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Trophic environments influence size at metamorphosis and recruitment performance of Pacific oysters

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Abstract :

Reproduction and recruitment of benthic invertebrates are influenced by the climate and by the ecological structure of marine ecosystems, along with local anthropogenic pressures such as eutrophication or oligotrophication. Using the Pacific oyster Crassostrea gigas as a biological model, we tested the hypothesis that the variability in prodissoconch II (PII) size (i.e. size at metamorphosis) depends on ecological functioning. Settlement and recruitment were assessed at 5 sampling sites on the French Mediterranean shellfish farmed Thau lagoon during the main summer recruitment events in 3 consecutive years (2012-2014). Hydrobiological and planktonic analyses were conducted at 3 sampling sites. Our results showed that recruitment was extremely heterogeneous, ranging from 0 to 260 ± 27 SE ind. dm⁻² throughout the ecosystem and was linked with variability in PII size, which ranged from 180 to 296 µm. The annual temporal pattern of PII sizes appeared to be controlled by temperature during the settlement period, whereas the spatial pattern depended on phytoplankton biomass and on the trophic functioning of the ecosystem. Smaller PII sizes were significantly correlated with the highest phytoplankton biomass, while larger PII sizes were positively correlated with mixotrophic cryptophyte abundance. We found an inverse relationship between PII size and survival after metamorphosis, showing that recruitment success was associated with smaller PII sizes. Regional climate conditions and local trophic functioning appear to be key factors in metamorphosis and consequently contribute to recruitment heterogeneity. Further studies should be performed in other ecosystems following an oligotrophication trajectory to generalize this result.

Keywords : Larval ecology, Recruitment, Prodissoconch II, Crassostrea gigas, Oligotrophication, Cryptophytes, Thau lagoon
INTRODUCTION

While the impacts of eutrophication were a preoccupation in the 2000s (Nixon 1995, De Jonge & Elliott 2001, Smith & Schindler 2009), increased demand for the recovery of ecosystem services (Bullock et al. 2011) and good environmental status (Vethaak et al. 2017) have raised new questions about the repercussions of oligotrophication on community structure (Lie et al. 2011, Saeck et al. 2013) and about the function of some coastal ecosystems (Jeppesen et al. 1998, 2005, Duarte et al. 2009, Yanagi 2015). French Mediterranean lagoons (Collos et al. 2009, Leruste et al. 2016), like some Japanese shellfish farmed estuaries and bays (Yanagi 2015), are subject to oligotrophication processes caused by wastewater management. A decrease in nutrient inputs into Mediterranean lagoons led to a decrease in phytoplankton biomass and hence to a shift in the structure of phytoplankton community from diatoms, cryptophytes and green algae to mixotrophic dinophytes (Gowen et al. 2015, Leruste et al. 2016), toxic dinoflagellates and picocyanobacteria (Collos et al. 2009). Changes in the ecological structure or ecological function of phytoplankton communities may have consequences for the food web (Alvarez-Cobelas & Rojo 2000, Cloern 2001). A reduction in phytoplankton biomass could cause major problems and could jeopardize the carrying capacities of coastal ecosystems that are exploited for shellfish farming, like in the Thau lagoon and hence their sustainability (Dame 2012).

We suggest that these impacts may be accentuated in the first development stages of bivalves, as larvae and newly settled juveniles have been widely shown to be the most susceptible to stressful conditions (Qiu et al. 2002, Rayssac et al. 2010, Jenewein & Gosselin 2013). Their susceptibility can be expressed in many ways, but is usually reflected in growth and survival. In the Pacific oyster *Crassostrea gigas*, newly referred to as *Magallana gigas*, a planktotrophic stage corresponds to a pelagic free-swimming period during which the veliger larvae feed, grow and
disperse via water currents generally for a period of between two and four weeks (Kennish et al. 1995, Bhaud 2000). Near the end of its planktonic period, the advanced veliger develop competent characteristics, like feet for territorial prospecting behavior (pediveliger stage) and become able to settle (Pechenik & Heyman 1987, Hadfield et al. 2001) followed by metamorphosis, when they are transformed into post larval benthic mollusks (Zardus & Martel 2002, Bishop et al. 2006, Pechenik 2006). In marine invertebrates, metamorphosis integrated early ontogenic factors and environmental selection (Bishop et al. 2006) and is generally achieved in oysters within 1-3 days (Baker & Mann 1998). In bivalves, metamorphosis is defined as the morphological change associated with the attachment of the individual to a substrate and the secretion of a dissoconch shell (Bayne 1976). Metamorphosis involves four stages, 1) attachment to the substrate by the foot, 2) a change in the particle collecting structure from velum to completely developed heterorhabdic gills (Cannuel & Beninger 2006), 3) shell growth beyond the prodissoconch 4) loss of all larval organs to complete metamorphosis into a fully developed juvenile (Baker & Mann 1994). Metamorphosis is triggered by chemical and/or physical cues when conditions allowing the morphological transformation are favorable, e.g. a suitable habitat, thermal and hyaline requirements, the quantity and quality of available food (Pechenik 1990, Bishop et al. 2006, Toupoint et al. 2012). The lack of settlement/metamorphosis cues may delay metamorphosis, thereby prolonging the larval stage until a suitable environment is found (Coon et al. 1990, Pechenik 2006). Thus, size at metamorphosis estimated by the prodissoconch II (PII) measurements can be highly variable (Coon et al. 1990, Martel et al. 1995, 2014). Although significant research has been conducted on Mytilus edulis and suggests that trophic factors affect settlement/size at metamorphosis (Martel et al. 2014), to date, no study has been conducted on oysters, one of the most economically important groups of invertebrates. The
purpose of our study was thus to test the hypothesis that the size of *C. gigas* at metamorphosis is linked to specific environmental/trophic conditions that may affect the success of subsequent recruitment. The Thau lagoon was selected to test this hypothesis because of its high temporal and spatial ecological heterogeneity that affect oyster recruitment (Lagarde et al. 2017). The specific objective of this study was to characterize the spatial and temporal variability of oyster spat recruitment to (i) test the existence of variation of PII size in the Thau lagoon, (ii) evaluate the relationship between this variation and environmental and/or trophic conditions and (iii) assess its impact on recruitment success. The originality of this study is the simultaneous monitoring of larvae, juveniles (abundance and survival), size at metamorphosis, hydrobiological conditions and planktonic characteristics in the same species in three different years (2012-2014) at five different sampling sites.

**MATERIALS AND METHODS**

**Thau lagoon and sampling sites**

The Thau lagoon is the largest nanotidal lagoon in the Occitanie region (formerly known as Languedoc-Roussillon) in southern France. It covers an area of 7,500 ha (19 km × 4.5 km) orientated along a north-east / southwest axis and has a mean depth of 3.5 m. Seawater from the Mediterranean Sea enters through narrow channels. Five sampling sites were monitored to assess pre-settled oyster larvae and post-settled spat abundances in pelagic and benthic habitats: two sites with collectors suspended inside shellfish farming structures (Bouzigues and Marseillan) and three with collectors suspended outside structures anchored on specially designed mooring systems (Listel, Meze_osfz and Balaruc) (Figure 1). Hydrological and planktonic data were monitored at the three sampling sites Bouzigues, Marseillan and Listel (Figure 1).
Abundance of young settlers and survival rate at metamorphosis

As reported by Lagarde et al. (2017), young oyster abundances were estimated every two weeks at three different settler stages: pre-settled larvae (pediveliger), young postlarvae and newly settled juveniles (Arakawa 1990) from June to September in 2012, 2013 and 2014. An original method with a temporal overlap of collector deployment was used (Hughes et al. 2000, Arnold & Steneck 2011, Lagarde et al. 2017). Pediveligers showed prodissococonch 2 (PII) shell sizes ranging from 180 to 300 μm, young postlarvae were strictly benthic and cemented with a dissoconch shell ranging from 300 to 1,000 μm and newly settled juveniles (maximum four weeks old) measured from 1 to 8 mm. To collect these settler stages, the sites were equipped with three replicated sets of two collectors each measuring 110 cm composed of 44 white plates (15 cm diameter; surface area 250 cm², Figure 2a).

The collectors were vertically submerged 2 m below the surface, suspended inside shellfish farming structures, or outside farming structures (see Figure 2(a) in Lagarde et al. (2017) for more details). The systems were designed to support two sets of collectors immersed for two or four weeks. Pediveliger and post larva abundance was assessed on the 2-week collectors and oyster juvenile abundance was assessed on the 4-week collectors. After being sampled, the 4-week collector was replaced with a new one, i.e. a replacement every two weeks throughout the summer. Each collector was sampled at three vertical levels: close to the top (39th plate), in the middle (22th plate) and at the bottom (5th plate). Both faces of the plates (above and below) were examined under a binocular microscope to assess the mean abundance of pediveliger, post-larvae and newly settled juveniles, or macroscopically when possible. On each occasion, counting was evaluated on subunits, i.e. from one to four basic subunits (15 to 60 cm³), replicated three times per face (Figure 2b). The abundance of each stage per plate was averaged from subunit counts.
and converted into total individual abundances per plate, and then expressed in relation to the sampling surface per dm². Survival rates at metamorphosis corresponded to the ratio of the abundance of oyster juvenile survivors on the collectors immersed for four weeks divided by the pediveliger abundance on the collectors immersed for two weeks.

**Prodissoconch II size**

To assess prodissoconch II (PII) size, 10 postlarvae per plate were removed from three plates (at the top, middle and bottom of the collectors) and placed side by side on the microscope blade to obtain an optimal quantity of 30 measurements per site and per year for the main recruitment event in 2012, 2013 and 2014. Postlarvae were individually detached from their plate, placed and oriented on the plasticine flange of a holding blade (Martel et al. 2014) of an Olympus SZ61 binocular microscope. A digital microscope Keyence, VHX 2000 (200-300× magnification and set in high dynamic range mode with light shifts) was used to measure PII sizes. Based on a lateral view, PII heights were measured in accordance with a maximum dorsoventral dimension from the umbo to the most distant part of the clear demarcation formed by a growth line at the boundary layer between PII and dissoconch (Figure 3).

**Environmental measurements**

Hydrological and plankton samples were collected every Monday morning from June 1 to the September 30 in 2012, 2013 and 2014. Samples were collected at Listel and Mèze sites. Bouzigues and Marseillan sites were located less than 1 km from where environmental measurements are permanently monitored (REPHY – French Observation and Monitoring program for Phytoplankton and Hydrology 2017). For Bouzigues and Marseillan, hydrological and plankton samples associated with juvenile sampling sites were in biocoenosis, characterized
by the same phytoplankton, zooplankton and benthic populations (Jarry et al. 1990, Jouffre et al. 1991, Guelorget et al. 1994).

Temperature and salinity were measured twice a week with WTW® probes positioned between 1 and 1.5 m below the surface. Oxygen concentrations were measured once a week at the bottom of the water column at the Bouzigues site..

The three sampling campaigns provided a total of 135 observations of plankton characteristics: 3 sampling sites x 15 weeks x 3 years. The Bouzigues, Meze_osfz and Marseillan sites were sampled in 2012 and the Bouzigues, Listel and Marseillan sites were sampled in 2013 and 2014.

Each phytoplankton and protozooplankton sample was collected at each sampling site and date as subsamples of a 4 liter sample. Samples were collected weekly using a Standard Water Sampler acc. to Ruttner (Hydro-Bios Apparatebau GmbH) without analytical replicates or sampling replicates.

For total chlorophyll a [Total Chl. a] measurements, seawater samples (200 mL) were filtered (Bec et al. 2005, 2011) under vacuum (<10 cm Hg) on Whatman GF/F membranes (0.7 μm porosity) and stored in glass tubes at –20 °C. The filters were ground in 90% acetone and extracted for 24 h at 4 °C in the dark. Chlorophyll a biomass was also determined after size fractioning (200 ml for picophytoplankton and 200 ml for nanophytoplankton from the 4 l sample) through Nuclepore membranes (3 and 20 μm, respectively) to determine the contribution of picophytoplankton (PICO, < 3 μm), nanophytoplankton (NANO, 3-20 μm) and microphytoplankton (MICRO, > 20 μm) to total phytoplankton biomass. The pigment content (µgChla l⁻¹) was measured using a spectrofluorometer (Perkin-Elmer LS50b)(Neveux & Lantoine 1993).

The abundances of picocyanobacteria (CYAN, < 1 μm), autotrophic picoeukaryotes (PEUK, < 3 μm) and nanophytoplankton (NANO TOT, 3-20 μm) were measured on the basis of a sampled
volume of 1 ml with a Becton Dickinson FACSCalibur flow cytometer (Bec et al. 2011). Total picophytoplankton (PICO TOT) abundances were estimated as the sum of the CYAN and PEUK abundances. Among nanophytoplankton, Cryptophytes (CRYPTO) were distinguishable from other photosynthetic organisms (NANO) by their strong orange fluorescence associated with phycoerythrin pigment and their size. The abundances are expressed as $10^6$ cells l$^{-1}$.

To measure bacterial abundance, samples (1 ml taken from the 4 l sample) were fixed with prefiltered (0.2 µm) buffered formaldehyde (final concentration 2%) and stored in liquid nitrogen. The abundances were determined using the Becton Dickinson method with a FACSCalibur flow cytometer (Marie et al. 1997). The procedure was slightly modified as higher concentrations of fluorochrome (SYBR Green I) were used (Bouvy et al. 2016). One milliliter of fixed samples was incubated with SYBR Green I (Molecular Probes) at a final concentration of 1/375 for 15 min at 4 °C in the dark. Stained bacterial cells excited at 488 nm were determined according to their side-scattered light (SSC) and green fluorescence (FL1) and collected using a 530/30 nm filter. Fluorescent beads (0.94 µm, Polysciences Inc.) were added to each sample.

The taxonomic composition of protozooplankton was estimated (heterotrophic flagellates in a 30 ml sample and ciliates in a 100 ml sample) and phytoplankton (in a 10 ml sample) using the standard Utermohl method NF-EN-152014, 2006. For the phytoplankton, two main classes, “Diatoms” (aggregating 52 taxa) and “Dinoflagellates” (aggregating 38 taxa) were first used to explore the dataset. Taxonomic composition is expressed as the number of individuals per liter.

The taxonomic composition of phytoplankton was not monitored in 2012 at the “Meze_osfz” sampling site. For analysis of heterotrophic flagellates (HF), the 30 ml samples were preserved in an 8% formaldehyde solution and stored in a cold room at 4 °C in the dark until analysis. A 10 ml subsample was stained using 4’,6-diamidino-2-phenylindole (DAPI) at a final concentration
of 2.5 µg ml⁻¹. The HF counts were performed using an epifluorescence microscope (Olympus AX70) with UV illumination (Sherr et al. 1993). For the determination of naked ciliates and tintinnids, 100 ml samples were preserved in 2% Lugol’s iodine solution and kept in a cold room at 4 °C in the dark until analysis. Naked ciliates and tintinnids were identified, measured and counted with an inverted microscope (Olympus IX70) after a 100 ml sample was left to settle in an Utermöhl chamber for 24 h (Utermöhl 1931).

**Data analysis**

Data analyses and graphics were performed with R statistical software (R Core Team 2015). Decimal logarithm transformations were used to tend towards linearity of response variables and to linearize the relationship with explanatory variables (Table 1). The environmental data from our three annual hydrobiological and plankton survey were averaged over a 14 day period before the retrieval of the collector during the main recruitment event.

The main recruitment events were identified each year by considering the highest oyster juvenile abundances observed at the different sampling sites. A non-parametric one way ANOVA (Kruskall test, ‘pgirmess’ package) was performed using data pooled for each date and associated with a multiple comparison test of Siegel and Castellan (Siegel & Castellan 1988) to target the best sampling period for oyster recruitment at each sampling site each year. Oyster juvenile abundance was graphically described using comparison of means with standard error intervals.

A two-way cross Permanova (Permanova-Primer-E7.0.12 Permanova Plus; Primer-E Ltd, Plymouth, UK) was performed to compare PII size measured during the main oyster recruitment events among sampling sites (5 fixed levels: Marseillan, Listel, Meze_osfz, Bouzigues or Balaruc), year (3 fixed levels: 2012, 2013 and 2014) and their interaction (Site x Year).
Resemblance matrixes were calculated on Bray-Curtis distances. Permanova was used rather than ANOVA because of non-orthogonality of our larval settler databases. This approach using similarities, like ANOVA, is more accurate than non-parametric analysis, because it uses the permutation method (9999 permutations). Homoscedasticity was verified by using the Permdisp test (Anderson et al. 2008).

A graphical representation of PII measurements and environmental variables was made with a principal component analysis (PCA) with the factor ‘year and sampling site’ (Lê et al. 2008). This PCA was used to explore and illustrate the heterogeneity of the three sampling sites according to the year and interactions between the PII sizes and their environment. The means of PII sizes were calculated using individuals from the collector replicates (1 collector is represented by 3 replicates of plates, each replicate is intended to optimally represent about 30 PII measurements). Each average PII size was compared with the averages of the environmental data acquired over the two weeks prior to the retrieval of the collector at the site. The broken stick model and Kaiser criterion were used to compare the eigenvalues and performed to interpret axes whose eigenvalues were higher than mean of all eigenvalues (Borcard et al. 2011, Oksanen et al. 2015). Linear models were used to fit regression and single stratum analysis of variance was performed following assumptions of random sampling, homoscedasticity (tested and confirmed with the Fligner-Killeen test) and normality (verified with Shapiro–Wilk tests), respectively to test the influence of year, temperature and survival at metamorphosis on the means of PII sizes.
RESULTS

Oyster spat abundance and survival after metamorphosis

In 2012, two significant recruitment events were characterized (Figure 4). The first harvest date (August 13) was characterized by a juvenile abundance of 45 ± 4 ind. dm\(^{-2}\) at Listel and of 8 ± 1 ind. dm\(^{-2}\) at Bouzigues. The second significant recruitment event (September 25) was characterized by 14 ± 2 ind. dm\(^{-2}\) at Bouzigues, 12 ± 2 ind. dm\(^{-2}\) at Balaruc and 7 ± 1 ind. dm\(^{-2}\) at Listel. In 2013, the highest oyster juvenile abundances were recorded on August 13 and August 28 (Figure 4) highlighting excellent recruitment at the Meze_osfz sampling site with a density of 68 ± 17 ind. dm\(^{-2}\) and 92 ± 17 ind. dm\(^{-2}\). On August 28, the Listel site had an average juvenile oyster abundance of 32 ± 5 ind. dm\(^{-2}\) harvested, and a high juvenile abundance was also recorded at Meze_osfz on October 9, 2013 (172 ± 19 ind. dm\(^{-2}\)). The 2014 profile revealed that the recruitment period lasted two months (Figure 4) with four consecutive recruitment events (August 13 and 27, and September 10 and 24). The Meze_osfz site showed remarkable juvenile abundances (75 ± 6 ind. dm\(^{-2}\), 260 ± 27 ind. dm\(^{-2}\), 73 ± 18 ind. dm\(^{-2}\), 30 ± 6 ind. dm\(^{-2}\)) during these four consecutive events. The Listel site showed the same range of juvenile abundance as in 2012 and 2013 with 24 ± 6 ind. dm\(^{-2}\) on August 27 and 39 ± 11 ind. dm\(^{-2}\) on September 10. In 2014, it should be noted that experimental stations in the shellfish aquaculture zones (Bouzigues, Meze_osfz and Marseillan) had, on average, extremely low recruitment levels of between 0 and 2 ind. dm\(^{-2}\). Taken together, these results showed that the main oyster recruitment events occurred on August 13 in 2012, August 28 in 2013 and August 27 in 2014 (Figure 4).

Prodissoconch II size

PII size was determined during the main oyster recruitment event each year, i.e. on August 13, 2012, August 28, 2013 and August 27, 2014 (Figure 4). Permanova revealed a significant year
and site interaction effect ($df_{year \times site} = 7$, $df_{total} = 404$, Pseudo-$F = 3.99$, $p < 0.001$). In 2012, the pairwise Permanova showed significant differences in PII size depending on the site, with larger PII size (mean ± se) at the Marseillan site ($p < 0.03$; $247.0 \pm 5.2 \, \mu m$, $n=12$) and smaller PII size at Balaruc ($p < 0.02$; $213.7 \pm 4.1 \, \mu m$, $n=31$). In 2013, four size categories were observed (Figure 5), the largest PII size at Marseillan ($p < 0.003$; $270.7 \mu m \pm 3.3 \mu m$, $n=35$) and the smallest at Meze_osfz ($p < 0.01$; $222.1 \pm 3.4 \mu m$, $n=35$). In 2014, the PII size was categorized in two groups with larger sizes ($p < 0.001$) at the Marseillan site ($273.2 \pm 5.2 \mu m$, $n=11$), Bouzigues ($265.9 \pm 3.8 \mu m$, $n=24$) and Balaruc ($275.0 \pm 2.6 \mu m$, $n=15$) and smaller ($p < 0.001$) in Listel ($254.8 \pm 4.5 \mu m$, $n=30$) and Meze_osfz ($243.8 \pm 4.3 \mu m$, $n=35$).

**Relations between PII size, hydrology and plankton data**

A principal component analysis was conducted on the hydrological measurements, plankton data and PII sizes to explore their relationship and to characterize structured ecological gradients and spatial patterns (Figure 6). Applying the “broken stick” model and Kaiser criterion, four axes were mainly explanatory but only the first three axes are shown because they each individually represent more than 10% of the total inertia (Figure 6a). On the Dim1/Dim2 plot (Figure 6b), the first axis opposed autotrophic organism (bottom right; biomass and abundance of nanophytoplankton and biomass of total chlorophyll $a$ with hetero/mixotrophic organisms (top left; fh, tintinnids, ciliates and crypto). The second axis was mainly driven by abundances of picoplanktonic organisms such as ciliates, peuk and crypto. The first two axes accounted for 64.0% of variance. Although weakly represented on the factorial plane of axes 1 and 2, the PII size appeared to be positively correlated with hetero/mixotrophic organisms such as Crypto and Ciliates and anti-correlated with the variables representing autotrophic organisms (biomass and abundance of nanophytoplankton and total chlorophyll $a$ biomass. The low representation of the
PII size variable on these first two axes reveals the marked influence of ecological heterogeneity and gradients in the ecosystem in response to the observed biological effect. The Dim1/Dim3 plot (Figure 6c) shows that in planes 1 and 3, the right part of plot showed high and positive contributions of autotrophic organisms (diatoms^A, nano^A, nano^B, total chlorophyll a^B and cyan^A) and the left part showed negative contributions by fh^A, crypto^A and ciliates^A. The Dim2-Dim3 plot (Figure 6d) shows the correlations between the variables on axes 2 and 3 with axis 3 (12.6%) opposing PII size and Tintinnids^A, fh^A, temperature and bacteria^A. The distribution of sampling sites in factorial planes 1-2 indicates that the sites expressed their heterogeneity differently between years with respect to the autotrophic or hetero/mixotrophic regime (Figure 6e, f, g). The sites Marseillan 2012, Marseillan 2014, and Bouzigues 2014 had the biggest PII, the highest heterotrophic abundances and the lowest phytoplankton biomass and abundance. The Listel site had the smallest PII size. Overall, the right to left progression from autotrophic to heterotrophic is clear on the first two axes whereas the temperature has a structuring effect and is related to the sizes of PII on the first two axes of this ACP.

Annual averages (mean ± se) of size at metamorphosis increased significantly (Anova, F value = 48.5, p < 0.0001, Figure 7a) from 2012 (226.7 ± 23.3 µm, n = 113) to 2013 (248.0 ± 25.99 µm, n = 177) and 2014 (258.2 ± 24.50 µm, n=115).

Water temperature (mean ± se) during the main oyster recruitment events was on average 25.7 ± 0.1 °C in 2012, 24.8 ± 0.1 °C in 2013, 23.3 ± 0.1 °C in 2014 (Figure 7) with a significant anticorrelation with PII size (Spearman’s correlation coefficient = -0.74 and p < 0.001) (Figure 7b). Figure 8 shows six trophic parameters significantly correlated with PII size (p < 5%); Cryptophyte abundances were positively correlated (Corr. Coef. =0.69 and p < 0.001) and anticorrelated with total chlorophyll-a biomass (Corr. Coef. =-0.51 and p < 0.01), micro-
phytoplankton biomass (Corr. Coef. = -0.77 and p < 0.001), nanophytoplankton biomass (Corr. Coef. = -0.57 and p < 0.05), picocyanobacteria (corr. Coef. = -0.62 and p < 0.05) and tintinnid abundances (Corr. Coef. = -0.41 and p < 0.01).

**Relation between PII size and survival after metamorphosis**

Figure 9 shows a negative linear relation between PII sizes and survival after metamorphosis (cor. Coef. = -40.8%, df. = 379, p < 0.0001). The significant negative slope (b = -40.9, p < 0.0001) shows that juvenile survival decreased with an increase in PII size (Table 2). The value of 254.8 µm (p < 0.0001) at the intercept for PII size indicates low survival compared to 213.9 µm for high juvenile survival. The proportion of variance explained by the regression is relatively low (multiple R-squared = 0.167) compared to the total dispersion because of the great variability of the PII sizes measured.

**DISCUSSION**

In a new context of oligotrophication, our overall objective was to explore variability of recruitment of *Crassostrea gigas*, the most highly exploited bivalve species in the world (FAO 2012). This is the first time variability of prodissoconch II size has been assessed during the main oyster recruitment events in *C. gigas* in a semi-enclosed nanotidal ecosystem, Thau lagoon in southern France. Likewise, the hypothesis that, in *C. gigas*, larger size at metamorphosis is linked to lower juvenile survival rates was confirmed. The retention of competent larvae in the water column was mainly related to temperature and environmental/trophic conditions. Contrariwise, rapid settlement and metamorphosis at a smaller size increased the survival probability of Pacific oyster juveniles and had a positive impact on recruitment success.

The recruitment variability of benthic invertebrates has already been studied to ensure the sustainability of the established populations (Pechenik 2006, St-Onge et al. 2015, Barbier et al.)
In our case, monitoring in 2012, 2013 and 2014 revealed spatial and temporal patterns that had an environmental influence on different stages, i.e. spawning, settlement and metamorphosis (Lagarde et al. 2017, Ubertini et al. 2017). This shows that the intrinsic ecological functioning of the lagoon combined with heterotrophic/mixotrophic vs. autotrophic regimes (Leruste et al. 2016) can influence gametogenesis, spawning behavior, settlement of larvae, and hence recruitment success. As already described for lakes, these changes in functioning affect the trophic chain through a cascade of interactions extending from bacteria to fish (Jeppesen et al. 1998, 2005, Ozkan et al. 2016). The concept of a time window for recruitment, as described by Pineda et al. (2006), is fully applicable to the recruitment abundances in our study context. The ecological specificities of Thau lagoon are expressed in the heterogeneous oyster recruitment within the narrow recruitment windows defined by specific spatial and temporal patterns (Lagarde et al. 2017). The temporal component of Pacific oyster recruitment in the lagoon according to three determinants: (i) The recruitment determinant is the temperature of the ecosystem that results in different ecological functioning (hypoxic / normoxic, autotrophic / heterotrophic); (ii) the determinant of pediveligers abundance is the abundance of diatoms Chaetoceros spp; (iii) the determinant of metamorphosis survival is the abundance of nanophytoplankton. It is known that Thau lagoon is spatially heterogeneous due to its topogeography, currents, confinement and its intensive exploitation for shellfish farming. The effects of shellfish farming on the ecosystem are well known, particularly significant depletions of biomass and abundance of plankton in shellfish growing areas due to bivalve filtration (Deslous-Paoli et al. 1993, Lam-Hoai et al. 1997, Mazouni, Deslous-Paoli, et al. 1998). Lagarde et al. (2017) showed that plankton depletion, including Chaetoceros spp and nanophytoplankton, are not favorable for a larval cycle up to recruitment, particularly in periods of top-down trophic
control of shellfish areas by filter feeders during recruitment windows.

The larval stage and particularly the steps of metamorphosis may be delayed depending on environmental functioning (Lutz & Jablonski 1978, Pechenik 1990, 1999, 2006). Martel et al. (2014) highlighted the relation between a longer delay before metamorphosis, an increase in the PII size of the blue mussel *Mytilus edulis* and a decrease in settlement and recruitment success.

In the present study, *in situ* spatial and temporal patterns of prodissococonch II sizes were identified for the first time in *C. gigas* based on environmental cues. Prodissoconch II heights were measured during the best recruitment windows each year as representative of the functioning scenarios of the entire Thau basin exploited for shellfish farming. The variation in size was on average 20% per sampling site with individual maxima of up to 40%, i.e., from 180 to 296 µm. Our results suggest that the yearly temporal pattern is mainly driven by temperature, while the higher spatial variability of PII size is related to the quantity and quality of available food (autotrophic vs. heterotrophic regime) for larvae and juveniles (Lagarde et al. 2017, Ubertini et al. 2017). Further study is needed on the impacts of delayed metamorphosis on the duration of larval pelagic stage, changes in PII sizes in terms of physiological states or indicators such as energy reserves, total lipids and essential fatty acids.

Temperature controls activities that affect the kinetics of the biological and ecological processes (Frontier & Pichod-Viale 1998). It is known that temperature determines the patterns of distribution of organisms as a function of gradients and discontinuities (Hochachka et al. 2002). At an individual scale, the growth rates of poikilotherm organisms are governed by temperature. Temperature has a direct effect on the physiology of oyster larvae by enabling high metabolism rates (Rico-Villa et al. 2010) thereby accelerating development, but temperature may not affect total metabolic demand (Hoegh-Guldberg et al. 1991). In the present study, the annual means of
PII size were anti-correlated with water temperature (2012: 25.7 °C, 2013: 24.8 °C, 2014: 23.3 °C), which contradicts the physiological rules and disagrees with the results obtained by Arakawa (1990), who studied the eutrophicated Hiroshima Bay in the 1990s. However, this negative correlation between seawater temperature and PII size has already been reported in *Mya arenaria* (Lutz & Jablonski 1978), *Mytilus edulis* (Bayne 1965) and *Crassostrea gigas* (Flores-Vergara et al. 2004). This relationship could be due to the longer time available for feeding and growth at lower temperatures. At high temperatures, the transition between feeding stages, such as velum resorption, is shortened, and could result in smaller PII sizes (Lutz and Jablonski, 1978).

The temperature of the sea water has known effects on the activation of spat oyster pathogens such as the Ostreid herpes virus OsHV-1 and bacteria of the group *Vibrio splendidus* (Pernet et al. 2012, Petton, Boudry, et al. 2015, Petton, Bruto, et al. 2015). OsHV-1 mortality in France started in early May and lasted until late September, with an interruption in July and August when seawater temperatures increased from 16 °C to more than 23 °C (Pernet et al. 2014). In our study context, reproduction and recruitment processes were mainly studied in a temperature window ranging from 23 °C to 27 °C. We thus presume that pathogen agent OsHV-1 was not active during our recruitment window.

Temperatures drastically affect the whole ecological functioning of the Mediterranean lagoon in summer (Chapelle, Lazure, et al. 2000, Chapelle, Ménesguen, et al. 2000). In the case of high summer temperatures, diatoms, cryptophytes and chlorophyta are known to represent phytoplankton communities in this mesotrophic Mediterranean lagoon (Collos et al. 2009, Bec et al. 2011, Leruste et al. 2016). Moreover, a spatial gradient in ecological functioning was revealed with smaller PII sizes in the middle of the ecosystem (Listel and Meze_osfz) and larger sizes at
the eastern (Marseillan) and western (Balaruc) ends. Analysis of environmental variables showed that the smaller PII sizes associated with better survival were driven by higher biomass of autotrophic organisms such as picocyanobacteria, nano and micro-phytoplankton and total chlorophyll a biomass. In contrast, larger PII sizes with lower survival rates were associated with lower autotrophic biomass. The significant negative correlation between PII size and nanophytoplankton and micro-phytoplankton biomass highlights the importance of the planktotrophic relationship during settlement and metamorphosis (Toupoint et al. 2012). Our results are in agreement with those of Toupoint et al. (2012), who showed in the blue mussel that high cyanobacteria, pico- and nano-eukaryote biomasses were correlated with better recruitment. Considering a context of heterogeneity caused, on the one hand, by the effects of depletion due to shellfish farming areas and on the other hand, by geographical gradients, we hypothesize that pico-organisms (cyanobacteria eukaryotes), nano- and micro-phytoplankton biomasses act as a trigger for trophic settlement, as already demonstrated in mussels (Toupoint et al. 2012, Martel et al. 2014), and given their impact on PII size, probably also trigger metamorphosis. Our results show that high cryptophyte abundances correspond to large PII sizes. Cryptophytes are mixotrophic nano-flagellates that play a role in the remineralization of organic matter and in predation on picoplankton such as the *Synechococcus sp.* picocyanobacteria (Yoo et al. 2017) and bacteria (Klaveness 1989, Pastoureaud et al. 2003). The appearance of picocyanobacteria (mostly *Synechococcus sp.*) has been reported to be related to a reduction in nutrient loading and to an increase in water temperature (Collos et al. 2009). Cryptophyte abundance and biomass respond rapidly to both nutrient loads and picocyanobacteria prey abundance due to their high growth rate (Yoo et al. 2017). These mixotrophic features (Cloern & Dufford 2005) make cryptophytes revealers of a transitional state during oligotrophication. What is more, thanks to
their high nutritional values, digestibility and nanophytoplankton size, cryptophytes are considered as good prey for filter feeders like adults oysters (Klaveness 1989, Pastoureaud et al. 2003). However, cryptophytes have ejectile organelles named trichocysts/ejectosoms (Klaveness 1989) that may play a defensive role against predation by protozoa (Yamagishi et al. 2012). It has also been reported that, in the case of Raphidophyceae in lake ecosystems, trichocysts may also act as repellents to grazers (Lebret et al. 2012). Here, we hypothesize that the anatomy of cryptophytes with trichocysts/ejectosoms would be inappropriate for larval development and would thus be metamorphosis inhibitors/delayers. Of course, this hypothesis needs to be tested in experimental studies. Tintinnids are heterotroph organisms, predators of phytoplankton, specifically of nano-phytoplankton, including cryptophytes, COD and bacteria (Dolan 2000, Sitran et al. 2009). Tintinnid abundance probably reflects picocyanobacteria populations and cryptophyte dynamics and acts as a booster of settlement or metamorphosis or perhaps just as a neutral trophic competitor of oyster larvae, revealing mixo/heterotrophic traits of oligotrophication process. These results fill some of the many gaps in our knowledge of the interactions between cryptophytes, ciliates and oyster larvae.

Bacteria have been shown to facilitate the settlement of many invertebrate larvae, including oysters (Fitt et al. 1989, 1990, Coon et al. 1990). In the present study, we show that high picocyanobacteria abundance corresponds to small PII size. We hypothesize that picocyanobacteria abundance boosts settlement or metamorphosis in the presence of an efficient trophic chain.

The functional approach (heterotrophic vs. autotrophic) has been used to understand how environmental changes or gradients drive phytoplankton community structure (Litchman et al. 2010, Leruste et al. 2016). Some morphological and physiological traits, in particular cell size,
maximum growth rate and trophic regime reflect how phytoplankton adapt to nutrient availability (Litchman et al. 2007, 2010). During oligotrophication, the reduction in nutrient inputs could thus favor small cells, which compete more effectively for nutrient uptake and have high growth rates (Chisholm 1992, Kamenir & Morabito 2009, Litchman et al. 2010), and mixotrophic species, which have some advantages over strictly autotrophic cells (Anneville & Pelletier 2000). In our case, an ecosystem with heterotrophic functioning indeed resulted in sizes at metamorphosis ranging from 250 to 300 μm, while autotrophic functioning resulted in sizes ranging from 200 to 250 μm. This ecological heterogeneity expresses a specificity of Thau lagoon with regard to PII size, i.e., an west-east gradient that tended to be linear in 2012 (with the largest sizes in the west and the smallest in the east) and another gradient as a reverse parabolic curve in 2013 and 2014 that recalls the optimal production curves of Shelford’s law of tolerance (Begon et al. 2007). The central zone of the lagoon represented by the "Meze_osfz" site appears to be the zone of ecological preference (with small PII size, high survival and high recruitment rates) and, at both west and east ends of the lagoon, tolerance limits of the species with high PII sizes, lower survival and recruitment rates.

Concerning the impact of a shellfish exploitation zone, the influence of filter feeders (oysters and their epibiota) on the spatial distribution of particulates and dissolved compounds determines the biogeochemical processes both in the water column (Mazouni, Gaertner, et al. 1998, Chapelle, Lazure, et al. 2000, Chapelle, Méneguen, et al. 2000) and in the sediment (Mazouni et al. 1996, Gilbert et al. 1997). The biocoenosis caused by shellfish farming results in specific ecological functioning and in a tenfold reduction in nanophytoplankton, and micro-phytoplankton biomass (Tournier & Pichot 1987, Jarry et al. 1990, Souchu et al. 2001) as well as in microzooplankton included tintinnids, rotifers, anthozoan larvae and crustacean and mollusk larvae compared to
values measured in the middle of the lagoon (Lam-Hoai et al. 1997). This ecological heterogeneity, as exemplified by the Thau basin, should affect some marine invertebrate larvae (Borsa & Millet 1992) and affect oyster recruitment at biocoenosis scale.

Our results showed that the best metamorphosis survival rate was associated with small PII size. Our results are in agreement with those of Martel et al. (2014) on Mytilus edulis and support the hypothesis that inter-annual variations in the adult population of most benthic invertebrates are not only related to variability in larval supply, but even more to post-settlement processes (Gosselin & Qian 1997, Hunt & Scheibling 1998, Fuchs et al. 2013).

In Hiroshima bay, the first industrial spatfall basin of C. gigas in Japan, the ratio of the nano- and pico-phytoplankton (>10-20 micro m) to total chl \(a\) in 2010 clearly decreased when the lack of natural oyster recruitment became serious (Hori, \textit{com. pers.}). In comparison with the 1990s, when recruitment was successful, the ratio of nano- and pico-phytoplankton to total chlorophyll \(a\) was more than 90\% from May to September (Sik Lee et al. 1996) whereas in 2017, the ratio was less than 30-60\% (Matsubara, unpublished data). In France, temperature related nanophytoplankton have been shown to have a significant influence on the success of recruitment in both the Arcachon basin and Thau lagoon (Auby & Maurer 2004, Lagarde et al. 2017). These results in France and Japan reinforce the hypothesis that the origin of the variability in the success of larval development, metamorphosis and recruitment in natural spatfall basins is mainly driven by a combination of temperature and the quality and quantity of phytoplankton. Other studies conducted in Atlantic coastal ecosystems also support this hypothesis (Pouvreau 2015).
CONCLUSION

In the presence of ecological heterogeneity, plasticity responses in larval traits can affect size at metamorphosis, juvenile development, and recruitment survival. Ecological structuring offers different kinds of pelagic and benthic habitats that correspond to recruitment windows (Pineda et al. 2006, Buckley et al. 2010) some of which may be optimal (Cury & Roy 1989, Pineda et al. 2006, Lagarde et al. 2017). Pechenik (1990) emphasized the absolute need for field studies to assess variability of PII size, delays in metamorphosis and their impact on juvenile survival and development. Our work helps characterize interactions between planktonic larvae and trophic and environmental conditions, including the impact of filter-feeders on the recruitment of C. gigas. This study highlights for the first time the existence of variability in prodissoconch II size in C. gigas during major oyster recruitment events in the French Mediterranean Thau lagoon in 2012, 2013 and 2014. PII size varied with the temperature and with trophic conditions and was smaller under high water temperature conditions combined with high nano and micro-phytoplankton biomass, and with tintinnid and picocyanobacterial abundances. Inversely, when cryptophytes were more abundant and water temperatures were lower, the larvae that settled and metamorphosed were bigger. We also demonstrated that the survival rate of smaller prodissoconch II shells was higher while the larger PII sizes had lower survival rates. Under oligotrophication, we hypothesize that in a favorable autotrophic context, it is better for larvae survival to metamorphose early and to be smaller whereas in unfavorable heterotrophic/mixotrophic conditions, the larvae were bigger with potential metamorphosis delays. Links with trophic conditions need to be confirmed by studying the relation between the energetic values of nano-son and postlarvae during major recruitment events. Further laboratory experiments are recommended to test the link between
size at metamorphosis, energetic reserves of larvae and the delay in metamorphosis in
different trophic conditions. The negative influence of cryptophytes on metamorphosis also
needs to be confirmed in lab experiments. These innovative results lead us to hypothesize that
the shift from an autotrophic to a heterotrophic system could increase the variability of {C.}
gigas recruitment and possibly jeopardize the sustainability of shellfish farming in coastal
ecosystems undergoing oligotrophication.

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their effects in the marine environment. Mar Environ Res 124:11–20


Table 1: Variables characterizing interactions between the environment and larvae. Each environmental variable was averaged over a 2-week period preceding the retrieval of the collectors to characterize the environment for metamorphosis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Description</th>
<th>Unity</th>
<th>Abbreviation</th>
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</thead>
<tbody>
<tr>
<td><strong>Target variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prodissococonch II height</td>
<td>measure</td>
<td>µm</td>
<td>P2 size</td>
</tr>
<tr>
<td>oyster spat</td>
<td>abundance</td>
<td>ind. dm(^{-2})</td>
<td>oyster spat</td>
</tr>
<tr>
<td>pediveligers</td>
<td>abundance</td>
<td>ind. dm(^{-2})</td>
<td>pedi</td>
</tr>
<tr>
<td><strong>Environmental variables</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>spat / pediveliger survival</td>
<td>ratio of oyster spat to pediveliger abundance</td>
<td>-</td>
<td>metamorphosis survival</td>
</tr>
<tr>
<td>oxygen concentration</td>
<td>daily average</td>
<td>mg l(^{-1})</td>
<td>oxygen</td>
</tr>
<tr>
<td>temperature</td>
<td>daily average</td>
<td>°C</td>
<td>temperature</td>
</tr>
<tr>
<td>salinity</td>
<td>daily average</td>
<td>No unit</td>
<td>salinity</td>
</tr>
<tr>
<td>bacteria</td>
<td>abundance</td>
<td>10(^6) cell l(^{-1})</td>
<td>bacteria(^A)</td>
</tr>
<tr>
<td>total picoeukaryotes</td>
<td>abundance</td>
<td>10(^6) cell l(^{-1})</td>
<td>peuk(^A)</td>
</tr>
<tr>
<td>total picocyanophyceae</td>
<td>abundance</td>
<td>10(^6) cell l(^{-1})</td>
<td>cyan(^A)</td>
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<tr>
<td>nanophytoplankton</td>
<td>abundance</td>
<td>10(^6) cell l(^{-1})</td>
<td>nano(^A)</td>
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<tr>
<td>cryptophytes</td>
<td>abundance</td>
<td>10(^6) cell l(^{-1})</td>
<td>crypto(^A)</td>
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<td>heterotrophic flagellates</td>
<td>abundance</td>
<td>cell l(^{-1})</td>
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<tr>
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<td>abundance</td>
<td>cell l(^{-1})</td>
<td>ciliates(^A)</td>
</tr>
<tr>
<td>tintinnids</td>
<td>abundance</td>
<td>cell l(^{-1})</td>
<td>tintinnids(^A)</td>
</tr>
<tr>
<td>diatoms</td>
<td>abundance</td>
<td>cell l(^{-1})</td>
<td>diatoms(^A)</td>
</tr>
<tr>
<td>dinoflagellates</td>
<td>abundance</td>
<td>cell l(^{-1})</td>
<td>dinoflagellates(^A)</td>
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<td>biomass</td>
<td>µg Chla l(^{-1})</td>
<td>Total_chlo_(a)^(B)</td>
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<tr>
<td>picophytoplankton</td>
<td>biomass</td>
<td>µg Chla l(^{-1})</td>
<td>pico(^B)</td>
</tr>
<tr>
<td>nanophytoplankton</td>
<td>biomass</td>
<td>µg Chla l(^{-1})</td>
<td>nano(^B)</td>
</tr>
<tr>
<td>micro-phytoplankton larger than 20 µm</td>
<td>biomass</td>
<td>µg l(^{-1})</td>
<td>microphyto(^B)</td>
</tr>
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</table>
Table 2: Analysis of variance of the linear regression between the survival after metamorphosis and prodisoconch II size. Significant values are in **bold**.

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<tr>
<th></th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F</th>
<th>p</th>
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<td>Response: PII size</td>
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<tr>
<td>Survival after metamorphosis</td>
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<td>47975</td>
<td>47975</td>
<td>75.9</td>
<td>&lt; 2.2e-16</td>
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<tr>
<td>Residuals</td>
<td>379</td>
<td>239600</td>
<td>632</td>
<td></td>
<td></td>
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</tbody>
</table>

Linear model: P2 size ~ Survival after metamorphosis

<table>
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<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>T</th>
<th>p</th>
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</thead>
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<td>Intercept</td>
<td><strong>254.7</strong></td>
<td>1.817</td>
<td>140.237</td>
<td>&lt;2e-16 ***</td>
</tr>
<tr>
<td>Survival after metamorphosis</td>
<td><strong>-40.947</strong></td>
<td>4.700</td>
<td>-8.71</td>
<td>&lt;2e-16 ***</td>
</tr>
</tbody>
</table>

Residual standard error 25.14 on 379 degrees of freedom

Multiple R-squared: **0.1668**
Adjusted R-squared: 0.1646

F: 75.89 on 1 and 379 DF **p < 2.2e-16**
Figure captions

Figure 1: (a) The Mediterranean Thau lagoon in France and (b) sampling sites in Thau lagoon. The five sampling sites where benthic Pacific oyster larvae and juvenile abundances were monitored are (Marseillan, green cross; Meze_osfz, cyan diamond; Bouzigues, black circle; and Listel, blue cross). Red circles show the location of the hydrological and plankton sampling sites. Grey boxes indicate the location of shellfish farms.

Figure 2: The breeding structure carried a set of 3 replicate collectors within the shellfish farming zone. Outside the shellfish farming zone, a mooring system as described by Lagarde et al. (2017) was used. (b) Top view of a collector plate with counting subunits in yellow, in this case: 14 cm².

Figure 3: Measurement of maximum shell height along maximal (H-H’) dorsoventral axis of larvae and juvenile oysters (Bayne 2017) in Thau lagoon. (a) Prodissoconch II and dissoconch shells showing demarcation of the growth line delimiting metamorphosis. (b) Prodissoconch II size (H-H’= 228.9 µm, 2012 Bouzigues, Replicate 1, middle plate, measurement 8).

Figure 4: Means (± standard error) Pacific oyster spat abundance per dm² observed at the 5 sampling sites on a west-east gradient (Marseillan, Listel, Meze_osfz, Bouzigues and Balaruc), at two-week intervals throughout the summer in 2012, 2013, and 2014. Juvenile abundances were estimated after four weeks of immersion (n=27 per date & sampling site). Letters indicate significant groups resulting from the Siegel and Castellan multiple comparisons test (p ≤ 0.05). The squares indicate the main annual recruitment event used to characterize variations in prodissoconch II size.
Figure 5: Mean (± standard error) of prodissocconch II size as a function of the sampling site on a west-east gradient (Marseillan, Listel, Meze_osfz, Bouzigues and Balaruc) and of the year (2012, 2013 and 2014) during the main annual recruitment events with the associated strength. Letters indicate significant differences according to the two-way-cross permutation Anova, pair-wise tests 'Year x Sampling site' for pairs of levels of the factor 'Sampling site'. Numbers at the bottom of the histograms indicate the number of samples per year and per sampling site.

Figure 6: Principal component analysis (a) Comparison of the broken stick model and eigenvalues, (b) Biplots with correlation circle Dim 1 / Dim 2 of averaged environmental data for a 14 day period preceding the retrieval of the collector (superscript A: abundance, B: biomass), (c) Correlation circle Dim 1 / Dim 3, (d) Correlation circle Dim 2 / Dim 3 and the Site factor per year (e) 2012, (f) 2013, (g) 2014.

Figure 7: (a) Increasing trend in PII size versus year, (b) Daily temperatures during the 2 week metamorphosis period preceding retrieval of the collector (August 13, 2012, August 28, 2013, and August 27, 2014), (c) Significant relationship between PII size and temperature means over the duration of the metamorphosis period. Mid-line: median; box: 25th and 75th percentiles, whiskers: 1.5× the interquartile range; circle: outliers; line: least square regression; crosses: means.

Figure 8: Relationship between PII size (mean of 10 measurements per circle, 3 replicated plates per collector) with plankton during the metamorphosis period, (a) total chlorophyll-a biomass, (b) micro-phytoplankton biomass, (c) nanophytoplankton biomass, (d) cryptophyte abundance (e) picocyanobacteria abundance (f) tintinnid abundance. Significant values * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure 9: Prodissoconch II size (Anova p < 0.001) as a function of metamorphosis survival expressed in percent (n = 333, censured data 100+ for over 100% survival); black line: regression line, red dotted line: ± 95% confidence interval.
Figure 1
Figure 2
Figure 3.3
Figure 4

Kruskall-Wallis, p < 0.0001

2012

Oyster spat dm²

2013

Kruskall-Wallis, p < 0.0001

2014

Kruskall-Wallis, p < 0.0001

Figure 4
Figure 5
Figure 6
Figure 7
Figure 8