Does Encapsulation Protect Embryos from the Effects of Ocean Acidification? The Example of Crepidula fornicata

Fanny Noisette, Thierry Comtet, Erwann Legrand, François Bordeyne, Dominique Davoult, Sophie Martin

To cite this version:

Fanny Noisette, Thierry Comtet, Erwann Legrand, François Bordeyne, Dominique Davoult, et al.. Does Encapsulation Protect Embryos from the Effects of Ocean Acidification? The Example of Crepidula fornicata. PLoS ONE, Public Library of Science, 2014, 9 (3), pp.e93021. 10.1371/journal.pone.0093021. hal-01097262

HAL Id: hal-01097262
https://hal.sorbonne-universite.fr/hal-01097262
Submitted on 19 Dec 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Does Encapsulation Protect Embryos from the Effects of Ocean Acidification? The Example of Crepidula fornicata

Fanny Noisette1,2*, Thierry Comtet1,2, Erwann Legrand1,2, François Bordeyne1,2, Dominique Davoult1,2, Sophie Martin1,2

1 Sorbonne Universités, UPMC Univ. Paris 06, UMR 7144, Station Biologique de Roscoff, Roscoff, France, 2 CNRS, UMR 7144, Station Biologique de Roscoff, Roscoff, France

Abstract

Early life history stages of marine organisms are generally thought to be more sensitive to environmental stress than adults. Although most marine invertebrates are broadcast spawners, some species are brooders and/or protect their embryos in egg or capsules. Brooding and encapsulation strategies are typically assumed to confer greater safety and protection to embryos, although little is known about the physico-chemical conditions within egg capsules. In the context of ocean acidification, the protective role of encapsulation remains to be investigated. To address this issue, we conducted experiments on the gastropod Crepidula fornicata. This species broods its embryos within capsules located under the female and veliger larvae are released directly into the water column. C. fornicata adults were reared at the current level of CO2 partial pressure (pCO2) (390 µatm) and at elevated levels (750 and 1400 µatm) before and after fertilization and until larval release, such that larval development occurred entirely at a given pCO2. The pCO2 effects on shell morphology, the frequency of abnormalities and mineralization level were investigated on released larvae. Shell length decreased by 6% and shell surface area by 11% at elevated pCO2 (1400 µatm). The percentage of abnormalities was 1.5- to 4-fold higher at 750 µatm and 1400 µatm pCO2, respectively, than at 390 µatm. The intensity of birefringence, used as a proxy for the mineralization level of the larval shell, also decreased with increasing pCO2. These negative results are likely explained by increased intracapsular acidosis due to elevated pCO2 in extracapsular seawater. The encapsulation of C. fornicata embryos did not protect them against the deleterious effects of a predicted pCO2 increase. Nevertheless, C. fornicata larvae seemed less affected than other mollusk species. Further studies are needed to identify the critical points of the life cycle in this species in light of future ocean acidification.

Introduction

Early life history stages of marine species, including embryos and larvae, are of crucial importance in population dynamics as they ensure dispersion, colonize new areas and sustain populations [1]. Their success in development and final recruitment are essential for the persistence of viable populations. Early stages of marine invertebrates are in general morphologically and ecologically distinct from the adult stage and are generally thought to be more sensitive to environmental stress [1] although, in some cases, they may be more tolerant than adults, e.g. some Antarctic species exposed to warming [2]. In the context of climate change, early development may be affected by various factors, such as temperature increases, hypoxia zones or ocean acidification. Due to the increase in atmospheric pCO2 predicted for the end of the century (from 475 to 1531 µatm according to the Intergovernmental Panel on Climate Change (IPCC)), pH in surface seawaters is likely to decline by 0.6 – 0.32 units [3], leading to a decrease in carbonate ion concentrations ([CO3\(^{2-}\)]\) and a reduction in the calcium carbonate saturation state (Ω) [4]. Due to these changes in seawater carbonate chemistry, ocean acidification is considered a major threat to calcifying marine species, affecting their physiology and impairing their ability to build calcium carbonate shells and skeletons [8,9,10,11], which can ultimately modify their behavior and distribution [5,6,7]. Early life stages (embryos, larvae and juveniles) of calcifying species are thus expected to be highly affected by ocean acidification [12,13], as opposed to non-calcifying larvae which are predicted to be more tolerant [14,15]. This relatively higher vulnerability is likely due to fragile larval skeletons [9] and their high ratio of exposed surface-to-body mass compared to adults [16]. Identifying life history stages that are the most vulnerable to global change is needed to determine bottlenecks for species persistence and addressing their sensitivity to acidification is a major issue in a changing ocean [9].

Responses to near-future (end of century) levels of pCO2 depend on species, populations, habitats and developmental stages [9,17,18,19] and understanding these effects on the early life stages requires taking into account the complete developmental cycle, from egg to juvenile [12]. In particular, the impact of elevated pCO2/decreased pH on early life stages has been investigated in a broad range of species, including corals [20,21], echinoderms [22,23,24], crustaceans [15,16,25], mollusks [26,27],
and fish [28,29]. In mollusks, which have been studied intensively (see refs. [26,30] for a review), deleterious effects of increased pCO2 have been demonstrated on fertilization success [31,32], hatching success [33,34], larval survival [35,36], growth [37,38], shell formation [39,40], development duration [41,42], and settlement [43,44].

Most of the species studied are broadcast spawners, which may be considered particularly vulnerable to ocean acidification because fertilization and complete pelagic larval life occur in the water column [9,45]. Whether alternative reproductive modes are affected in a changing ocean is still poorly documented. Brooding and/or egg laying in egg masses or capsules are typically assumed to confer protection to developing embryos [46,47]. For example, it has been shown that encapsulated embryos of some gastropod species survive better in conditions of salinity stress than embryos removed from their capsule [48,49]. A few studies have explored the effects of decreased pH on embryos brooded and/or laid in benthic gelatinous egg masses or in egg capsules in bivalve [50], gastropod [35,41,51,52] or cephalopod [27,33] mollusks. Depending on the study, reduced pH has different effects that are related to the range of species habitats and the strategy to protect embryos (brooding, egg masses, capsules) studied as well as the source of pH change (pCO2 increase, salinity stress). Encapsulation has been suggested to protect embryos against ocean acidification [41,54], whereby the buffer capacity of intracapsular fluids may reduce the potential effect of extracapsular elevated pCO2 seawater.

To study this issue in a non-broadcast-spawner species, we chose the slipper limpet Crepidula fornicata, Linné 1758 (Gastropoda) as our biological model. Native to the northeast American coast, this species was introduced in Europe at the end of the 19th century, primarily via oyster farming [53], and has now become invasive in bays and estuaries where it reaches very high densities [54]. It has a benthic-pelagic life cycle, with a number of original features. Benthic adults form stacks with males at the top and females at the bottom. After internal fertilization, females brood their embryos in egg capsules for 3 to 4 weeks [55,56]. Capsules are protected between the neck and the propodum of the female parent and attached to the substratum to which the female is fixed [55]. Each female spawns between 28 and 64 capsules, each containing 300 to 600 larvae [56,57]. Larvae are released in the water column where they spend between 2 and 7 weeks [59,60]. Whether alternative reproductive modes are considered particularly vulnerable to ocean acidification scenarios predicted for the end of the century by the IPCC [3].

The pCO2 was adjusted by bubbling CO2-free air (current control pCO2) or pure CO2 (two elevated pCO2 treatments) in three 100 L header tanks supplied with unfiltered seawater pumped at the foot of the Station Biologique de Roscoff. Each of the three pCO2 treatments had six 10 L replicate aquaria. This was an open system, and CO2-treated seawater from the mixing header tanks was continuously supplied to the 18 aquaria (6 per pCO2 condition), at a rate of 9 L h−1 (i.e. a renewal rate of 90% h−1). Aquaria were placed in a thermostatic bath where temperature was controlled to ±0.2 °C using 150 to 250 W submersible heaters. C. fornicata adults were grown at four successive temperature levels (10, 13, 16 and 19 °C) which corresponded to the range of in situ temperatures typically encountered in our study area (Service d’Observation de la Mer et du LitToral data). Adults were reared for four weeks at each temperature level. Changes in temperature were implemented slowly, with increases of 0.2 °C day−1 over a period of two weeks.

pCO2 and temperature were monitored and controlled by an off-line feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header tanks and the on/off heater switch in the thermostatic bath. The pH values of the system were adjusted from daily measurements of pH in each of the 10 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris/HCl and 2-aminoypyridine/HCl buffers [64]. Slipper limpets were fed three times a week with a mix made from a stock solution of Chaetoceros gracilis (~15 × 10⁶ cells mL⁻¹) and Isochrysis affinis galbana (~26 × 10⁶ cells mL⁻¹). This algal mix (400 mL) was distributed in each aquarium. Seawater flow was stopped for two hours to allow the limpets to feed.

Seawater parameters were monitored throughout the experiment in each of the 18 aquaria. pH in each of the 10 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris/HCl and 2-aminoypyridine/HCl buffers [64]. Slipper limpets were fed three times a week with a mix made from a stock solution of Chaetoceros gracilis (~15 × 10⁶ cells mL⁻¹) and Isochrysis affinis galbana (~26 × 10⁶ cells mL⁻¹). This algal mix (400 mL) was distributed in each aquarium. Seawater flow was stopped for two hours to allow the limpets to feed.

Seawater parameters were monitored throughout the experiment in each of the 18 aquaria. pH in each of the 10 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris/HCl and 2-aminoypyridine/HCl buffers [64]. Slipper limpets were fed three times a week with a mix made from a stock solution of Chaetoceros gracilis (~15 × 10⁶ cells mL⁻¹) and Isochrysis affinis galbana (~26 × 10⁶ cells mL⁻¹). This algal mix (400 mL) was distributed in each aquarium. Seawater flow was stopped for two hours to allow the limpets to feed.

Encapsulation in an Acidified Ocean

Methods

Crepidula fornicata adult collection and culture

C. fornicata stacks were collected by SCUBA divers on 30 November 2011, after the end of the reproductive period [61] in Morlaix Bay (northwestern Brittany, France), at the “Barre des Flots” site (3°53.015′W; 48°40.015′N). No specific permissions were required for sampling at the selected location, as it is not privately-owned or protected. Field sampling did not involve endangered or protected species.

After being held 6 weeks in natural ambient unfiltered seawater, C. fornicata adults were randomly distributed into 18 aquarium tanks of 10 L each (adapted from [62]) and reared for 24 weeks (12 January 2012 to 28 June 2012) in three pCO2 treatments selected according to the recommendations of Barry et al. [63]: (1) 390 μatm (pH on the total scale (pHT) = 8.07) as the current pCO2 (control), (2) 750 μatm (pHT = 7.82) and (3) 1400 μatm (pHT = 7.56); the former two pCO2 levels are pessimistic scenarios predicted for the end of the century by the IPCC [3].

The pCO2 was adjusted by bubbling CO2-free air (current control pCO2) or pure CO2 (two elevated pCO2 treatments) in three 100 L header tanks supplied with unfiltered seawater pumped at the foot of the Station Biologique de Roscoff. Each of the three pCO2 treatments had six 10 L replicate aquaria. This was an open system, and CO2-treated seawater from the mixing header tanks was continuously supplied to the 18 aquaria (6 per pCO2 condition), at a rate of 9 L h−1 (i.e. a renewal rate of 90% h−1). Aquaria were placed in a thermostatic bath where temperature was controlled to ±0.2 °C using 150 to 250 W submersible heaters. C. fornicata adults were grown at four successive temperature levels (10, 13, 16 and 19 °C) which corresponded to the range of in situ temperatures typically encountered in our study area (Service d’Observation de la Mer et du LitToral data). Adults were reared for four weeks at each temperature level. Changes in temperature were implemented slowly, with increases of 0.2 °C day−1 over a period of two weeks.

pCO2 and temperature were monitored and controlled by an off-line feedback system (IKS Aquastar, Karlsbad, Germany) that regulated the addition of gas in the header tanks and the on/off heater switch in the thermostatic bath. The pH values of the system were adjusted from daily measurements of pH in each of the 10 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris/HCl and 2-aminoypyridine/HCl buffers [64]. Slipper limpets were fed three times a week with a mix made from a stock solution of Chaetoceros gracilis (~15 × 10⁶ cells mL⁻¹) and Isochrysis affinis galbana (~26 × 10⁶ cells mL⁻¹). This algal mix (400 mL) was distributed in each aquarium. Seawater flow was stopped for two hours to allow the limpets to feed.

Seawater parameters were monitored throughout the experiment in each of the 18 aquaria. pH in each of the 10 aquaria using a pH meter (826 pH mobile, Metrohm AG, Herisau, Switzerland) calibrated with Tris/HCl and 2-aminoypyridine/HCl buffers [64]. Slipper limpets were fed three times a week with a mix made from a stock solution of Chaetoceros gracilis (~15 × 10⁶ cells mL⁻¹) and Isochrysis affinis galbana (~26 × 10⁶ cells mL⁻¹). This algal mix (400 mL) was distributed in each aquarium. Seawater flow was stopped for two hours to allow the limpets to feed.
### Table 1. Seawater parameters.

| Temperature (°C) | n (except $A_T$) | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
|------------------|------------------|------|----|------|----|------|----|------|----|------|----|------|----|
|                  | &nbsp; &nbsp; &nbsp; | &nbsp; &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| 10°C             |                  |      |    |      |    |      |    |      |    |      |    |      |    |
| 390 μatm 23      | 9.7              | 0.2  |    | 8.14 | 0.01| 323  | 7  | 2365 | 2  | 2138 | 4  | 2.47  | 0.04|
| 750 μatm 23      | 9.8              | 0.2  |    | 7.82 | 0.01| 729  | 19 | 2369 | 2  | 2270 | 4  | 1.33  | 0.03|
| 1400 μatm 23     | 9.5              | 0.2  |    | 7.55 | 0.03| 1487 | 75 | 2377 | 3  | 2366 | 11 | 0.78  | 0.08|
| 13°C             |                  |      |    |      |    |      |    |      |    |      |    |      |    |
| 390 μatm 27      | 12.9             | 0.2  |    | 8.12 | 0.02| 356  | 25 | 2418 | 2  | 2167 | 8  | 2.76  | 0.07|
| 750 μatm 27      | 13.0             | 0.1  |    | 7.81 | 0.01| 781  | 20 | 2416 | 2  | 2304 | 3  | 1.48  | 0.03|
| 1400 μatm 27     | 12.8             | 0.1  |    | 7.53 | 0.01| 1557 | 43 | 2422 | 2  | 2405 | 4  | 0.82  | 0.02|
| 16°C             |                  |      |    |      |    |      |    |      |    |      |    |      |    |
| 390 μatm 28      | 15.9             | 0.1  |    | 8.08 | 0.01| 376  | 11 | 2379 | 5  | 2127 | 5  | 2.80  | 0.05|
| 750 μatm 28      | 16.1             | 0.1  |    | 7.82 | 0.00| 748  | 8  | 2369 | 5  | 2238 | 2  | 1.66  | 0.01|
| 1400 μatm 28     | 16.0             | 0.1  |    | 7.55 | 0.01| 1492 | 19 | 2380 | 5  | 2345 | 2  | 0.94  | 0.01|
| 19°C             |                  |      |    |      |    |      |    |      |    |      |    |      |    |
| 390 μatm 23      | 18.4             | 0.5  |    | 8.02 | 0.01| 550  | 10 | 2391 | 2  | 2152 | 5  | 2.70  | 0.05|
| 750 μatm 23      | 18.6             | 0.5  |    | 7.77 | 0.01| 858  | 19 | 2395 | 3  | 2266 | 4  | 1.68  | 0.04|
| 1400 μatm 23     | 18.4             | 0.5  |    | 7.51 | 0.01| 1652 | 41 | 2394 | 3  | 2359 | 4  | 0.96  | 0.03|

Legend: Mean parameters of carbonate chemistry in each $pCO_2$ treatment at each temperature level. The pH on the total scale ($pH_T$) was measured daily and total alkalinity ($A_T$) was measured every 4 weeks. Other parameters were calculated with the CO2SYS software [65]. $pCO_2$: CO2 partial pressure; DIC: dissolved inorganic carbon; $\Omega_{Ar}$: saturation state of aragonite.

doi:10.1371/journal.pone.0093021.t001
200 μm mesh sieve, rinsed with seawater and preserved in 96% ethanol.

Pools of larvae from adults acclimated to the different $\rho$CO$_2$ levels since January were collected from the different $\rho$CO$_2$ conditions at the temperature level of 19°C between 8 and 24 June 2011. Only samples with enough intact larvae were used. Thus two viable samples per $\rho$CO$_2$ condition were studied.

### Morphological variables

Morphological measurements were performed on a random subsample of 40 larvae when possible or at least 20 larvae from each of the 6 larval pools. Larvae with unbroken shells were isolated in sterile, flat-bottom, 96-well plates and preserved in pure glycerol as described in Auzoux-Bordenave et al. [68]. Each larva was placed on its right side and photographed under light microscopy using an Olympus Camedia C-7070 camera attached to an Olympus SZX 12 dissecting microscope. Pictures were taken without autofocus at ×90 magnification. Maximum length, height and projected surface area of the left side (Figures 1A, B, and C, respectively) were measured by analyzing images with ImageJ software [69], after calibration with a stage micrometer.

### Abnormalities

In each subsample used for morphological measurements, veliger larvae with abnormal shells were counted and the percentage of abnormal larvae was estimated per $\rho$CO$_2$ treatment. To be considered as a shell abnormality and not as a broken shell, deformities had to be devoid of fracture lines. A “deformity index” was calculated to quantify the intensity of the shell deformity. It was defined as the ratio between the angle formed by the abnormality and its “depth”, which is the distance between the theoretical curve of the shell and the forest point (extreme point) of the deformity (Figure 1D).

---

**Figure 1. Morphological variables.** All measurements were taken on larvae lying on their right side. A: maximum shell length (L) (in μm); B: maximum shell height (h) (in μm); C: projected surface area of the left side (in mm$^2$); D: deformity index, a is the angle of the abnormality (in degrees) and $d$, the depth of the abnormality (in μm).

doi:10.1371/journal.pone.0093021.g001

**Figure 2. Morphological variables.** Mean shell length, height and left surface area (± SE) in the different $\rho$CO$_2$ treatments. Different letters above bars indicate significant differences between treatments (p<0.05, Dunn post-hoc test), n = 51 to 92.

doi:10.1371/journal.pone.0093021.g002

**Shell mineralization**

For each $\rho$CO$_2$ treatment, 5 to 8 larvae were randomly chosen among the previous subsamples and observed under polarized light to determine birefringence patterns with an Olympus dissecting microscope equipped with polarizing filters. All polarized images were acquired with an Olympus camera at ×100 magnification with 40 ms light exposition. Birefringence under
polarized light is due to the mineral phase composing the shell [40,68,70,71]. In the absence of mineralized structures, there is no birefringence and the picture looks totally black. Under identical light conditions, areas appearing more birefringent contain a much larger proportion of crystalline calcium carbonate [70,72]. The intensity of birefringence of each shell was used as a proxy for mineralization level for the three $p$CO$_2$ treatments. It was quantified from pictures by using ImageJ software [69]. Pictures of polarized shells were first transformed into grayscale images. A mean gray value (in pixels) was determined for each birefringent zone. All birefringent zones of the shell were compiled to obtain a global mean gray value, giving the intensity of the birefringence of the whole shell.

**Statistics**

All statistical analyses were performed using the free software R 2.15.0 version [73]. Normality and homoscedasticity of the data were first checked using Shapiro and Levene tests, respectively. Due to the non-normality and heterogeneity of variance, the influence of $p$CO$_2$ on morphological variables, deformity indices and birefringence intensity was analyzed using the non-parametric Kruskal-Wallis test followed by the Dunn post-hoc test [74]. A Chi-squared ($\chi^2$) test followed by G-tests (likelihood-ratio test) [75] were used to compare percentages of anomaly between the three $p$CO$_2$ conditions.

**Results**

**Morphological variables**

$p$CO$_2$ significantly affected length, height and surface area of the hatched larvae (Figure 2, Table 2). These morphological variables are related to each other and were generally influenced in the same way by $p$CO$_2$. Length and height were the highest at 390 $\mu$atm and significantly decreased with increased $p$CO$_2$. Larvae collected at 750 and 1400 $\mu$atm $p$CO$_2$ showed a decrease of 5.3% and 5.9% in length, respectively, and 2.6% and 4.5% in height, respectively, compared to control larvae (390 $\mu$atm).

Similarly, the greatest shell surface area was observed at 390 $\mu$atm $p$CO$_2$, but then significantly decreased by 6.2% and 11.2% at 750 and 1400 $\mu$atm $p$CO$_2$, respectively.

**Abnormalities**

Abnormalities in larvae were observed as notches located close to the shell aperture (Figure 3). The percentage of abnormal larvae increased with increased $p$CO$_2$ and ranged from 6.7 to 26.5% (Figure 4; $\chi^2$ test, p<0.05). Abnormalities were 1.5- and 4-fold at $p$CO$_2$ levels of 750 and 1400 $\mu$atm, respectively, than at

---

**Table 2. Effect of $p$CO$_2$ on morphological variables, abnormality indices and intensity of birefringence.**

|                      |                 |   |     |
|----------------------|-----------------|---|-----|
|                      | **Kruskal-Wallis Test** | df | H   | p    |
| Length               | 2               | 37.353 | < 0.001 |
| Height               | 2               | 16.235 | < 0.001 |
| Surface area         | 2               | 30.106 | < 0.001 |
| Abnormality index    | 2               | 6.046  | 0.049  |
| Intensity of birefringence | 2         | 14.562 | < 0.001 |

Legend: Summary of the non-parametric Kruskal-Wallis tests testing the effect of $p$CO$_2$ on each morphological variable, abnormality index and birefringence intensity.

doi:10.1371/journal.pone.0093021.t002

**Figure 3. Shell abnormalities.** Different intensities of shell abnormalities observed among samples. A, B, C, and D show whole larvae whereas E, F, G, and H show the detail of their respective abnormalities.

doi:10.1371/journal.pone.0093021.g003
Furthermore, different intensities of shell abnormalities were observed with variation in notch acuteness. The deformity index varied between 0.03 (390 μatm $p$CO$_2$) and 0.17 (1400 μatm $p$CO$_2$). Although the Kruskal-Wallis test showed a marginally significant $p$CO$_2$ effect ($p = 0.049$; Table 2), the pairwise Dunn post-hoc test did not detect significant differences between the three $p$CO$_2$ treatments ($p > 0.05$).

Among the abnormal larvae observed under polarized microscopy (see below), some showed abnormalities which appeared less birefringent, and even not mineralized, as revealed by the lack of birefringence in these parts of the shell (Figure 5).

Shell mineralization

Pictures taken under polarized light (Figure 6) suggest that the intensity of the birefringence decreased with increased $p$CO$_2$. The measure of birefringence intensity using the mean gray values estimated for each shell clearly confirmed this relationship (Table 2). Mineralization was greatest at 390 μatm $p$CO$_2$, intermediate at 750 μatm $p$CO$_2$ and lowest at 1400 μatm $p$CO$_2$ (Figure 6).

Discussion

In our study, the effects of elevated $p$CO$_2$ were integrated from embryo formation and throughout embryonic stages up until the release of veliger larvae. The integration of $p$CO$_2$ effects across all developmental stages, from fertilization to settlement and beyond, is particularly instructive because early life stages may respond to environmental stressors in a different way than adults. Acute exposures of early life stages have shown various effects in growth or feeding performances [76,77], but these may not represent field conditions. Results obtained from acclimation to high $p$CO$_2$ across different life stages often differ from those arising from acute exposure of a given stage. For example, it has been shown that oyster D-veliger larvae grown from eggs fertilized at elevated $p$CO$_2$ were more drastically affected than those first produced at ambient $p$CO$_2$ and then reared later (embryo stages) at high $p$CO$_2$ [78]. Keeping the parents under different $p$CO$_2$ conditions before fertilization and until larval release allowed embryos to develop entirely under a given level of stress. To our knowledge, only Dupont et al. [79] on sea urchins, Parker et al. [80] on mollusks and Vehmaa et al. [81] on copepods acclimated adults to high $p$CO$_2$ during reproductive conditioning before studying larvae in the same $p$CO$_2$ conditions.

$p$CO$_2$ effects were first investigated on the shell morphology of the hatched larvae. The size (length, height and surface area) of the released larvae decreased with increased $p$CO$_2$. Length and height were not significantly different between the $p$CO$_2$ levels of 750 and 1400 μatm, whereas the shell surface area progressively decreased with increased $p$CO$_2$ from 390 to 1400 μatm. Although in some rare cases, elevated $p$CO$_2$ does not affect larval morphology and growth [82,83], a correlation between high $p$CO$_2$ and smaller size has been demonstrated in most bivalve and gastropod species studied to date (see review in Table 3), with $p$CO$_2$ sometimes affecting the shape of the larval shell [41]. As observed here for length and height (ca. –5% in the two high $p$CO$_2$ treatments), shell lengths of *Crassostrea gigas* veliger larvae are smaller under elevated $p$CO$_2$, but are similar in conditions of pH lowered by 0.4 and 0.7 pH units, a range corresponding to our pH values [32]. Smaller size and delayed shell growth can be
attributed to problems in shell deposition, delayed periostracum formation and/or increased shell dissolution, as hypothesized by Watson et al. [84].

Such processes may lead to developmental abnormalities and to an increase in their frequency under elevated pCO2. Some (7%) C. fornicata larvae in the control group (390 μatm pCO2) showed mild shell abnormalities in the form of a notch close to the aperture. The frequency of this abnormality increased under high pCO2, being 1.5-fold more frequent at 750 μatm pCO2 and reaching 26% at 1400 μatm pCO2. The intensity of shell abnormality, estimated using the deformity index, did not vary significantly with increasing pCO2, although more pronounced shell deformities were detected at the highest pCO2 condition (1400 μatm). The occurrence of abnormal shells is a common response in mollusk larvae exposed to elevated pCO2. In bivalves for example, abnormalities can occur as shell hinge and edge deformities [85], irregular-shaped shells [40] or protruding mantles [86]. The frequency of abnormalities can reach 40% of shell deformities in Pecten maximus larvae reared at 1250 μatm pCO2 [31]. In gastropods, larval shells are considered abnormal when shells are too small to fully cover the soft body [34] or when dissolution zones are observed at the edge of the aragonitic larval shell [33], with frequencies of abnormality ranging from 20% in Haliotis discus hannai at 1650 μatm pCO2 [33] to 40% in Haliotis kamtschatkana at 800 μatm pCO2 [36]. At extremely high pCO2 (> 1700 μatm), some abalone larvae are even unable to precipitate a calcareous shell [36,44].

Such abnormalities may be due to different processes: (i) the production of amorphous CaCO3 may be affected by damage to embryonic ectodermic cells and/or (ii) seawater corrosion may induce shell dissolution, affecting the strength and calcification of some parts of the shell [32]. Here, the mineralization level of larval shells was investigated at each pCO2 level by observing the veliger aragonitic shell under polarized light [70]. The characteristic dark cross observed in each larval shell indicated a radial arrangement of aragonite crystals [72] and did not have been considered as non-crystalline zones. The intensity of birefringence was used as a proxy for mineralization because increases in birefringence reflect increases in crystalline structure and calcification of the shell. Observed under polarized light, abnormalities appeared less birefringent than the rest of the shell, suggesting that deformities were likely less calcified as proposed by Barros et al. [32]

The birefringence intensity of the larval shells decreased with increased pCO2, and was significantly lower at 1400 μatm pCO2. This drop in birefringence revealed a decrease in calcification, which may be related to a less mineralized matrix [87], or more likely to a reduction in shell thickness [72]. Our data did not allow us to discriminate between these two possibilities, but previous studies have already reported a decrease in shell thickness under high pCO2 in bivalve larvae. For example, using scanning electron microscopy measurements, Gazeau et al. [39] showed a decrease in thickness of 12% in Mytilus edulis larvae at 745 μatm pCO2. Talmage and Gobler [43] report a decrease in thickness of Mercenaria mercenaria (~43%) and Argopecten irradians (~47.5%) larval shells after 17 days of development at 1500 μatm pCO2, which was associated with an impact on the integrity and the
connectedness of the hinge structure. A decrease of 5.7% in shell thickness of brooded larvae of the oyster Ostrea chilensis has also been observed following a decrease in pH (down to 6.56) within the mother’s pallial cavity due to valve closure under salinity stress [50].

The decrease in larval size and mineralization level of the shell may be due to reduced CaCO₃ saturation or hypercapnic suppression of metabolic pathways involved in the calcification process [9]. Very little is known about the conditions occurring during intracapsular development and how acidified seawater can affect encapsulated embryos. Previous studies have shown that egg capsules of some gastropods, including C. fornicata, are permeable to water and ions (e.g. [48,71,88]) and it can be assumed that the capsule wall in C. fornicata is almost impermeable to gas because of its low O₂ conductance [89]. Under “normal” conditions, embryos of C. fornicata will progressively be exposed to hypoxia [89] and hypercapnia via their respiration. This may lead to low intracapsular pH, as reported in other gastropod species [90],

| Reproductive mode | Species (veliger stage) | Measured parameter | pH conditions | pCO₂ conditions (patm) | % decrease due to pCO₂ | Study |
|-------------------|-------------------------|--------------------|--------------|-----------------------|------------------------|-------|
| Broadcast spawner | Crassostrea gigas      | shell length       | pH₅BS 7.4    | 2268                  | decrease               | [40]  |
| Broadcast spawner | Crassostrea gigas      | shell length       | pH₅BS 7.8    | 1000                  | 16%                    | [78]  |
| Broadcast spawner | Crassostrea gigas      | shell length and height | pH₅BS 7.76 – 7.37 | 1386 – 3573 | 10.6%               | [32]  |
| Broadcast spawner | Crassostrea gigas      | shell area         | pH₅BS 7.7 – 7.4 | 1497 – 2386 | 18.7 – 29%            | [101] |
| Broadcast spawner | Crassostrea gigas      | shell area         | pH₅BS 7.8 7.4 | ~ 650 – 1500 | 16.7%                | [38]  |
| Broadcast spawner | Crassostrea virginica  | shell area         | pH₅BS 7.76 7.8 | 840                   | 16%                    | [94]  |
| Broadcast spawner | Saccostrea glomerata   | shell length       | -            | 1000                  | 22%                    | [31]  |
| Broadcast spawner | Saccostrea glomerata   | shell length       | pH₅BS 7.8    | 1000                  | 34%                    | [78]  |
| Broadcast spawner | Saccostrea glomerata   | shell length       | pH₅BS 7.9    | 856                   | 31.6 – 1.3%            | [80]  |
| Broadcast spawner | Saccostrea glomerata   | shell length       | pH₅BS 7.8 – 7.6 | 508.8 – 775.6 | 8.7 – 6.3%           | [84]  |
| Broadcast spawner | Saccostrea glomerata   | shell height       | pH₅BS 7.8 – 7.6 | 508.8 – 775.6 | 7.5 – 5.1%            | [84]  |
| Broadcast spawner | Mytilus edulis         | shell length       | pH₅BS 7.8    | 1200                  | 4.5 – 6%               | [39]  |
| Broadcast spawner | Mytilus edulis         | shell thickness   | pH₅BS 7.8    | 1200                  | 12%                    | [39]  |
| Broadcast spawner | Mytilus edulis         | shell area         | pH₅BS 7.6    | 1388 – 1493 | 7 – 8%               | [16]  |
| Broadcast spawner | Mytilus galloprovincialis | shell length and height | pH₅BS 7.4 | 2000 | 26 – 20% | [86] |
| Broadcast spawner | Capparina dentata      | shell length       | pH₅BS (?) 7.5 | 1627                  | 10%                    | [85]  |
| Broadcast spawner | Macoma balatica       | shell length       | pH₅BS 7.7 – 7.2 | 1700 – 4400 | 4.3 – 8.5%           | [103] |
| Broadcast spawner | Mercenaria mercenaria | shell length       | pH₅BS 7.8 – 7.5 | 1500                  | 16.2 – 16.9%          | [42]  |
| Broadcast spawner | Mercenaria mercenaria | shell thickness   | pH₅BS (?) 7.5 | 1500                  | 43%                    | [43]  |
| Broadcast spawner | Mercenaria mercenaria | shell diameter    | pH₅BS (?) 7.5 | 1500                  | 43%                    | [43]  |
| Broadcast spawner | Hallois discus hannai | shell length       | pH₅BS 7.71   | 1050                  | 25.0%                  | [33]  |
| Broadcast spawner | Ostrea lurida          | shell growth       | pH₅BS 7.76   | 1000                  | 5 – 14%                | [104] |
| Egg masses         | Littorina obtusata     | shell length       | pH₅BS (?) 7.6 | -                     | 10%                    | [41]  |
| Egg masses         | Stylochelus striatus   | shell area         | pH₅BS (?) 7.6 | -                     | 24 – 36%               | [52]  |
| Egg                | Sepia officinalis      | total weight       | pH₅T 7.84 – 7.60 | 750 – 1430 | no change           | [53]  |
| Encapsulation + brooding | Crepipatella dilatata | shell thickness   | pH₅BS (?) 6 | -                     | 30%                    | [51]  |
| Encapsulation + brooding | Crepidula fornicata | shell length       | pH₅T 7.8 – 7.6 | 750 – 1400 | 5.3 – 10.7% | Present study |
| Encapsulation + brooding | Crepidula fornicata | shell height       | pH₅T 7.8 – 7.6 | 750 – 1400 | 2.6 – 13.1% | Present study |

p₅BS: pH on the NBS scale; pH₅T: pH on the total scale; pH₅SW: pH on the seawater scale.
doi:10.1371/journal.pone.0093021.t003

The decrease in larval size and mineralization level of the shell may be due to reduced CaCO₃ saturation or hypercapnic suppression of metabolic pathways involved in the calcification process [9].
without altering development [57]. Under elevated \( pCO_2 \), diffusion of more protons (\( H^+ \)) from external seawater to the intracapsular medium may alter intracapsular carbonate chemistry, thus enhancing metabolic acidosis. Similar acidosis can be observed under low salinity stress. For example, a decrease in pH to 6.4 recorded within the pallial cavity of the calyptradid Cerastoderma dilatata [47,51] led to the partial shell decalcification of the brooded encapsulated embryos [51]. This decalcification may cause the release of some carbonate ions (\( CO_3^{2-} \)), which could bind to free \( H^+ \) to form bicarbonate ions (\( HCO_3^- \)), thus buffering the intracapsular acidosis and limiting the drastic pH effects on larval metabolism. This potential buffering role, in combination with a decrease in the \( CaCO_3 \) saturation state, is likely to affect shell mineralization and calcification. Such processes have been suggested to buffer acidosis resulting from anaerobiosis in C. fornicata [91], and may also explain our observations. Alternative mechanisms that can decrease the intracapsular acidosis, such as the active excretion of \( H^+ \) out of the capsule through a proton pump, as shown in the cephalopod Sepia officinalis [92], need to be investigated.

Altogether, our results show that, despite the potential protective role provided by encapsulation and brooding, elevated seawater \( pCO_2 \) affected the shells of the released larvae in C. fornicata. Embryos of C. fornicata were affected by high \( pCO_2 \) during their intracapsular development. However, the overall low abnormality rate and low decrease in size suggested they were likely less affected than other mollusk early life stages. The natural exposure of embryos to low intracapsular pH as demonstrated in cephalopod eggs (pH on the seawater scale of the perivitelline fluid of ca. 7.35 at 16°C [53]) and gastropod capsules (pH of the intracapsular fluid lower than 7 [90]) could confer to C. fornicata larvae some resilience to elevated \( pCO_2 \) levels. Indeed, it has been shown that bivalves naturally exposed to high \( pCO_2 \) conditions in their habitat (due to high levels of benthic respiration or to seawater naturally enriched in \( CO_2 \)) are less affected by ocean acidification than other mollusk species [93,94]. Further studies are however needed to determine the pH of the intracapsular fluid in C. fornicata, and how it will be affected under future scenarios of ocean acidification.

The effects of elevated \( pCO_2 \) observed on C. fornicata larvae released from capsules suggest critical ecological consequences for their subsequent planktonic life and benthic settlement. Production of smaller larvae with weaker shell strength may increase vulnerability of larvae to predation and physical damages [37]. Furthermore, larval physiologically stressed during their development; for various abiotic factors may delay metamorphosis and settlement [94], staying longer in the water column which lead them to be more exposed to predators and diseases [94,95,96]. In addition, reduced size in early developmental stages may affect the juvenile survivorship and fitness [97,98]. Given these consequences on the early life stages of C. fornicata, \( pCO_2 \) may influence its invasion dynamics in its introduction range via reproductive success, larval survival and dispersal, and settlement success. Further studies are required to fully understand the interactions between climate change and biological invasions [99,100]. In particular, more studies on early life stages and particularly the transition processes between them (e.g. metamorphosis) are needed to identify the potential tipping points, the demographic bottlenecks and the global resistance of non-native species in the context of ocean acidification.

**Acknowledgments**

The authors thank the “Marine Operations and Services Department” at the Station Biologique de Roscoff for underwater sampling. We also thank the “Multicellular Marine Models” staff for providing microalgae and their help for building the aquarium system. We are grateful to Stéphanie Auzoux-Bordenave and Nathalie Wessel for hosting us at the Station de Biologie Marine de Concarneau and for their help in acquiring the polarized light images. We also thank Frédérique Viard and Thomas Broquet for helpful discussions at various stages of this work. We are grateful to the editor, Pauline Ross, and the two anonymous reviewers for their helpful and constructive comments which greatly improved this manuscript.

**Author Contributions**

Concepted and designed the experiments: FN TC DD SM. Performed the experiments: FN EL FB. Analyzed the data: FN EL. Contributed reagents/materials/analysis tools: FN EL TC SM. Wrote the paper: FN EL TC FB DD SM.

**References**

1. Pechenik JA (1999) On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. Mar Ecol-Prog Ser 177: 349–367.
2. Peck LS, Souster T, Clark MS (2013) Juveniles are more resistant to warming than adults in 4 species of Antarctic marine invertebrates. PLoS One 8: e66033.
3. IPCC (2013) Working Group I Contribution to the IPCC Fifth Assessment Report - Climate Change 2013: The Physical Science Basis - Summary for Policymakers.
4. Feely RA, Doney SC, Cooley SR (2009) Ocean acidification: present conditions and future changes in a high-CO2 world. Oceanography: 22: 36–47.
5. Portner HO (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist’s view. Mar Ecol-Prog Ser 373: 203–217.
6. Widdicombe S, Spicer JI (2008) Predicting the impact of ocean acidification on marine invertebrates early life-history - What we know, what we need to know and what we can do. Biogeosciences Discuss. 6: 3109–3131.
7. Po¨rtner H-O (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol-Prog Ser 373: 203–217.
8. Putnam HM, Mayfield AB, Fan TY, Chen CS, Gates RD (2013) The effects of elevated \( pCO_2 \) on invertebrates early life-history - What we know, what we need to know and what we can do. Biogeosciences Discuss. 6: 3109–3131.
9. Byrne M (2011) Impact of ocean warming and ocean acidification on marine invertebrates. Mar Ecol-Prog Ser 475: 85–92.
10. Bechmann RK, Taban RC, Westerlund S, Godal BF, Armborg M, et al. (2011) Effects of ocean acidification on early life stages of shrimp (Pandalus borealis) and mussel (Mytilus edulis). J Toxic Environ Health-Part A 74: 424–438.
11. Range P, Pilø D, Ben-Hamoudou R, Chicharo M, Matias D, et al. (2012) Seawater acidification by \( CO_2 \) in a coastal lagoon environment: effects on life history traits of juvenile mussels Mytilus galloprovincialis. J Exp Mar Biol Ecol 424: 89–98.
12. Dupont S, Pourry M, Thordyke M (2009) Impact of \( CO_2 \)-driven ocean acidification on invertebrates early-life-history - What we know, what we need to know and what we can do. Biogeosciences Discuss. 6: 3109–3131.
13. Kurihara H (2008) Effects of \( CO_2 \)-driven ocean acidification on the early developmental stages of invertebrates. Mar Ecol-Prog Ser 373: 275–284.
14. Nguyen HD, Doo SR, Sears NA, Byrne M (2012) Noncalcifying larvae in a changing ocean: warming, not acidification/hypercapnia, is the dominant stressor on development of the sea star Mitheda calcar. Glob Change Biol: 2466–2476.
15. Panec C, Schölgl P, Havenhand J (2013) Larval development of the barnacle Amphibalanus imparvis responds variably but robustly to near-future ocean acidification. ICES J Mar Sci 70: 803–811.
16. Dupont S, Pourry N, Thordyke M (2010) What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? Estuar Coast Shelf Sci 89: 182–185.
17. Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, et al. (2004) Impact of anthropogenic \( CO_2 \) on the \( CaCO_3 \) system in the oceans. Science 305: 362–366.
18. Kroecker KJ, Kordal RL, Cim MN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett 13: 1419–1434.
32. Barros P, Sobral P, Range P, Chicharo L, Matias D (2013) Effects of seawater acidification on early development and molecular plasticity in the Mediterranean sea urchin Paracentrotus lividus exposed to CO2-driven acidification. J Exp Biol 216: 1537–1568.

25. Egilsdottir H, Spicer JI, Rundle SD (2009) The effect of CO2 acidified seawater on larval settlement of mussels. J Exp Mar Biol Ecol 373: 240–246.

27. Gutowska MA, Melzner F (2009) Abiotic conditions in cephalopod (Sepia officinalis) eggs: embryonic development at low pH and high pCO2. Mar Biol 156: 515–519.

28. Moran D, Stottrup JG (2011) The effect of carbon dioxide on growth of the European abalone Haliotis tuberculata. Aquat Toxicol 102: 24–30.

29. Munday PL, Gagliano M, Donelson JM, Dixson DL, Thorrold SR (2011) Acclimation to elevated CO2 does not enhance survival or growth of the reef fish species Plectorhinchus garricki. J Mar Biol Assoc UK 91: 807–821.

44. Byrne M, Ho M, Wong E, Soars NA, Selvakumaraswamy P, et al. (2010) Unshelled abalone and corroded urchins: development of marine calcifiers in a high CO2 world. P Roy Soc Lond, B Biol 278: 2376–2383.

49. Pechenik JA (1983) Egg capsules of the slipper limpet Crepidula fornicata (L.) protect against low-salinity stress. J Exp Mar Biol Ecol 72: 2287–2295.

76. Chan KYK, Grunbaum D, O'Donnell MJ (2011) Effects of ocean-acidification-induced morphological changes on larval swimming and feeding. J Exp Biol 214: 3857–3867.
78. Parker LM, Ross PM, O'Connor WA (2010) Comparing the effect of elevated pCO2 and temperature on the fertilization and early development of two species of oysters. Mar Biol 157: 2435–2452.

81. Vehmaa A, Brutenmark A, Engstrom-Ost J (2012) Maternal effects may act as an adaptation mechanism for copepods facing pH and temperature changes. PLoS One 7: e48538.

82. Gazeau F, Gattuso J-P, Greaves M, Elderfield H, Peene J, et al. (2011) Effect of carbonate chemistry alteration on the early embryonic development of the Pacific oyster (Crassostrea gigas). PLoS One 6: e23010.

88. Maeda-Martinez AN (2008) Osmotic and ionic concentration of the egg capsule fluid of Crassostrea gigas (Gastropoda, Prosobranchia, Volutidae). Cah Biol Mar 15: 215–227.

95. Hickman CS (2001) Evolution and development of gastropod larval shell morphology: experimental evidence for mechanical defense and repair. Evolution & Development 3: 18–23.

100. Lenz M, da Gama BAP, Gerner NV, Gobin J, Groner F, et al. (2011) Non-native marine invertebrates are more tolerant towards environmental stress than taxonomically related native species: Results from a globally replicated study. Environ Res 111: 943–952.

101. Ko GWK, Chan VBS, Dineshram R, Choi DKS, Li AJ, et al. (2013) Larval development and metamorphosis in Balanus amphitrite Darwin (Cirripedia; Thoracica): significance of food concentration, temperature and nucleic acids. J Exp Mar Biol Ecol 263: 125–141.

102. White MM, McCorkle DC, Mullineaux LS, Cohen AL (2013) Early exposure to elevated pCO2 induces physiological tolerance to ocean acidification in a marine mussel. PLoS One 8: e5661.

103. Jansson A, Norkko J, Norkko A (2013) Effects of reduced pH on the growth, metabolism, and metabolic rates of bay scallops (Argopecten irradians) to multiple climate change stressors. Aquat Biol 4: 225–233.

104. Hettinger A, Sanford E, Hill T, Hosfeld J, Russell A, et al. (2013) The influence of food supply on the response of Olympia oyster larvae to ocean acidification. Biogeosciences 10: 6161–6184.