Impact of seawater carbonate chemistry on the calcification of marine bivalves

J. Thomsen\textsuperscript{1,2}, K. Haynert\textsuperscript{1,3}, K. M. Wegner\textsuperscript{4}, and F. Melzner\textsuperscript{1}

\textsuperscript{1}Marine Ecology, GEOMAR Helmholtz Centre for Ocean Research, Kiel, Germany
\textsuperscript{2}Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 93092-0202, USA
\textsuperscript{3}J. F. Blumenbach Institute for Zoology and Anthropology, Georg August University Göttingen, 37073 Göttingen, Germany
\textsuperscript{4}Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Wadden Sea Station Sylt, 25992 List, Germany

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Correspondence to: J. Thomsen (jothomsen@ucsd.edu)

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Abstract

Bivalve calcification, particular of the early larval stages is highly sensitive to the change of ocean carbonate chemistry resulting from atmospheric CO$_2$ uptake. Earlier studies suggested that declining seawater [CO$_3^{2-}$] and thereby lowered carbonate saturation affect shell production. However, disturbances of physiological processes such as acid-base regulation by adverse seawater $p$CO$_2$ and pH can affect calcification in a secondary fashion. In order to determine the exact carbonate system component by which growth and calcification are affected it is necessary to utilize more complex carbonate chemistry manipulations. As single factors, $p$CO$_2$ had no and [HCO$_3^-$] and pH only limited effects on shell growth, while lowered [CO$_3^{2-}$] strongly impacted calcification. Dissolved inorganic carbon (C$_T$) limiting conditions led to strong reductions in calcification, despite high [CO$_3^{2-}$], indicating that [HCO$_3^-$] rather than [CO$_3^{2-}$] is the inorganic carbon source utilized for calcification by mytilid mussels. However, as the ratio [HCO$_3^-$]/[H$^+$] is linearly correlated with [CO$_3^{2-}$] it is not possible to differentiate between these under natural seawater conditions. Therefore, the availability of [HCO$_3^-$] combined with favorable environmental pH determines calcification rate and an equivalent of about 80 $\mu$mol kg$^{-1}$ [CO$_3^{2-}$] is required to saturate inorganic carbon supply for calcification in bivalves. Below this threshold biomineralization rates rapidly decline. A comparison of literature data available for larvae and juvenile mussels and oysters originating from habitats differing substantially with respect to prevailing carbonate chemistry conditions revealed similar response curves. This suggests that the mechanisms which determine sensitivity of calcification in this group are highly conserved. The higher sensitivity of larval calcification seems to primarily result from the much higher relative calcification rates in early life stages. In order to reveal and understand the mechanisms that limit or facilitate adaptation to future ocean acidification, it is necessary to better understand the physiological processes and their underlying genetics that govern inorganic carbon assimilation for calcification.
The release of CO$_2$ by fossil fuel combustion and its subsequent absorption by the ocean has a fundamental impact on its carbonate chemistry. CO$_2$ uptake increases the dissolved inorganic carbon (C$_T$) in particular concentrations of seawater CO$_2$ (or partial pressure, pCO$_2$) and HCO$_3^-$- . These changes cause an acidification of the oceans and results in a decline of [CO$_3^{2-}$]. Numerous studies demonstrated that ocean acidification interferes with the calcification process in many marine organisms (e.g. Kroeker et al., 2010; Gazeau et al., 2014). It has been hypothesized that calcifiers are mainly impacted by the decline in [CO$_3^{2-}$] and the corresponding decrease of the calcium carbonate saturation state $\Omega$. Strong undersaturation ($\Omega < 1$) with respect to calcium carbonate is expected to cause dissolution of existing calcium carbonate structures or can impact shell formation directly (Miller et al., 2009; Thomsen et al., 2010; Rodolfo-Metalpa et al., 2011; Pansch et al., 2014).

However, whereas a large number of studies investigated the general response of calcifiers to ocean acidification, only few tried to disentangle the mechanistic response to specific carbonate chemistry species to test this hypothesis (Jury et al., 2010; Bach et al., 2011; de Putron et al., 2011; Suffrian et al., 2011; Gazeau et al., 2012; Keul et al., 2013; Haynert et al., 2014). In fact, studies performed with multicellular heterotrophs that do not compensate the ocean acidification induced decline in extracellular pH by means of HCO$_3^-$ accumulation, revealed a strong correlation of calcification rate with ambient seawater [CO$_3^{2-}$]. In contrast, calcification rate increased as a result of higher [CO$_3^{2-}$] in the extracellular/calcifying fluids in pHe regulating animals (Gutowska et al., 2010, Maneja et al., 2013). Although these findings match the general hypothesis of the sensitivity of calcifiers to ocean acidification it is unclear why CO$_3^{2-}$ plays such an important role in the biomineralization process in marine organisms (Bach, 2015). [CO$_3^{2-}$] only contributes less than 10% to the oceanic C$_T$ pool, whereas HCO$_3^-$ contributes >90%. Furthermore, its availability is highly variable due to the strong dependency on seawater pH and concentrations drastically decline at pH
values below 8.5. Whereas the change in $[\text{CO}_3^{2-}]$ and the related change in saturation state $\Omega$ has been suggested to impact calcification directly, reductions in seawater pH and increases in $p\text{CO}_2$ affect physiological processes such as acid-base regulation. It may thereby impact calcification in a secondary fashion via reductions in scope for growth (Melzner et al., 2013; Dorey et al., 2013).

Calcification involves intracellular production of an amorphous calcium carbonate (ACC) precursor which is exocytosed from the calcifying epithelia and transported to the site of shell formation (Weiner and Addadi, 2011). The precursor is then integrated into an organic matrix framework and remains either transiently in the amorphous state or crystallizes into a specific polymorph such as aragonite or calcite depending on the specific properties of matrix proteins (Weiss et al., 2002; Jacob et al., 2008). For the intracellular production of ACC relatively large amounts of carbonate equivalents need to be transported into the cells. This transport may potentially be accomplished by either uptake of seawater via endocytosis as suggested for foraminifera (Bentov et al., 2009) or direct $\text{HCO}_3^- / \text{CO}_3^{2-}$ carbonate transport across the cell membranes performed by a set of specific proteins and coupled to anion co-transport or cation exchange (Parker and Boron, 2013). Independent of the exact mechanisms, calcification of bivalves in general and their larval stages in particular is especially sensitive to ocean acidification (Talmage and Goble, 2010; White et al., 2013; Gazeau et al., 2014).

Due to the high sensitivity of calcification to external seawater carbonate chemistry it is important to consider the environmental conditions the organism is exposed to. In open ocean habitats, $p\text{CO}_2$ and pH conditions are relatively stable (Hofmann et al., 2011). Furthermore, under fully saline conditions ($S = 32–37$) seawater titratable alkalinity ($\text{A}_T$) with its main components $[\text{HCO}_3^-]$ and $[\text{CO}_3^{2-}]$ is nearly linearly correlated with salinity, ranging between 2200 and 2400 µmol kg$^{-1}$ for most ocean regions (Millero et al., 1998). In contrast, much more variable carbonate chemistry ($p\text{CO}_2$, pH and $\text{A}_T$) is encountered in many coastal ecosystems and variability will increase even further in future (e.g. Hofmann et al., 2011; Cai et al., 2011; Melzner et al., 2013). In
estuaries freshwater inputs lead to significantly lower salinity which reduces alkalinity (Miller et al., 2009). The Baltic Sea is an example of a brackish water habitat with eastward declining salinity and alkalinity due large freshwater inputs form the surrounding land masses. Although salinity decreases to almost 0, the high riverine $A_T$ load causes relatively high $A_T$ values that are significant higher than expected from dilution of seawater with distilled water (1200–1900 µmol kg$^{-1}$, Beldowski et al., 2010). Nevertheless, due to the comparatively low $A_T$ even small increases in atmospheric $pCO_2$ will cause low saturation or even undersaturation with respect to aragonite in the Baltic and estuaries in general (Miller et al., 2009; Melzner et al., 2013). Coastal, brackish habitats might therefore be hotspots for bivalve vulnerability to future ocean acidification.

This study contributes to an understanding of the mechanisms and sensitivities of calcification in bivalves, with a focus on larval stages. For this purpose, experiments with strong modifications of the specific carbonate system parameters and meta-analyses of the calcification response of bivalves exposed to changes in carbonate chemistry have been conducted. We hypothesize that the calcification process in bivalves is highly dependent on external seawater carbonate chemistry and in particular on $HCO_3^-$ availability as a substrate and favorable pH conditions.

2 Material and methods

2.1 Animal collection and maintenance

Adult and juvenile *M. edulis* specimens were collected from 1 m depth in Kiel Fjord, Baltic Sea. For experiments with larvae, adults were transferred into a flow-through setup over night and spawning was induced the next day. Juveniles were directly placed in the experimental units after measurement of initial length and wet mass. All experiments were conducted in constant temperature rooms at GEOMAR in Kiel, Germany. Larvae or juveniles were placed in 500 mL experimental units which were aerated with humidified air with constant $pCO_2$ levels (see details below).
2.2 Experimental set up

2.2.1 Exp. 1: juvenile experiment

For the experiment on the calcification response of juvenile mussels, individuals with an initial mean shell length of 706 ± 37 µm were collected in Kiel Fjord and transferred into experimental units filled with 0.2 µm seawater. The experiment lasted for three weeks and specimens were fed twice a day with a *Rhodomonas* suspension resulting in initial concentrations of 25 000 cells mL\(^{-1}\). Algae were cultured in artificial seawater supplemented with Provasiolis’ enriched seawater (PES) in 7 L plastic bags under constant illumination and aeration (for details see Thomsen et al., 2010). The densities of algae cultures was measured daily using a particle counter (Coulter Counter, Beckmann GmbH, Germany) in order to calculate the volume which was needed to be added to reach desired densities in experimental units. Water was exchanged twice a week in order to avoid accumulation of waste products and significant influence of microbial activity and calcification on seawater alkalinity. The experiment was terminated by removing specimens from the experimental units after 15 days. Somatic tissues and shells were separated, dried at 60°C over night, shell lengths were measured by taking pictures using a stereo microscope (Leica F165, Leica Microsystems GmbH, Wetzlar, Germany) which were analyzed using ImageJ 1.43u. Shell mass was determined using a balance (Sartorius, Germany). Initial shell mass was calculated from a regression of measured shell length and shell mass (shell mass (mg) = 23.8 × SL(mm)^2.75, \(R^2 = 0.95\), \(n = 31\), shell length range 6–12 mm). Calcification was calculated by subtraction of the initial shell mass from final shell mass. The organic content of shells was not considered which leads to a minor overestimation of calcification rates (<10%). During the experiment, control mussel shell length and mass increased by a factor of 1.6 and 2.6, respectively.
2.2.2 Exp. 2 + 3: larval experiments

For the larval experiments, adult individuals were placed in separate 800 mL beakers filled with 0.2 µm filtered seawater and gently aerated with pressurized air. Spawning was induced by rapidly increasing seawater temperature by 5 °C above ambient temperate using heaters. Spawning usually started after 20 to 40 min. following heat shock treatment. Egg densities were determined by counting three replicated sub samples using a stereomicroscope. Embryos were transferred into the experimental units once the 4–8 cell stage was reached at an initial density of 10 embryos mL⁻¹. Experimental duration of the larval experiment was restricted to the lecithotrophic phase and larvae were not fed. After the D-veliger stage was reached in all treatments (day 4), larval samples were taken and preserved with 4 % paraformaldehyde and buffered using 10 mM NaHCO₃.

2.3 Carbonate chemistry manipulation

The dependency of juvenile and larval calcification on seawater carbonate chemistry speciation was determined by adjusting seawater alkalinity using 1 M HCl and 1 M NaHCO₃ (see details Table 1) and aeration with different pCO₂ levels (juveniles, Exp. 1: 390 and 4000 µatm, larval: Exp. 2: 390 and 2400 µatm, larval Exp. 3: 0 and 390 µatm). pCO₂ treatments were realized using the central gas mixing facility of GE-OMAR (390, 2400 and 4000 µatm), CO₂ free air was generated by using a soda lime CO₂ scrubber (Intersorb Plus™, Intersurgical, Germany).

Carbonate chemistry was constrained by measuring seawater pH and either A_T in the juvenile (Exp. 1) or C_T in the larval experiment (Exp. 2 + 3). pH was determined either on NBS scale using a WTW 340i pH m or on the total scale using seawater buffers mixed for a salinity of 15 and measured using a 626 Metrohm pH m. A_T was determined with a 862 Compact Titrosampler (Metrohm, USA), C_T using an AIRICA C_T analyzer (Marianda, Germany). Carbonate chemistry parameters were calculated using the CO2sys program. For calculations, the KHCO₃ dissociation constant (Dickson
et al., 1990) and the carbonate system dissociation constants K1 and K2 (Mehrbach et al., 1973, refitted by Dickson and Millero, 1987) were used.

### 2.4 Calculation of larval and juvenile *Mytilus* calcification and metabolic rates

Calcification rates were calculated for ontogenetic stages ranging from the formation of the first larval $D$ shell to the juvenile stage two years after settlement. Larval calcification was calculated (1) assuming a total of 24 h required for $D$ shell formation and (2) for later veliger stages using a shell length and mass correlation for *M. edulis* larvae (Sprung, 1984a) and the maximal increment of larval shell length during the planktonic phase under optimal feeding and temperature conditions (11.8 µm day$^{-1}$, Sprung, 1984a). Respiration rates of similar sized larval stages were calculated from the oxygen consumption rates published by Sprung (1984b) and converted into nmol ind$^{-1}$ h$^{-1}$. Other studies have obtained similar relationships for calcification (Jespersen and Olsen, 1982) and respiration rate (Riisgard and Ranglov, 1981) in the same species. Calcification rates of metamorphosed settled mussels were calculated from shell mass increments published for *M. edulis* kept under control $p$CO$_2$ (<550 µatm) and optimized feeding conditions (Thomsen et al., 2010; Thomsen and Melzner, 2010; Melzner et al., 2011; Thomsen et al., 2013) without considering the organic content of shell mass and its small ontogenetic change during the early benthic stage (Jörgensen, 1976; Thomsen et al., 2013).

### 2.5 Meta-analysis of bivalve calcification in ocean acidification experiments

A meta-analysis was performed in order to compare the calcification performance of larvae and juveniles over a range of calculated seawater [CO$_3^{2-}$]. Published data including the measurements from this study were used. The increment of shell mass (juveniles) and $D$ shell length (larvae) was considered as a measure for calcification performance. For the analysis of larval calcification only data published for unfed lecitotrophic mytilid (*M. edulis, trossulus, galloprovincialis, californianus*) and oyster
larvae (*Crassostrea gigas*) were considered (Gazeau et al., 2010, 2011; Kurihara et al., 2007, 2009; Sunday et al., 2011; Vitahkari et al., 2013; Frieder et al., 2014, this study). In order to be able to compare the published data which differed in absolute sizes of larvae (potentially due to slightly differing experimental duration, temperatures, species size, maternal/paternal effects) and weight in juveniles (due to age), values are expressed as the relative calcification of a treatment compared to control conditions (= 100 %). Carbonate chemistry parameters were either read from tables or recalculated from the provided data published in the manuscripts according to experimental temperature and salinity conditions using CO2sys and the settings described above (Tables S1 and S2 in the Supplement).

### 2.6 Statistics

Data were analysed using ANOVA and Tukey Posthoc test following tests for normal distribution using Shapiro Wilks test with Statistica 8. If assumption for parametric testes were not given, non-parametric Kruskal-Wallis test was applied. Regression analyses were performed using Sigma Plot 10. Data points in graphs depict mean ± standard deviation.

### 3 Results

#### 3.1 Impact of carbonate chemistry speciation on bivalve calcification

Calcification rates of juveniles (Exp. 1) kept under elevated $p\text{CO}_2$ (4000 µatm) and control alkalinity were lower (17.7 ± 2.3 mg) in comparison to those obtained under control $p\text{CO}_2$ (27.0 ± 4.9 mg, Fig. 1). Reduction of alkalinity resulted in lowered shell growth under control $p\text{CO}_2$ (13.4 ± 1.1 mg) and increased alkalinity at high $p\text{CO}_2$ enabled higher calcification rates that were similar to those of control animals (28.6 ± 2.5). Maximum shell mass growth of juveniles depended on seawater $[\text{CO}_3^{2-}]$ and was reduced at low concentrations (Table 2).
Depending on water temperature, formation of the first larval shell in *Mytilus* is completed after about 2 days whereby adverse carbonate system conditions can cause a substantial delay. In Exp. 2 ($pCO_2$: 390 and 2400 µatm, control $A_T$: 1950–2000 µmol kg$^{-1}$) larvae were sampled after four days in order to ensure fully developed shells in all treatments. Larvae kept under low $pCO_2$ had a mean shell length of 117.4 ± 8.4 µm when raised under control alkalinity conditions. In comparison, shell size decreased significantly to 92.3 ± 9.0 µm in the treatment with elevated $pCO_2$ (Fig. 2). Lowering [CO$_3^{2−}$] under control $pCO_2$ by means of HCl addition resulted in a similar decline of larval shell size. In contrast, high $pCO_2$ treatment and NaHCO$_3$ addition increased seawater [CO$_3^{2−}$] and larval shell sizes were similar to animals from control $pCO_2$ and alkalinity. In summary, seawater [CO$_3^{2−}$] had a significant effect on shell length (Table 2).

In Exp. 3 ($pCO_2$: 0 and 390 µatm) larvae were exposed to low C$_T$ treatments by aeration with 0 µatm $pCO_2$ air and either control (low C$_T$ 1) or reduced alkalinity (low C$_T$ 2). The treatment with CO$_2$ free air increased seawater pH$_{NBS}$ to 8.90 ± 0.06 (low C$_T$ 1) and 8.75 ± 0.11 (low C$_T$ 2) at $A_T$ values of 1.471 ± 75 and 405 ± 66 µmol kg$^{-1}$, respectively and simultaneously decreased seawater C$_T$ (Table 1). As a consequence, [HCO$_3^{−}$] was reduced to 1.169 ± 95 and 342 ± 67 µmol kg$^{-1}$. However, due to the high seawater pH, [CO$_3^{2−}$] remained relatively high at 300 ± 20 and 62 ± 8 µmol kg$^{-1}$. Shell length of larvae was greatest under control conditions (111.9 ± 6.8 µm) and was significantly reduced in the low C$_T$ treatments with 98.8 ± 10.0 µm (low C$_T$ 1) and 92.1 ± 1.2 µm (low C$_T$ 2, ANOVA, $F$: 8.26, $p < 0.01$, Table 2). Plotting shell lengths against seawater [CO$_3^{2−}$] revealed no correlation of calcification with [CO$_3^{2−}$] when [HCO$_3^{−}$] was low at the same time (Fig. 3).

### 3.2 Meta-analysis

The comparison of published data on larval calcification revealed the strong correlation of shell size and seawater [CO$_3^{2−}$] in all investigated mytilid species and
the oyster C. gigas and can be described best by an exponential rise to maximum function \((554 \pm 66) + 455 (\pm 60) \times (1 - e(-0.029 (\pm 0.005) \times [\text{CO}_3^{2-}]))\), \(r^2 = 0.84\), \(F = 673.8, p < 0.01\). The critical \([\text{CO}_3^{2-}]\) has been estimated applying broken stick regression (BSR, two asymptotes (calcification response compared to control: < 97%: \(0.28 (\pm 0.06) \times [\text{CO}_3^{2-}] + 75.4 (\pm 3.4)\), > 97%: \(0.01 (\pm 0.005) \times [\text{CO}_3^{2-}] + 98.3 (\pm 0.7)\)), which approximates the exponential function. The two linear regressions intercept at a \([\text{CO}_3^{2-}]\) of about 85 µmol kg\(^{-1}\). Calcification appears to be relatively unaffected at higher \([\text{CO}_3^{2-}]\), but drastically declines below this critical threshold (Fig. 4a). In agreement, shell mass increment of juvenile, settled M. edulis followed a similar relationship (Fig. 4b). Regressions of relative calcification rates of both ontogenetic stages, larvae and juveniles, did not significantly differ from each other (ANCOVA, factor \(\text{CO}_3^{2-}\), \(F: 35.2, p < 0.01\), factor ontogenetic stage \(p\text{CO}_2\ F: 0.96, p > 0.05\), Table 2).

Absolute calcification rates of bivalves increase during ontogeny from planktonic larval to benthic life stages from 0.01 to 958 nmol ind\(^{-1}\) h\(^{-1}\) (Fig. 5b). However, mass specific calcification rate (per mg drymass) was highest during D shell formation with 767 nmol h\(^{-1}\) mg\(^{-1}\) and decreased with age to about 58.4 nmol h\(^{-1}\) mg\(^{-1}\) in juveniles (Fig. 5c). The high calcification rate during D shell formation is also depicted in Fig. 5a. During this period, calcification rate is much higher than during the next days and comparable rates are only reached at the end of the planktonic life phase (Fig. 5a). Calcification rates are compared with overall metabolic processes depicted as oxygen consumption rates. In contrast to calcification, individual based respiration rates are similar in trochophora and early shelled veliger, relatively lower than calcification during D shell formation and steadily increase with biomass in growing larvae (Fig. 5a).

4 Discussion

While the result of the present study confirms the strong correlation of shell formation and seawater \([\text{CO}_3^{2-}]\) in bivalves (Gazeau et al., 2011), under non \(C_T\) limiting conditions
is becomes evident for the first time that not CO$_3^{2-}$ but HCO$_3^-$ is the substrate used for calcification. The results support the general hypothesis that calcification is the process primarily affected by ocean acidification. In our laboratory experiments, seawater pCO$_2$ and pH as single factors explained no or only to a small degree the observed decline of calcification rates. High pCO$_2$ causes acidification of intra- and extracellular fluids as [CO$_2$] levels need to increase to the same extend in order to maintain a diffusion gradient between animal and ambient seawater. Low seawater pH causes higher passive proton leakage into the cytosol and thereby elevates costs for proton removal from the animal tissues by means of active transport (Boron et al., 2004). However, increased costs for regulation of intracellular acid-base homeostasis in somatic tissues seem to be of minor importance for the overall performance of these bivalve genera. This speaks for a cost efficient acid-base regulation system in bivalves, which is potentially related to the fact that control of acid-base homeostasis is limited to the intracellular space. The pH of the much larger extracellular compartment remains un-regulated and declines in acidified seawater (Thomsen et al., 2010, 2013). In contrast, a substantial fraction of the bivalve energy budget is dedicated to biomineralization processes, particularly the production of shell organic matrix (Palmer, 1992; Thomsen et al., 2013). Adverse conditions for calcification may then secondarily affect growth by reducing the energy available for protein biosynthesis or space limitation within the shell (Waldbusser et al., 2013).

The critical CO$_3^{2-}$ concentration for calcification is at about 80 µmol kg$^{-1}$, below this threshold calcification starts to decline drastically. On the other hand, higher [CO$_3^{2-}$] does not lead to a further increase in calcification, which suggests a $C_T$ saturation of the calcification mechanism. Future levels of elevated CO$_2$ concentrations may have a substantial effect on calcification, in particular at low alkalinity conditions whereas high alkaline water may potentially partially buffer negative effects (Miller et al., 2009; Fernandez-Reiriz et al., 2012; Thomsen et al., 2013). Nevertheless the result of the larval experiment conducted under $C_T$ limiting conditions suggests that not the CO$_3^{2-}$ concentration itself is determining calcification rates. Similar results were obtained for
corals and the coccolithophore *Emiliana huxleyi* (Jury et al., 2010; Bach, 2015). Instead, calcification seems to depend on external HCO$_3^-$ concentrations as calcification significantly declined at lowered HCO$_3^-$ (< 1000 µmol kg$^{-1}$) despite high [CO$_3^{2-}$]. This suggests that, most probably, HCO$_3^-$ is the substrate used for calcification. Its availability in seawater is about 10 fold higher compared to CO$_3^{2-}$ and its concentration does not significantly change within the naturally prevailing pH conditions observed in seawater (cf. Bach, 2015). Intracellular calcification requires a concentration mechanism for Ca$^{2+}$ and HCO$_3^-$ in specialized membrane enclosed intracellular vesicles to produce the amorphous calcium carbonate (ACC) precursor (Weiner and Addadi, 2011). Enrichment of HCO$_3^-$ in the extracellular lumen of calcifying vesicles is potentially performed via solute carrier (SLC) transporters of the families SCL4 and SLC26 such as Cl$^-$/HCO$_3^-$ exchangers (AE) or Na$^+$/HCO$_3^-$ co-transporters (NCBT) (Parker and Boron, 2013). A study carried out over a wide range of seawater [HCO$_3^-$] confirmed its important role in the calcification process compared to [CO$_3^{2-}$] (Jury et al., 2010). Nevertheless, in a realistic ocean acidification scenario seawater [HCO$_3^-$] slightly increases due to elevated seawater C$_T$, but calcification in general declines. Therefore, the explanatory power of [HCO$_3^-$] under natural conditions (e.g. HCO$_3^-$ > 1000 µmol kg$^{-1}$) is low as HCO$_3^-$ is not limiting and the dependency of calcification on its availability is barely visible. However, the conversion of bicarbonate into carbonate generates an equimolar number of protons which need to be removed from the vesicles and subsequently excreted from calcifying cells. Thus, lowered seawater pH diminishes the H$^+$ gradient between the calcifying epithelia and the ambient water which needs to be counterbalanced by up regulation of active H$^+$ extrusion mechanisms (Stumpp et al., 2012). If the regulatory capacities can not fully compensate for the adverse ambient conditions calcification rates remain reduced. pH is a good predictor of the calcification response under normal A$_T$ conditions (> 2000 µmol kg$^{-1}$, e.g. Frieder et al., 2014). In experiments with strong carbonate chemistry modifications, such as lowered A$_T$, the close correlation disappears as the reduced HCO$_3^-$ availability is not considered.
Therefore, the combination of both parameters, carbon availability and H\(^+\) gradient, expressed as the ratio \([\text{HCO}_3^-]/[\text{H}^+]\) which is linearly correlated to \([\text{CO}_3^{2-}]\) predicts the calcification response best (Bach, 2015). The reduction in calcification rate in marine organisms in response to reduced \([\text{CO}_3^{2-}]\) is potentially a misinterpretation of the complex chemical speciation of the carbonate system. Consequently, one should probably rather speak of “[\text{CO}_3^{2-}] equivalents”. Under natural conditions, high seawater \([\text{CO}_3^{2-}]\) corresponds to high \text{HCO}_3^- availability and relatively high pH of about 8, thus a large proton gradient between calcifying tissue and ambient seawater. These conditions provide enough \text{HCO}_3^- and enable fast extrusion of excess \text{H}^+ and are therefore beneficial for calcification.

The results of our experiments also suggest that the C\(_T\) accumulation mechanisms for calcification in mytilid bivalves do not seem to differ substantially between larval and benthic stages as the response to external carbonate chemistry is similar in both. The first shell (prodissoconch I) is secreted by the shell gland and, subsequently, the shell field (Kniprath, 1980, 1981). In later larval and juvenile stages, calcification is performed by the mantle tissue. Following settlement and metamorphosis, the mineralogy of the shell changes: while veliger prodissoconch I and II are exclusively composed of amorphous and aragonitic CaCO\(_3\) (Medakovic, 2000; Weiss et al., 2002; Weiss and Schönitzer, 2006), the newly formed shell of juveniles consists of calcite, which is a more stable polymorph (Medakovic, 1997). Nevertheless, this shift to a more stable polymorph does not seem to cause higher tolerance of the calcification process itself to adverse carbonate chemistry. It may, however, support the maintenance of calcified shells under very low \(\Omega\) in settled mussels.

In fact, the higher sensitivity of larval calcification seems to be primarily related to the much higher relative calcification rates per unit somatic bodymass. Thus, adverse carbonate system conditions have a much stronger effect in the early life stages. In relation to larval aerobic metabolic rates, calcification rates are especially high during the formation of prodissoconch I. This emphasizes the energetic importance of biomineralization in relation to all other vital processes at this life stage. Calcification rate strongly
declines in relation to metabolism in the later planktonic phases (Sprung, 1984b). The comparison of oxygen consumption rates with calcification rates also reveals that metabolic processes can not provide enough inorganic carbon for calcification – assuming a respiratory quotient of 0.7–1, i.e. generation of more or less equimolar amounts of CO\textsubscript{2} per O\textsubscript{2} respired. Therefore, larvae must take up seawater C\textsubscript{T} which is also an energetically more efficient source of HCO\textsubscript{3} than CO\textsubscript{2}, as only half of the protons are generated per mole of formed CaCO\textsubscript{3}. The high dependency of calcification on external C\textsubscript{T} from the ambient seawater is further supported by isotopic data which revealed only a minor fraction of metabolic CO\textsubscript{2} (5–15 %) but a large seawater signal in the shells of bivalves (McConnaughey and Gillikin, 2008; Waldbusser et al., 2013).

As a consequence of detrimental changes in seawater carbonate chemistry, costs for calcification are increased and more energy is required to produce a similar amount of calcium carbonate when compared to control conditions. This is of particular importance, as the formation of the first shell is exclusively fueled by the energy reserves provided by the egg as the larvae can start feeding only after they have reached the shelled veliger stage after ca. 2–3 days post fertilization (Waller, 1980; Widdows, 1991). The energy supply from the egg yolk enables maximal calcification rates and allows the early larvae to develop the D shell independent of the food concentrations of the ambient environment (Moran and Manahan, 2004). Once the first shell is produced, feeding larvae continue to calcify prodissoconch II but cease to grow if no food is available. The small remaining egg reserves may enable them to endure a short starvation period (Moran and Manahan, 2004). Starvation in the first days of the larval period does not induce high mortality during the subsequent days but eventually affects final settlement success (His and Seaman, 1992; Moran and Manahan, 2004). It has been suggested that the strong impairment of the larval energy budget under CO\textsubscript{2} stress might lead to an earlier depletion of their endogenous energy reserves which might eventually impact survival (Waldbusser et al., 2013). As low food concentrations limit larval growth, compensatory effects of higher food availability may play an important role in the planktonic phase similar to results reported for the benthic life phase (Sprung, 1984a; Melzner et
al., 2011; Thomsen et al., 2013). A recent study did not confirm this hypothesis for larvae of the oyster *Ostrea lurida*. Here, as a consequence of the limited larval clearance capacities, animals were potentially not limited by the provided food concentrations and growth rates leveled off in all treatments (Riisgard et al., 1980; Hettinger et al., 2013).

In conclusion, the meta-analysis of juvenile and larval calcification of mytilid mussel species and the oyster *C. gigas* revealed a similar response to lowered [CO$_3^{2-}$] in different species and populations. This uniform response suggests a kinetic, physico-chemical barrier of carbonate system speciation which limits calcification in marine bivalves and may potentially represent a barrier to rapid evolutionary adaptation of bivalves to abiotic conditions expected for the future ocean. More research is needed to understand the physiological basis of bivalve biomineralization machinery and its adaptability to adverse carbonate chemistry.

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**Author contributions.** J. Thomsen designed the study, J. Thomsen and K. Haynert conducted the experiments, meta-analyses and analyzed the data, K. M. Wegner supported the experimental work, J. Thomsen and F. Melzner wrote the manuscript with support of all co-authors.

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References


Table 1. Carbonate chemistry parameters of the four experiments (mean ± sd) calculated from measured $C_T$ (larval experiments) or $A_T$ (juvenile experiment) and pH (NBS or total scale).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Salinity (g kg$^{-1}$)</th>
<th>Temperature (°C)</th>
<th>Treatment</th>
<th>$A_T$ (µmol kg$^{-1}$)</th>
<th>$C_T$ (µmol kg$^{-1}$)</th>
<th>pH</th>
<th>$\rhoCO_2$ (µatm)</th>
<th>$[HCO_3^-]$ (µmol kg$^{-1}$)</th>
<th>$[CO_3^{2-}]$ (µmol kg$^{-1}$)</th>
<th>$\Omega$ (aragonite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>17.7 ± 1.3</td>
<td>17.3 ± 1.2</td>
<td>390/75</td>
<td>1976 ± 87</td>
<td>1886 ± 76</td>
<td>8.13 ± 0.03</td>
<td>501 ± 1</td>
<td>1784 ± 68</td>
<td>83 ± 11</td>
<td>1.33 ± 0.19</td>
</tr>
<tr>
<td>Juveniles</td>
<td></td>
<td></td>
<td>400/12</td>
<td>2052 ± 28</td>
<td>2104 ± 199</td>
<td>7.16 ± 0.01</td>
<td>5214 ± 81</td>
<td>1935 ± 132</td>
<td>10 ± 0</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>390/12</td>
<td>851 ± 34</td>
<td>847 ± 30</td>
<td>7.74 ± 0.08</td>
<td>564 ± 101</td>
<td>810 ± 29</td>
<td>15 ± 2</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400/75</td>
<td>5765 ± 74</td>
<td>5909 ± 58</td>
<td>7.57 ± 0.01</td>
<td>5649 ± 99</td>
<td>5613 ± 61</td>
<td>71 ± 7</td>
<td>1.14 ± 0.11</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>13.6 ± 0.1</td>
<td>17.7 ± 0.1</td>
<td>390/78</td>
<td>1943 ± 17</td>
<td>1863 ± 23</td>
<td>7.99 ± 0.04</td>
<td>510 ± 49</td>
<td>1766 ± 27</td>
<td>78 ± 6</td>
<td>1.26 ± 0.11</td>
</tr>
<tr>
<td>Larvae</td>
<td></td>
<td></td>
<td>2400/20</td>
<td>1998 ± 43</td>
<td>2032 ± 41</td>
<td>7.49 ± 0.04</td>
<td>1778 ± 149</td>
<td>1936 ± 40</td>
<td>27 ± 3</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>390/20</td>
<td>852 ± 11</td>
<td>848 ± 6</td>
<td>7.63 ± 0.07</td>
<td>544 ± 78</td>
<td>811 ± 6</td>
<td>16 ± 3</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2400/78</td>
<td>3418 ± 133</td>
<td>3398 ± 111</td>
<td>7.72 ± 0.06</td>
<td>1775 ± 207</td>
<td>3252 ± 104</td>
<td>77 ± 15</td>
<td>1.25 ± 0.24</td>
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<tr>
<td>Exp. 3</td>
<td>15.2 ± 0.2</td>
<td>16.0 ± 0.1</td>
<td>390/100</td>
<td>2056 ± 4</td>
<td>1942 ± 5</td>
<td>8.09 ± 0.01</td>
<td>404 ± 14</td>
<td>1825 ± 7</td>
<td>100 ± 3</td>
<td>1.60 ± 0.05</td>
</tr>
<tr>
<td>Larvae</td>
<td></td>
<td></td>
<td>0/300</td>
<td>1859 ± 47</td>
<td>1471 ± 75</td>
<td>8.76 ± 0.06</td>
<td>56 ± 13</td>
<td>1169 ± 95</td>
<td>300 ± 20</td>
<td>4.78 ± 0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0/60</td>
<td>540 ± 57</td>
<td>405 ± 66</td>
<td>8.61 ± 0.11</td>
<td>24 ± 11</td>
<td>342 ± 67</td>
<td>62 ± 8</td>
<td>0.99 ± 0.13</td>
</tr>
</tbody>
</table>
Table 2. Statistic: ANOVA, Kruskal-Wallis and ANCOVA of calcification rates against seawater [CO$_3^{2-}$], significant results in bold.

<table>
<thead>
<tr>
<th>Experiment 1 + 2</th>
<th>ANOVA</th>
<th>SS</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>p</th>
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</thead>
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<tr>
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<td>ANOVA</td>
<td>639</td>
<td>3</td>
<td>213</td>
<td>23.4</td>
<td>&lt;0.01</td>
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<tr>
<td>Exp. 2 larvae</td>
<td>ANOVA</td>
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<td>3</td>
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<td>11.8</td>
<td>&lt;0.01</td>
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<table>
<thead>
<tr>
<th>Experiment 3</th>
<th>Kruskal-Wallis</th>
<th></th>
<th>d.f.</th>
<th>n</th>
<th>sum of ranks</th>
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<tbody>
<tr>
<td>H: <strong>6.61</strong></td>
<td>p: &lt; <strong>0.05</strong></td>
<td></td>
<td>2</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metanalysis juvenile and larval calcification, ANCOVA</th>
<th>ANCOVA</th>
<th>SS</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_3^{2-}$ ontog. stage</td>
<td>ANCOVA</td>
<td>6285</td>
<td>1</td>
<td>6285</td>
<td>35.2</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>ANCOVA</td>
<td>172</td>
<td>1</td>
<td>172</td>
<td>1.0</td>
<td>&gt;0.05</td>
</tr>
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</table>
Figure 1. Exp. 1 Calcification response (measured as shell mass growth) of *M. edulis* juveniles kept under modified conditions for 15 days. Shell mass growth is plotted against seawater (a) $pCO_2$, (b) $[HCO_3^-]$, (c) $[CO_3^{2-}]$ and (d) pH. Data represent mean ± sd.
Figure 2. Exp. 2 Calcification response (measured as shell length) of *M. edulis* larvae kept under modified carbonate chemistry conditions for four days during the lecithotrophic phase. Shell length is plotted against seawater (a) $pCO_2$, (b) $[HCO_3^-]$, (c) $[CO_3^{2-}]$ and (d) pH. Data represent mean ± sd.
Figure 3. Exp. 3 Calcification response (measured as shell length) of M. edulis larvae kept under modified carbonate chemistry and C_T limiting conditions for four days during the lecithotrophic phase. Shell length is plotted against seawater (a) $p$CO$_2$, (b) [HCO$_3$$^-$. (c) [CO$_3^{2-}$] and (d) pH. Data represent mean ± sd.
Figure 4. Meta-analysis of the relative calcification response (as % of control) of (a) bivalve larvae of the genera *Crassostrea* and *Mytilus* during the lecithotrophic phase and (b) comparison of *Mytilus* larvae and juveniles plotted against calculated seawater \([\text{CO}_3^{2-}]\). Relative calcification rates were calculated from either shell length (larvae) or shell mass growth (juveniles).
Figure 5. Changes of physiological rates during the ontogeny of *M. edulis*. (a) respiration and calcification rates during the planktonic larval phase. (b) absolute calcification rates of larvae and juveniles (nmol h\(^{-1}\)). (c) relative calcification rates (nmol h\(^{-1}\) mg\(^{-1}\)) of larvae and juveniles. Data represent mean ±sd.