index</i>	0.008	152	3	2.682	0.049

<i>Table A11, ANOVA II Phase 2- overall</i>						
<i>Parameter</i>	<i>Response</i>	<i>Sum sq</i>	<i>Dfd</i>	<i>Dfn</i>	<i>F-value</i>	<i>p-value</i>
<i>pH</i>	<i>Growth rate</i>	0.009	77	6	0.401	0.878
	<i>Calcification rate</i>	26451	80	6	0.721	0.633
	<i>Allometry</i>	15.020	76	6	26.710	0.000
	<i>Symmetry index</i>	0.009	77	6	1.567	0.157
	<i>Mortality rate</i>	0.414	41	6	2.297	0.038
<i>Photoperiod</i>	<i>Growth rate</i>	0.001	77	1	0.222	0.638
	<i>Calcification rate</i>	17423	80	1	2.847	0.093
	<i>Allometry</i>	0.068	76	1	0.725	0.396
	<i>Symmetry index</i>	0.001	77	1	0.688	0.408
<i>pH:Photoperiod</i>	<i>Growth rate</i>	0.033	77	6	1.506	0.176
	<i>Calcification rate</i>	34429	80	6	0.938	0.468
	<i>Allometry</i>	0.297	76	6	0.529	0.786
	<i>Symmetry index</i>	0.009	77	6	1.509	0.176

Table A12, Post hoc H1

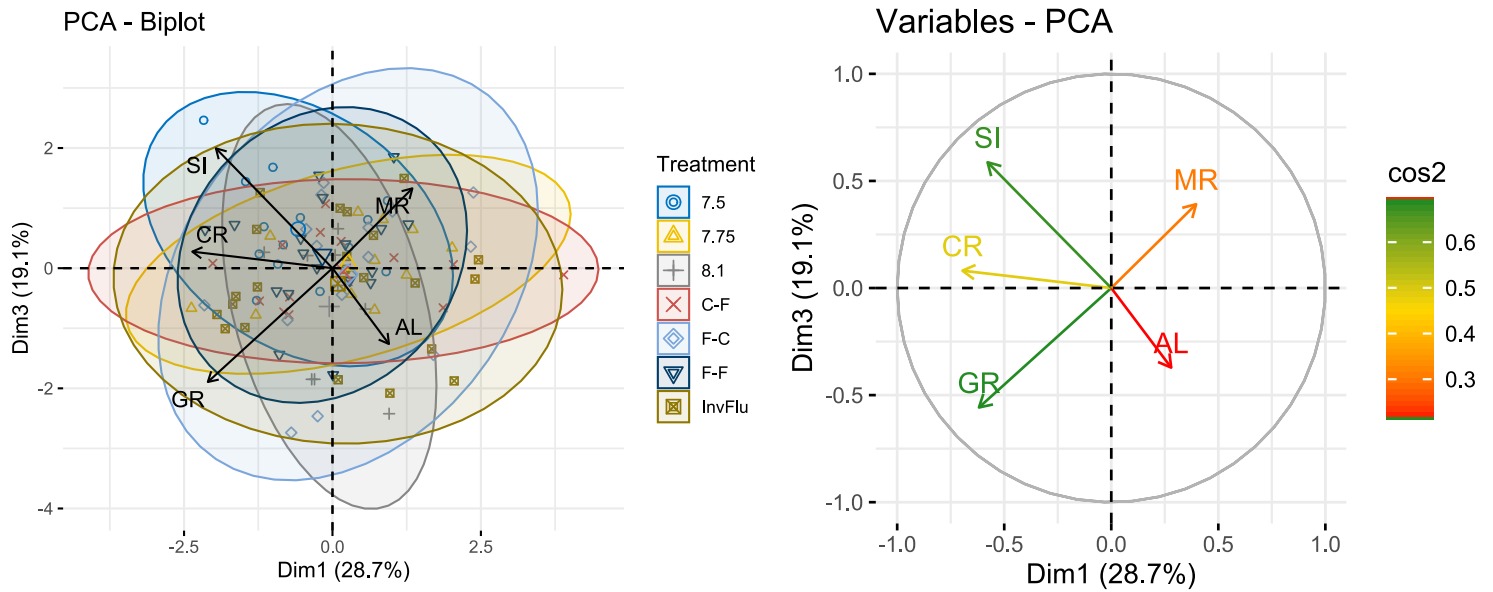
<i>Variable</i>	<i>Comparison</i>	<i>Phase 1</i>				<i>Phase 2</i>			
		<i>MORTALITY RATE</i>				<i>ALLOMETRY</i>			
		<i>Estimates</i>	<i>Conf.low</i>	<i>Conf.high</i>	<i>adj.pvalue</i>	<i>Estimates</i>	<i>Conf.low</i>	<i>Conf.high</i>	<i>adj.pvalue</i>
<i>pH</i>	7.75-7.5	0.067	-0.042	0.177	0.381	0.527	0.306	0.747	0.000
<i>pH</i>	8.1-7.5	0.049	-0.062	0.159	0.657	1.093	0.783	1.404	0.000
<i>pH</i>	F-F-7.5	-0.045	-0.154	0.063	0.696	0.311	0.103	0.520	0.001
<i>pH</i>	8.1-7.75	-0.018	-0.127	0.090	0.971	0.567	0.264	0.869	0.000
<i>pH</i>	F-F-7.75	-0.112	-0.218	-0.006	0.034	-0.215	-0.411	-0.019	0.025
<i>pH</i>	F-F-8.1	-0.094	-0.201	0.013	0.107	-0.782	-1.076	-0.488	0.000
<i>Photoperiod</i>	Night-Day					0.053	-0.068	0.175	0.386

Table A13, Post hoc H3

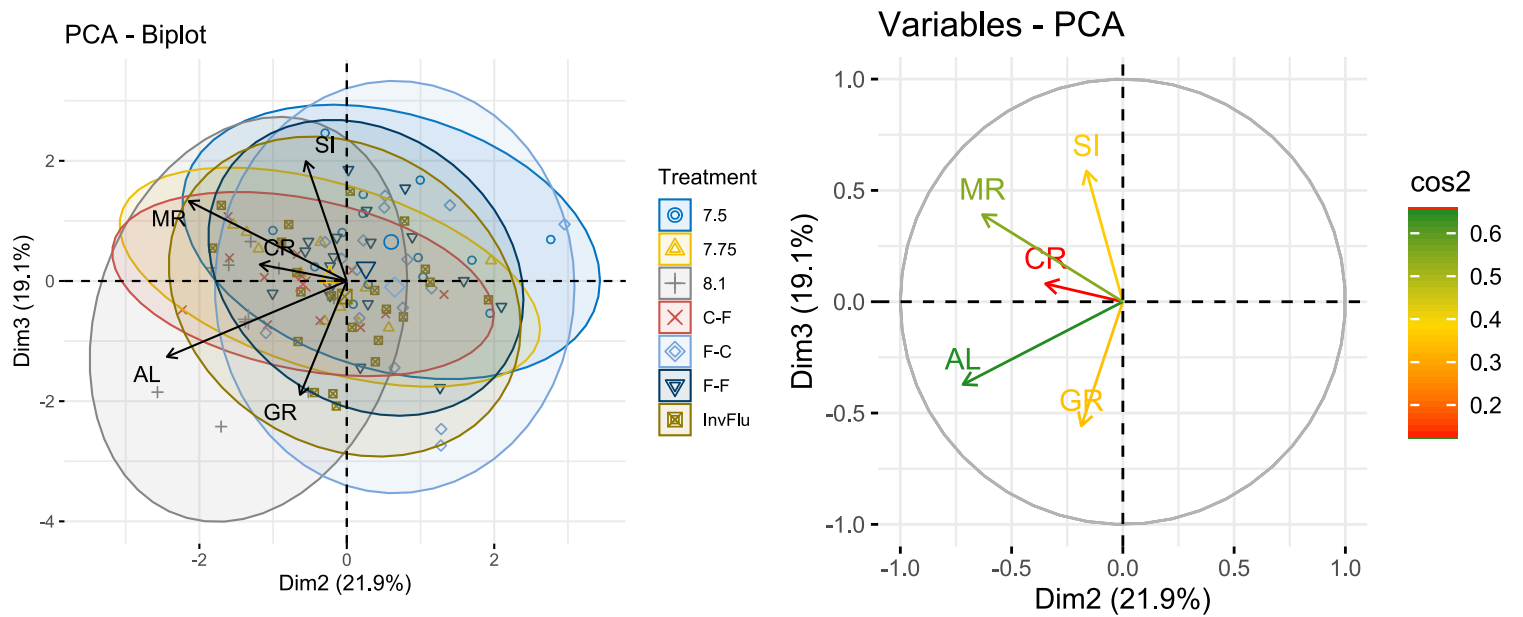
		Phase 1				Phase 2							
		MORTALITY RATE				ALLOMETRY				MORTALITY RATE			
Variable	Comparaison	Estimates	Conf.low	Conf.high	adj.pvalue	Estimates	Conf.low	Conf.high	adj.pvalue	Estimates	Conf.low	Conf.high	adj.pvalue
pH	C-F-7.75	-0.063	-0.173	0.047	0.450	0.110	-0.097	0.316	0.512	0.004	-0.130	0.138	1.000
pH	F-C-7.75	-0.051	-0.164	0.063	0.651	-0.336	-0.530	-0.142	0.000	-0.139	-0.275	-0.004	0.041
pH	F-F-7.75	-0.112	-0.222	-0.002	0.044	-0.215	-0.406	-0.025	0.020	-0.083	-0.222	0.055	0.397
pH	F-C-C-F	0.012	-0.101	0.125	0.992	-0.446	-0.643	-0.249	0.000	-0.143	-0.274	-0.012	0.026
pH	F-F-C-F	-0.050	-0.159	0.059	0.632	-0.325	-0.519	-0.131	0.000	-0.087	-0.221	0.047	0.328
pH	F-F-F-C	-0.062	-0.174	0.051	0.482	0.121	-0.060	0.301	0.307	0.056	-0.080	0.191	0.701
Photoperiod	Night-Day					0.029	-0.074	0.132	0.580				

Table A14, Post hoc H3 (continued)

		Phase 1 & 2							
		ALLOMETRY				MORTALITY RATE			
Variable	Comparaison	Estimates	Conf.low	Conf.high	adj.pvalue	Estimates	Conf.low	Conf.high	adj.pvalue
pHxPhase	C-F:Phase 2-C-F:Phase 1	0.374	0.085	0.663	0.003	0.113	-0.026	0.251	0.205
pHxPhase	F-C:Phase 2-F-C:Phase 1	0.037	-0.229	0.304	1.000	-0.042	-0.187	0.102	0.986



A6: ACP results over phase two according to dimensions 1 and 3, explaining respectively 28.7% and 19.1% of the total variance. **A.** Individual representation according to their respective treatment and studied responses. **B.** Cercle of correlations between the studied responses. CR = Calcification rate, GR = Growth rate, SI = Symmetry index, MR = Mortality rate, AL = Allometry



A7: ACP results over phase two according to dimensions 2 and 3, explaining respectively 21.9% and 19.1% of the total variance. **A.** Individual representation according to their respective treatment and studied responses. **B.** Cercle of correlations between the studied responses. CR = Calcification rate, GR = Growth rate, SI = Symmetry index, MR = Mortality rate, AL = Allometry