Ocean acidification impairs vermetid reef recruitment

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Vermetids form reefs in sub-tropical and warm-temperate waters that protect coasts from erosion,
regulate sediment transport and accumulation, serve as carbon sinks and provide habitat for other
species. The gastropods that form these reefs brood encapsulated larvae; they are threatened by rapid
environmental changes since their ability to disperse is very limited. We used transplant experiments
along a natural CO_2 gradient to assess ocean acidification effects on the reef-building gastropod
Dendropoma petraeum. We found that although *D. petraeum* were able to reproduce and brood at
elevated levels of CO_2, recruitment success was adversely affected. Long-term exposure to acidified
conditions predicted for the year 2100 and beyond caused shell dissolution and a significant increase in
shell Mg content. Unless CO_2 emissions are reduced and conservation measures taken, our results
suggest these reefs are in danger of extinction within this century, with significant ecological and
socioeconomic ramifications for coastal systems.

Surface ocean partial pressure of carbon dioxide (pCO$_2$) is rising in proportion to the increase in atmospheric
CO$_2$ caused by anthropogenic activities[1]. This is causing ocean acidification (OA) to occur, rapidly chan-
ging seawater chemistry by lowering pH and the concentration of carbonate ions, thus causing a dramatic
expansion in the global volume of seawater that is corrosive to biogenic calcareous reefs[2,3]. The rate of these
changes is unprecedented and driven by the rapid rise in CO$_2$ concentrations since the industrial revolution[4,5].
Here, we investigated reef-forming vermetids in the Mediterranean Sea where OA has already caused a 0.05–0.14
decrease in seawater pH since the pre-industrial period[6]. The magnitude and the rate of OA poses serious
challenges to marine species that must either tolerate or adapt to these new ocean conditions, or eventually
disappear[2,5].

Many reef-building species are adversely affected by increases in pCO$_2$ in short-term laboratory experiments
where the variability in carbonate chemistry is tightly constrained. However it is difficult to scale-up from such
studies to the effects of chronic exposures to the widely variable and gradually increasing pCO$_2$ levels found in
coastal environments[7–8]. For this reason, areas with naturally elevated levels of pCO$_2$, such as volcanic vents and
upwelling zones, are increasingly being used to study the long-term effects of OA on organisms, communities and
ecosystems[9]. In these areas, seawater pH is highly variable[10] although many marine ecosystems – including
tropical and temperate reefs – have wide diel fluctuations in seawater pH[10].

Work to date consistently reveals dramatic biodiversity loss along spatial gradients in pCO$_2$ where pH falls
from mean levels of 8.1 to <7.8 as this causes the loss of most of the calcareous habitat-forming species[9,11–14].
Transplant experiments along natural gradients in pCO$_2$ have shown that the effects of changes in seawater
chemistry are made worse by the increases in temperature that are expected to occur in the coming decades[12].

In sub-tropical and warm-temperate waters, vermetids form reefs that protect coasts from erosion, regulate
sediment transport and accumulation, serve as carbon sinks and provide habitat for fish and invertebrates of
commercial and recreational interest[15]. In the Mediterranean, these reefs occur on the lower shore and are
functionally similar to tropical coral fringing reefs (Fig. 1A). They are threatened since local extinctions have
begun to spread along the Eastern Mediterranean coast[16]. Vermetid reefs are built by the gastropod *Dendropoma
petraeum* (Monterosato, 1884) and the coralline alga *Neogoniolithon brassica-florida* (Harvey) Setchell & Mason
(1943) which cements the reef and triggers vermetid settlement[15,17] (Fig. 1B,D). The vermetid gastropod broods
encapsulated larvae and has highly restricted dispersal ability which hinders recovery from habitat loss[16,17]. This
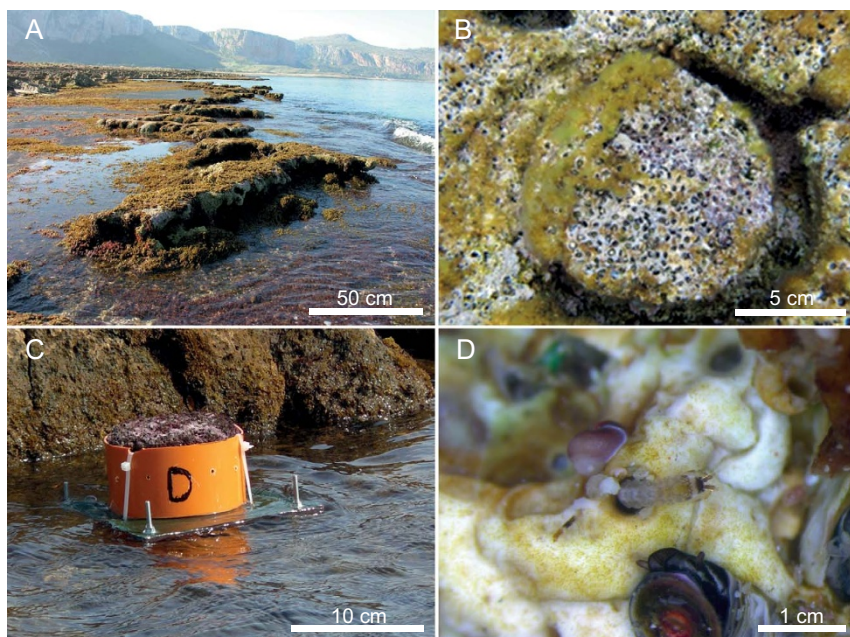
Figure 1 | Mediterranean vermetid reefs. (A) A pristine vermetid reef at low tide in NW Sicily, Italy. (B) Collection of a vermetid core in the outer rim of a vermetid reef; black spots are the shell openings of *Dendropoma petraeum* cemented by the coralline alga *Neogoniolithon brassica-florida*. (C) A vermetid core transplanted in the intertidal off Vulcano Island. (D) A recruit newly settled on the coralline alga (top left) and the shell opening with the operculum of a *D. petraeum* adult (below). Photo credits: R.C. (A); M.M. (B,C); M.M. and M.F. (D).

limited larval dispersal is useful for transplantation experiments along natural CO_2 gradients since it prevents recruitment from surrounding populations.

The juvenile stages of many marine calcifiers are vulnerable to OA as this can cause abnormal calcification, increased dissolution and it can create conditions that are unsuitable for settlement[18–22]. Many calcified organisms undergo changes in calcite/aragonite crystallography as well as changes in skeletal Ca/Mg/Sr ratios as CO_2 levels increase[22,23]. *Dendropoma petraeum* build their shells using aragonite[24], which dissolves easily due to OA. Here we examined recruitment success and shell composition in transplanted live reef cores in reference areas and along a CO_2 gradient off Sicily (Italy). These experiments were conducted to inform predictions of the impact of OA on these ecologically important coastal reefs.

Results

Throughout the experimental period (from Nov. 2010 to Nov. 2011), mean surface seawater pH decreased significantly with increasing proximity to CO_2 seeps (mean $\pm$ S.E., n = 95; High pH: 8.03 $\pm$ 0.01, Mid pH: 7.73 $\pm$ 0.02, Low pH: 7.31 $\pm$ 0.03 pH_{NBS} units) but did not differ between our reference site (CTL_Vent: 8.15 $\pm$ 0.01 pH_{NBS}) and the site where vermetid reef cores were taken (CTL_Core: 8.16 $\pm$ 0.01 pH_{NBS}) (Table 1; Supplementary Fig. S1 online). Temperature, salinity and total alkalinity (TA) remained relatively constant among sites (Table 1). The highest median values for pCO_2 were found at the Low pH (2797 µatm pCO_2) and at the Mid pH sites (990 µatm pCO_2), which had the lowest aragonite saturation medians (Ω_{ara}: 0.69 and 1.71, respectively) (Table 1; Supplementary Fig. S1 online). Periods of aragonite under-saturation occurred when pH was lowest at Low, Mid and High pH sites (minimum Ω_{ara}: 0.15, 0.62 and 0.76, respectively). Similar trends in the carbonate chemistry were recorded during the 6-month exposure from April to November 2011 (Table 1; Supplementary Fig. S1 online).

The vermetids brooded young all along the CO_2 gradient but recruitment success was clearly adversely affected at the Low and the Mid pH sites (1-way ANOVAs; pH: $F_{2,6}$ = 11.10, P = 0.009 after 6-months; pH: $F_{4,10}$ = 5.16, P = 0.016 after 12 months). After 12

months the number of newly settled recruits was significantly lower at the most acidified sites (SNK test: CTL_Core = CTL_Vent = High pH > Mid pH = Low pH), with >16.9 $\pm$ 6.4 ind. 100 cm^{-2} at High pH, CTL_Vent and CTL_Core sites, 5.3 $\pm$ 1.9 ind. 100 cm^{-2} at the Mid pH site, and 1 $\pm$ 0.8 ind. 100 cm^{-2} at the Low pH site (Fig. 2A). A similar response was recorded after a 6-month exposure (SNK test: High pH > Mid pH = Low pH; Fig. 2A). The number of living recruits was not affected by exposure time (2-way ANOVA; 6 vs 12 months: $F_{1,12}$ = 0.41, P = 0.532), differed between pH levels (2-way ANOVA; pH: $F_{2,12}$ = 18.36, P = 0.0002; SNK test: High pH > Mid pH = Low pH; Fig. 2A) and was not related to the number of *D. petraeum* adults (R^2 = 0.0387, P = 0.38, S.E.$_{res}$ = 15.12, n = 24; Fig. 2B) nor to coralline algal cover (R^2 = 0.0048, P = 0.76, S.E.$_{res}$ = 15.38, n = 24; Fig. 2C).

After 12 months, a lower post-settlement survival (%) of the vermetid recruits was recorded at extreme pCO_2/pH conditions (i.e., at the Low pH site) although these differences were not significant (1-way ANOVA, pH: $F_{4,10}$ = 1.98, P = 0.174; Fig. 2A). Similarly, no significant effects were observed in the 6-month exposure experiment (1-way ANOVA, pH: $F_{2,8}$ = 2.36, P = 0.175). Exposure time did not affect *D. petraeum* post-settlement survival (%) (2-way ANOVA; 6 vs 12 months: $F_{1,12}$ = 3.17, P = 0.100), whilst this was significantly lower at Low than Mid and High pH sites despite exposure duration (2-way ANOVA, pH: $F_{2,12}$ = 4.46, P = 0.035; SNK test) (Fig. 2A).

The aragonitic shell of new recruits (Supplementary Fig. S2 online) dissolved at pCO$_2$ levels expected by the end of this century and beyond (Fig. 3; Supplementary Fig. S3 online). The shells lost surface patterning at Low pH and exhibited abnormal accretion at Mid pH conditions (Fig. 3A–D). No signs of dissolution were recorded at present day and projected near future levels of pCO$_2$ (i.e., the High pH site: mean 633 $\pm$ 28 µatm pCO$_2$; mean $\pm$ S.E., n = 95) (Fig. 3E–L).

After 12 months, the Mg/Ca content of young recruits' shells was significantly different between sites (1-way ANOVA, $F_{3,9}$ = 7.118, P = 0.009), ranging on average from 5.6 to 9.5 mmol mol^{-1} Mg/Ca ratio in the High pH and reference sites, to 18.5 mmol mol^{-1} Mg/Ca

Table 1 | Seawater carbonate chemistry along a pH gradient off Vulcano Island CO_2 seeps and at a vermetid reef where cores were taken. Four sites at increasing distance from the main CO_2 venting area (Low pH, Mid pH, High pH, and CTL_Vent) were used to test the effect of pH on recruitment, and one site at the coring location (CTL_Core), 81 nautical miles from Vulcano Island, to control for any transplantation effect on recruitment. Results of one-way ANOVAs (F-ratios and P levels) and of post-hoc SNK tests are also reported for each variable. Means with different letters (a, b, c, d) are significantly different at P < 0.05 (SNK tests)

Nov 2010-Nov 2011 (12 months)

		Low pH	Mid pH	High pH	CTL_Vent	CTL_Core
		38°25.176′N 14°57.658′E	38°25.184′N 14°57.696′E	38°25.193′N 14°57.763′E	38°25.248′N 14°57.853′E	38°12.341′N 13°15.490′E
Distance from the vents (m)		240	300	390	850	–
Salinity $F_{4,470} = 7.52$, P < 0.001	mean ± S.E.	37.4(±0.2)[a]	37.2(±0.1)[b]	37.5(±0.1)[a]	37.6(±0.1)[a]	37.7(±0.2)[a]
	range	35.9–38.3	36.3–38.3	36.3–38.3	36.4–38.3	36.2–38.6
	median	37.6	37.1	37.7	37.7	37.9
Temperature (°C) $F_{4,470} = 1.27$, P = 0.28	mean ± S.E.	21.5(±0.5)	21.4(±0.5)	21.4(±0.5)	21.4(±0.4)	22.2(±0.5)
	range	13.6–28.9	13.7–29.2	13.9–28.6	13.8–28.8	13.9–29.8
	median	20.5	20.5	20.3	20.5	22.8
pH_{NBS} $F_{4,470} = 241.41$, P < 0.001	mean ± S.E.	7.31(±0.03)[a]	7.73(±0.02)[b]	8.03(±0.01)[c]	8.15(±0.01)[d]	8.16(±0.01)[d]
	range	6.75–7.94	7.39–8.14	7.52–8.24	7.93–8.28	7.92–8.29
	median	7.44	7.86	8.07	8.19	8.16
pCO_2(μatm) $F_{4,470} = 104.64$, P < 0.001	mean ± S.E.	3923(±307)[a]	1385(±91)[b]	633(±28)[c]	468(±15)[c]	462(±11)[c]
	range	840–14255	460–3249	353–2224	315–841	311–862
	median	2797	990	571	415	449
Total Alkalinity (μmol kg^{-1}) $F_{4,70} = 2.19$, P = 0.10	mean ± S.E.	2506(±8)	2517(±5)	2524(±4)	2525(±4)	2518(±2)
	range	2429–2556	2498–2551	2510–2567	2508–2559	2504–2530
	median	2509	2513	2517	2521	2518
Ω Aragonite $F_{4,470} = 174.86$, P < 0.001	mean ± S.E.	0.91(±0.06)[a]	1.89(±0.10)[b]	2.90(±0.08)[c]	3.44(±0.34)[d]	3.39(±0.07)[d]
	range	0.15–2.95	0.62–4.11	0.76–4.86	2.00–5.38	2.03–5.41
	median	0.69	1.71	2.89	3.33	3.35

Apr-Nov 2011 (6 months)

|		Low pH	Mid pH	High pH
Salinity $F_{2,102} = 0.27$, P = 0.15	mean ± S.E.	37.9(±0.1)	37.8(±0.1)	38(±0.1)
	range	37.1–38.6	36.8–38.4	37.5–38.5
	median	37.9	37.6	37.9
Temperature (°C) $F_{2,102} = 0.04$, P = 0.96	mean ± S.E.	25.6(±0.8)	25.6(±0.8)	25.3(±0.8)
	range	17.6–29	17.5–29	17.4–28.9
	median	26.7	26.8	26.1
pH_{NBS} $F_{2,102} = 66.42$, P < 0.001	mean ± S.E.	7.43(±0.05)[a]	7.87(±0.03)[b]	8.06(±0.02)[c]
	range	7.02–7.94	7.55–8.12	7.93–8.24
	median	7.49	7.96	8.08
pCO_2 (μatm) $F_{2,102} = 37.16$, P < 0.001	mean ± S.E.	2998(±354)[a]	985(±94)[b]	601(±27)[b]
	range	840–8251	478–2173	358–867
	median	2612	784	562
Total Alkalinity (μmol kg^{-1}) $F_{2,18} = 0.78$, P = 0.47	mean ± S.E.	2508(±12)	2520(±6)	2519(±3)
	range	2441–2533	2498–2537	2510–2531
	median	2523	2526	2517
Ω Aragonite $F_{2,102} = 60.09$, P < 0.001	mean ± S.E.	1.23(±0.11)[a]	2.64(±0.17)[b]	3.30(±0.12)[c]
	range	0.37–2.95	0.95–4.11	2.18–4.86
	median	1.12	2.86	3.31

Temperature data are daily averages from continuous logging. Salinity and pH were collected on different visits (Nov 2010-Nov 2011, n = 95; Apr-Nov 2011, n = 35). Average total alkalinity was calculated from water samples collected at each site (Nov 2010-Nov 2011, n = 15; Apr-Nov 2011, n = 7). See Boatta et al. (2013)[28] for further information on the geochemistry of the study area.

ratio in the Mid pH site (SNK test: Mid pH > High pH = CTL_Core = CTL_Vent) (Fig. 4A). In contrast, there were no differences in the shell Sr/Ca content between sites (1-way ANOVA, $F_{3,9} = 0.898$, P = 0.479; Fig. 4B).

Discussion

Our results show that the level of ocean acidification predicted to occur this century and beyond[1,25,26] impairs recruitment success, causes shell dissolution and alters the shell mineralogy of the reef-building gastropod *Dendropoma petraeum*. Post-settlement survival of new recruits did not decrease until very low pH conditions were reached where the results of our experiment were probably artificially enhanced by wide variability in CO_2 levels.
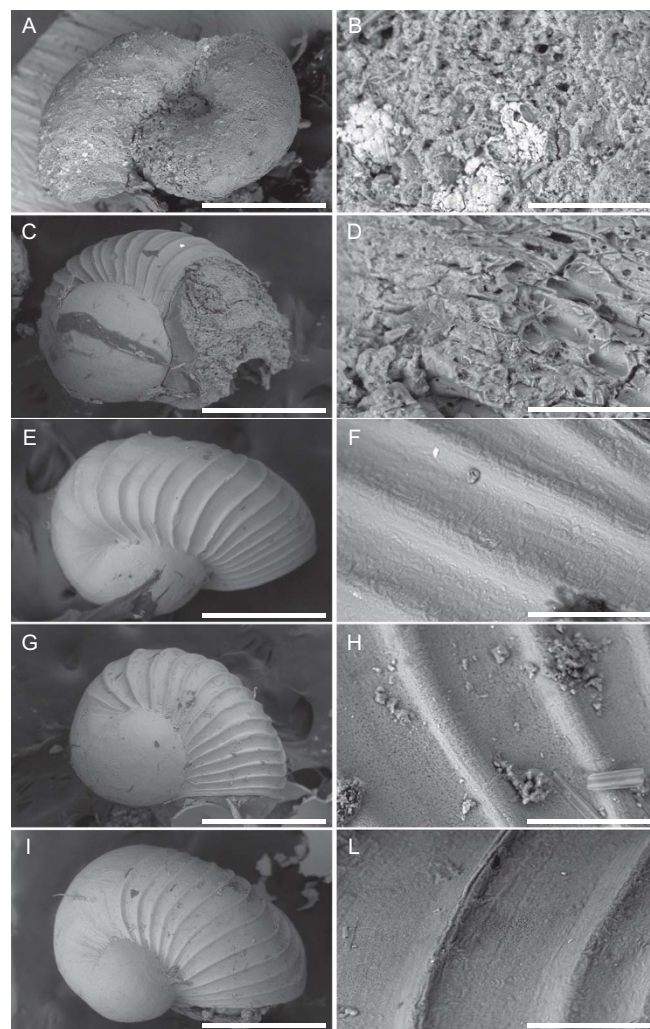
Average surface seawater CO_2 concentrations are expected to reach between 443 and 541 μatm in 2050, up to 936 μatm in 2100 and beyond 1900 μatm in 2300 causing present-day values in average global surface ocean pH to fall by 0.14 units by 2050, of 0.3–0.4 units by 2100 and >0.7 units by 2300[25–27]. Our vermetid cores were exposed to near-future pCO_2 and pH levels at the High pH site, to conditions expected by the end of this century at the Mid pH site, and to more extreme pCO_2 and pH at the Low pH site. The gradients in carbonate chemistry off Vulcano Island were consistent with previous observations of the area[28,29] and of other CO_2 seeps[10,11,30], and varied due to changes in wind-driven currents[28]. Background fluctuations in the seawater carbonate chemistry were the same at our reference site and the reef from which core samples were taken at 81.5
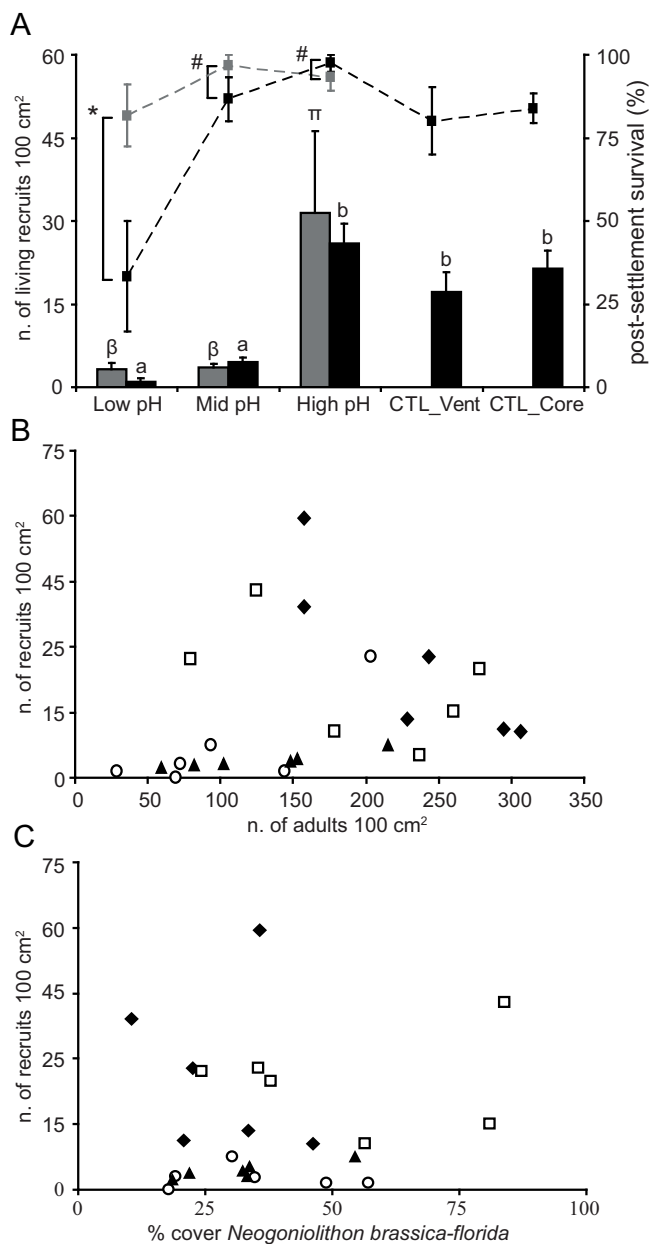
Figure 2 | **Effects of short- and long-term exposure to a pH gradient on the number and post-settlement survival of *Dendropoma petraeum* recruits.** (A) Average ($\pm$S.E.) number of living recruits 100 cm^{-2} (bars) and post-settlement survival (%) (lines) on the vermetid cores after 12-month (black) and 6-month (grey) exposures at different pH/site. Means with different symbols (*, #) and letters (a, b; β, π) are significantly different (SNK test; P < 0.05). (B–C) Linear regressions between the number of recruits settled and the density of *D. petraeum* adults (B) or the %cover of *N. brassica-florida* (C) at Low pH (circles), Mid pH (triangles), High pH (diamonds), and pooled CTL_Vent and CTL_Core (quadrates) sites.

Figure 3 | **Scanning Electron images of *Dendropoma petraeum* recruits after 12-month exposure to the pH gradient.** (A–B) Dissolution can be observed on recruits collected from the Low pH site (range of Ω_{ara}: 0.15-2.95; Table 1), the shell had lost its surface patterning. (C–D) Represent a sample from the Mid pH (Ω_{ara}: 0.62–4.11; Table 1), the protoconch is slightly dissolved whilst the biogenic carbonate accretion has a highly corroded appearance. (E–L) Vermetid recruits with no corroded appearance and typical accretion from High (Ω_{ara}: 0.76–4.86), CTL_Vent (Ω_{ara}: 2–5.38) and CTL_Core sites (Ω_{ara}: 2.03–5.41), respectively (Table 1). Scale bars are 150 μm in A,C,E,G, and I (left side) and 50 μm in B,D,F,H, and L (right side).

nautical miles distance. Given that the seawater carbonate chemistry of coastal marine ecosystems typically varies widely as a result of diel fluctuations in photosynthesis and respiration[10] is useful to incorporate such variability into OA studies[31].

The reef-building gastropod *D. petraeum* has a peculiar reproductive strategy and a highly specialised mode of development. Sperm are encapsulated in spermatophores and held for a couple of months in the female mantle cavity; internal fertilization occurs when the seawater starts to warm in late March-May[32]. Lecithotrophic larval development occurs in capsules within the female shell (usually each

female holds up to 25 capsules each containing 1–6 embryos) and larvae take a month to develop and hatch[17]. Crawling larvae settle a few hours after hatching[17] (Supplementary Video V1 online). We found that although recruits were produced all along the CO_2 gradient, there were 4 to 7 times fewer living young snails in reef cores exposed to CO_2 levels expected by 2100 (Mid pH site) and beyond (Low pH site) than at near-future (High pH) and reference conditions (CTL_Vent and CTL_Core). This trend was not related to the number of adult *D. petraeum* nor the amount of the coralline alga *Neogoniolithon brassica-florida*.

As shown for some marine gastropod species[33,34] and other invertebrate taxa[18,22], the decrease in recruitment we observed may be due to adverse effects of increased pCO_2 levels on *D. petraeum* early-life history stages (i.e. fertilization, larval development and settlement). To ensure that the female adults of the reef-building gastropod experienced fertilization and brooded fertilised eggs in capsules
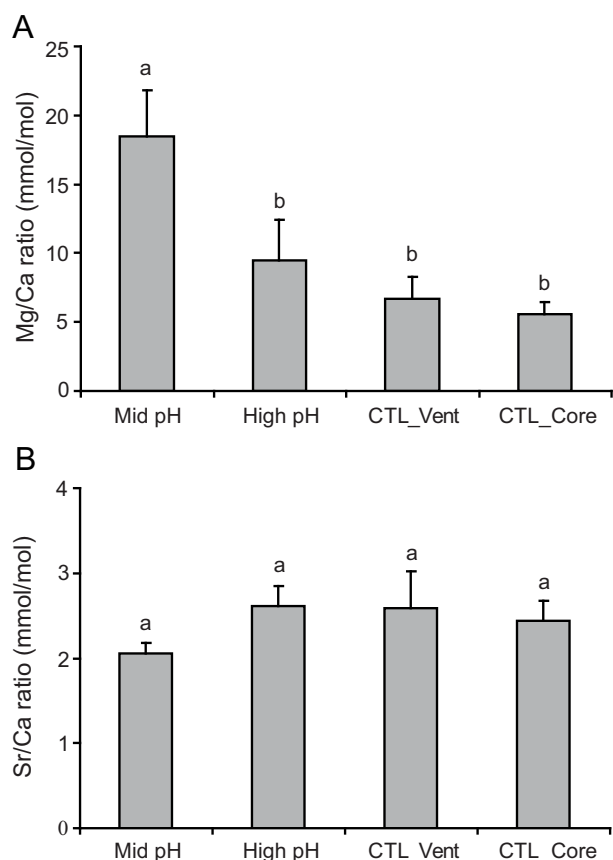
Figure 4 | **Shell mineralogy in the recruits of *Dendropoma petraeum* collected after 12-month exposure to a pH gradient.** (A) Mg/Ca levels of vermetid recruits shells increased in cores exposed to lowered pH conditions. (B) Sr/Ca levels were similar at sites with different pH values. Samples from the Low pH site were insufficient (with <3 recruits per core) to be analysed. Means ($\pm$S.E.; bars) with different letters (a, b) are significantly different (SNK test; $P < 0.05$).

along the Vulcano Island CO_2 gradient, we transplanted our vermetid cores at the end of the 2010 reproductive season (i.e. late November 2010) and before the next one started (April 2011)[17]. Under these circumstances all the vermetid females were simultaneously exposed for >1 month (in the short-term experiment) or for >6 months (in the long-term experiment) to the experimental pH conditions. At present it is unknown if vermetid larval development in egg casings within the maternal mantle cavity is affected by external environmental conditions. However, embryonic development within the egg capsules may be robust, since *Sepia officinalis* larvae are able to develop normally despite very high levels of CO_2 (i.e., 4000 μatm) within the egg capsules[35].

Upon hatching, vermetid larvae crawled out of the maternal shell and cemented themselves next to the mother using a flat aragonite disc. Although some molluscs can up-regulate calcification and tolerate acidified waters when a protective organic periostracum prevents shell dissolution[12], the vermetid settlement disc lacks this organic protective layer[17]. Therefore in areas subjected to periodic aragonite sub-saturation we found that the settlement discs were weakened by dissolution and that the new recruits were easily dislodged. We found no sign of shell dissolution of vermetid recruits at pCO_2 levels expected to occur in the next few decades (the High pH site) but their shells did dissolve at pCO_2 levels expected by the end of this century and beyond (in the Mid and Low pH site). Therefore, the lower post-settlement survival we recorded in the cores exposed to the most extreme pH conditions could be even overestimated as dissolution and dislodgement of dead shells under frequent periods

of aragonite under-saturation (with average values of pCO_2 above 3000 μatm and Ω_{ara} below 1) can occur. In estuaries, shell dissolution of newly settled juvenile bivalves exposed to under-saturated conditions can be a significant source of mortality that presents a bottleneck that can prevent successful recruitment[36]. The same is true for foraminifera as the high surface to volume ration of these small organisms means that they dissolve easily at 450 μatm pCO_2[37].

The vermetid shells laid down at elevated pCO_2 had significantly higher Mg/Ca ratios than shells grown in seawater with normal carbonate saturation states (Fig. 4A), as also shown in laboratory studies[38–41]. The incorporation of Mg has been used as temperature proxy, yet the role of other factors is poorly understood[38–41]. Variation in Mg/Ca ratio due to reduced carbonate saturation has been found in serpulid tubeworms[22,23] and some foraminifera[41–43]. The impact of seawater carbonate concentration on Mg/Ca, should therefore be taken into consideration when dealing with Mg/Ca thermometry[43]. As temperature, salinity and alkalinity were similar across our sampling site, we argue that it was differences in carbonate chemistry that altered the Mg/Ca ratio of the vermetid shells. Mg can substitute for Ca in carbonate, and many animals remove Mg from the calcification fluid since suboptimal Mg levels can weaken shells[44–46]. The increased Mg/Ca ratio we found in waters with lowered carbonate ion concentrations may reflect an impaired ability of the vermetids to remove Mg from haemolymph and extrapallial fluids. This inability to remove Mg from the calcification fluid may inhibit crystal nucleation[47]. In contrast, because no differences were observed on shell Sr/Ca ratios between transplant locations, it is likely that *D. petraeum* recruits, although growing in seawater with significantly different carbonate concentrations, were calcifying at similar rates (Fig. 4B)[48–50]. Incorporation of the larger cations of Sr plays a role in stabilization of the orthorhombic aragonite lattice[51], so an increased incorporation of the smaller Mg may interfere the lattice structure of aragonite. The effects of OA on material behaviour and chemical properties warrants further investigation.

In summary, our results show that vermetid reefs have reduced recruitment success and increased dissolution at expected levels of ocean acidification. Although vermetid recruits are resilient to near-future pCO_2 levels, it is likely that their reefs will not be able to withstand levels of acidification predicted for the end of this century. Sensitivity to ocean acidification seems to be higher in marine molluscs than in other marine taxa[52], especially at early lifestages[53]. Phenotypic plasticity in the short-term, and evolutionary adaptation over longer time periods may help vermetid survival[54,55] but it may be too late. Vast areas of vermetid reefs have recently died off in the Eastern Mediterranean which is thought to be due to widespread environmental changes in recent decades[16]. We fear that ocean acidification will accelerate this extinction process, exacerbating the effects of those additional stressors, such as pollution, that are known to damage these reefs[56]. Emergency conservation measures and a reduction in CO_2 emissions are both required to protect the reefs that remain. Ocean acidification may have far-reaching effects on the range of ecosystem services that pristine vermetid reefs provide, from coastal protection, carbon sequestration and habitat provision, with significant ramifications for coastal systems in the Mediterranean and other sub-tropical and warm-temperate regions.

Methods

(a) Study site, experiments set-up and analyses. This is the first reported successful transplantation of vermetid reefs. About the 80% of the transplanted cores remained after one year, withstanding wave action in the intertidal. At low tide, cores having similar abundances of *Dendropoma petraeum* adults were collected in November 2010 and April 2011 using a pneumatic drill (Airtec 478 SN, Italy) and a 13-cm corer (Fig. 1B,C) on pristine reefs at Cala Isola (NW Sicily). After collection, all the recruits of the year of *D. petraeum* ($\leq$2 mm shell size) were removed from each core with small forceps under a binocular microscope (Leica, MZ-APO). Newly attached recruits exhibit an evident protruding scar that constitutes the limit between the protoconch and the teleoconch, and rarely exceed 1 mm shell size at hatching and 2

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mm when 6 months old[17]. One year old specimens have 3–6 mm shell size and are often covered by the coralline *N. brassica-florida*[17].

In November 2010, the collected vermetid cores were randomly assigned to five intertidal sites: three sites along a pH gradient near Vulcano CO_2 vents (Low pH, Mid pH, and High pH at increasing distance from the vents), one control site at >800 m from the vents (CTL_Vent), and one site at the original coring location (CTL_Core) at Cala Isola, 81.5 nautical miles distance from Vulcano island, to control the transplant effect on recruitment. Tidal ranges are similar in the original coring location and in the vent sites, never exceeding 40 cm. Each core was included in a PVC tube, attached to a plastic plate and horizontally fixed at mean sea level (Fig. 1C). Fifteen vermetid cores (three per site) resisted after a 12-month exposure in the intertidal. To assess potential differences of exposure duration on vermetid recruitment, in April 2011 nine additional vermetid cores were collected, prepared as above and transplanted along the CO_2 gradient, but only at the Low pH, Mid pH and High pH sites. Therefore, the *D. petraeum* adults were simultaneously exposed for >1 month (in the short-term experiment, from April to November 2011) and for >6 months (in the long-term experiment, from November 2010 to November 2011) to the experimental pH conditions before the 2011 reproductive season started.

The cores were exposed to ocean pH and pCO_2 levels predicted in the next few decades (i.e., the High pH site) or by 2100 (Mid pH) and beyond (Low pH) respectively (Table 1)[25–27], whilst vermetids kept at CTL_Vent and CTL_Core sites were exposed at present-day pH and pCO_2 conditions (Table 1)[25–27].

All the cores were collected on November 2011, the top of each core was photographed and then they were frozen at −20°C. ImageJ software (open-access, National Institutes of Health) was used to estimate the total settlement surface available to recruits on each core (cm²), *D. petraeum* adult density (n. of ind. >3 mm shell size standardized to 100 cm²), and %cover of *N. brassica-florida*. Under a binocular microscope, the number of recruits' shells (i.e., distinguishing dead and living animals) was counted on each core collected along the pH/pCO_2 gradient and at the original coring site. Living recruits can be easily identified by the presence at the shell opening of the chitinous operculum, which is immediately lost after death. The number of living recruits (standardized in each core to 100 cm²) was used to assess the vermetid recruitment success along the gradient and references sites. In each core, the post-settlement survival (%) was calculated as the number of living recruits/total number of recruits' shells * 100. Therefore, post-settlement survival (%) can be overestimated if dissolution of dead shells and subsequent dislodgement by currents or waves occurred particularly in the most extreme pH conditions.

(b) Inductively coupled plasma–optical emission spectrophotometry (ICP-OES) and scanning electron microscopy (SEM) analyses. Shell elemental ratios for magnesium/calcium (Mg/Ca) and strontium/calcium (Sr/Ca) were quantified for living recruits of the 12-month experiment using inductively coupled plasma–optical emission spectrophotometry (ICP-OES, PE Optima 8300). Examined under the microscope, only newly attached recruits not covered by the coralline alga *Neogoniolithon brassica-florida*, which usually cement adults together, were used for compositional analysis therefore allowing to measure their shell isotopes elements without any potential contaminant. Unfortunately, because the newly settled recruits at the Low pH site were insufficient (with <3 recruits per core) to be analysed, samples from this site were not processed. To minimize the unbalanced sampling effects we used 5-20 individuals per core to analyze the average Mg/Ca ratio and Sr/Ca ratio. Recruits shells were treated with 5% bleach (NaOCl, Clorox TM) overnight (~17 h) to remove organic soft tissues from each sample. The cleaned tubes were rinsed with double distilled water twice, and were digested with 4 mL of 2% nitric acid. Analytes were measured for elemental presence of calcium (Ca, 396.847 nm), magnesium (Mg, 285.213 nm) and strontium (Sr, 407.771 nm) using ICP-OES. They were prepared in different dilutions (1-fold, 10-fold, 100-fold) enabling quantification of Ca, Mg and Sr within the calibrated ranges[57]. All glassware and containers involved in sample processing were soaked in 10% v/v HCl acid bath overnight, rinsed two times with double-distilled water and dried completely in an oven at 80°C. As well as quantifying the shell elemental composition using most of the available shells, 1–2 individuals from each core were selected at random and imaged on an SEM. Shells were air dried, mounted on SEM stubs with carbon tape for SEM observation without coating, and were imaged using Hitachi S-3400 Variable Pressure SEM at 20 kV.

(c) Seawater carbonate chemistry. At each field site the seawater carbonate system was characterized multiple times (Table 1) during the experiments. A 556 MPS YSI (Yellow Springs, USA) probe was used to measure salinity and pH. The pH sensor was calibrated using NBS scale standards buffers and then soaked in seawater for one hour. Hobo Onset loggers were also deployed to monitor seawater temperatures (°C) at 15-min interval for the whole duration of the experiment. For each site, average pH was calculated from hydrogen ion concentrations before reconverting back to pH values. Water samples for total alkalinity (TA) were filtered through 0.2 μm pore size filters, poisoned with 0.05 ml of 50% $HgCl_2$ to avoid biological alteration, and then stored in the dark at 4°C. Three replicate sub-samples were analyzed at 25°C using a titration system (Mettler Toledo, Inc.). The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0, as μEq Kg⁻¹ from the slope of the curve HCl volume versus pH. The pCO_2 and the saturation state of aragonite were calculated from pH$_{NBS}$, TA, temperature and salinity with the free-access CO_2 SYS package[58], using the constants of Roy et al.[59] and Dickson[60]. Temperature data are daily averages from continuous logging. Salinity and pH were collected on different visits (Nov 2010-Nov 2011, n =

95; Apr-Nov 2011, n = 35). Average Total Alkalinity was calculated from water samples collected at each site (Nov 2010-Nov 2011, n = 15; Apr-Nov 2011, n = 7).

(d) Statistical analyses. Analyses of variance were used to assess differences in recruits' responses in the 6-month and the 12-month exposure separately (one-way ANOVAs), and to compare potential consistencies in the results between the two experiments along the gradient sites (two-way-ANOVAs).

Specifically, one-way ANOVAs were used to compare the seawater chemistry parameters (see Table 1 for sample sizes), and both the number and the post-settlement %survival of *D. petraeum* recruits between sites with three levels (Low pH, Mid pH, and High pH) for the short-term (6 months) and five levels (Low pH, Mid pH, High pH, CTL_Vent and CTL_Core) for the long-term (12 months). A 2-way ANOVA was used to assess the number and the post-settlment %survival of *D. petraeum* recruits to exposure periods, with sites with three pH levels (Low pH, Mid pH, and High) as fixed factor and exposure periods (6 vs. 12 months) as fixed and orthogonal. One-way ANOVA was also used to assess the shell Mg/Ca and Sr/Ca ratio for new recruits shells obtained from the long term exposure (12-month) with sites as fixed factor with four levels (Mid pH, High pH, CTL_Vent and CTL_Core). Three replicates were considered for each analysis. Each replicate core was treated as a statistically independent replicate. Prior to run ANOVAs, normality was tested using Shapiro Wilk's test and homogeneity of variances was checked using Cochran's C-test, data were appropriately transformed whenever required. Differences among means at $P < 0.05$ were assessed using post-hoc SNK test.

Regression analyses were also run to assess the relationships between the numbers of recruits and either the number of *D. petraeum* adults or the %cover of *N. brassica-florida*. Data are presented as mean ± S.E. throughout the manuscript.

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Acknowledgments

Author contributions

M.M. conducted and conceived the experiments, analysed data and drafted the manuscript. R. R.-M., M.F. and C.A. conducted the field experiment. R. R.-M. carried out the TA analyses. V.B.S.C. and V.T. analysed the shell samples. J.M.H.-S. conceived the overall seep project. R.C. contributed to experimental design. All authors contributed to writing.

Additional information