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Digestion in sea urchin larvae impaired under ocean acidification

Meike Stumpp[1,2,3†], Marian Hu[1,2,3†], Isabel Casties[1], Reinhard Saborowski[4], Markus Bleich[2], Frank Melzner[3] and Sam Dupont[1*]

Larval stages are considered as the weakest link when a species is exposed to challenging environmental changes[1,2]. Reduced rates of growth and development in larval stages of calcifying invertebrates in response to ocean acidification might be caused by energetic limitations[3]. So far no information exists on how ocean acidification affects digestive processes in marine larval stages. Here we reveal alkaline (~pH 9.5) conditions in the stomach of sea urchin larvae. Larvae exposed to decreased seawater pH suffer from a drop in gastric pH, which directly translates into decreased digestive efficiencies and triggers compensatory feeding. These results suggest that larval digestion represents a critical process in the context of ocean acidification, which has been overlooked so far.

Ocean acidification as it is projected for the next century can affect vital functions of marine organisms. Larval stages are often particularly sensitive to ocean acidification. Decreased survival of larvae can directly affect population stability and could lead to decreased ecosystem integrity. As observed in several species, disturbances of extra- or intracellular acid–base homeostasis were correlated with energy budget reallocation and decreased scope for somatic growth and development[4,5]. However, little attention has been placed on whether digestive processes are impacted by decreased seawater pH, particularly in larval stages of marine invertebrates.

According to the preferred sources of nutrients and the necessary catabolic enzymes, digestive systems with distinct pH environments have evolved. For example, stomachs of most vertebrates operate at an acidic pH of ~2, corresponding to maximum activity of most gastric enzymes at low pH (ref. 6). On the other hand, the midgut of larvae of several insect species operates at a strongly alkaline pH of ~11 for the benefit of digestive enzymes (proteases, phosphatases) with a highly alkaline pH optimum[7]. To maintain high enzyme activities, digestive system pH is regulated by active ion transport processes through the net export or import of acid equivalents[8].

Here we investigated the effects of seawater acidification on digestive processes in green sea urchin pluteus larvae (*Strongylocentrotus droebachiensis*), which are keystone species in temperate and subpolar kelp ecosystems[9]. Owing to the fact that pluteus larvae cannot regulate the extracellular pH in their primary body cavity[3] the larval digestive system is directly exposed to changes in seawater pH. We reasoned that changes in seawater pH will directly influence the larval physiology by reducing stomach pH, digestive enzyme activity, and thus food assimilation and/or by challenging the acid–base regulatory machinery responsible for stomach pH maintenance. In terms of larval energy budgets, such challenges may be a critical reason for the reported reductions in growth and development of echinoid larvae in response to acidified sea water.

Using ion-selective micro-electrodes we found that the stomach pH of sea urchin pluteus larvae is alkaline, steadily increasing during ontogenesis from pH 8.9 in 5 day post-fertilization (dpf) to pH 9.6 in 9 dpf larvae (Figs 1a–d and 2a). Alkaline conditions in the digestive tract of herbivorous insects have been demonstrated to favour the solubility of plant proteins as well as the dissociation of phenol–protein complexes[10,11]. Furthermore, algal and higher plant chloroplasts are more efficiently digested at higher pH than at acidic or neutral pH (refs 12,13). Thus, alkaline conditions increase the ability to digest and dissolve dietary protein. This may also be advantageous to improve the utilization of algal proteins in an often nitrogen-limited trophic environment for most marine herbivores[14,15].

Chronic exposure to pH 7.7 or pH 7.4 sea water decreased stomach pH by 0.3 or 0.5 units indicating gastric alkalinity could not be maintained. (Fig. 1b,c). This effect was still evident when gastric pH values were corrected for developmental stage, demonstrating that gastric pH reduction can be attributed to ocean acidification rather than to developmental delay (Fig. 1d). Acute exposure (15–20 min) to acidified conditions (pH 7.3) could not change gastric pH (Supplementary Fig. S2C). The energetic consequence of the gastric pH reduction becomes even clearer when converting the pH gradients between stomach lumen and primary body cavity/sea water into proton concentrations. Under control conditions (sea water, pH 8.1) larvae maintain a H^+ concentration gradient of 7.9 nmol l^{-1}, whereas under acidified conditions (sea water, pH 7.4), this gradient increases to 39.8 nmol l^{-1}. These findings indicate that during chronic exposure to low pH, pluteus larvae are able to stabilize stomach pH at higher levels than expected by the observed decreases in seawater pH. We thus suggest the presence of well-developed ion-transporting gastric epithelia.

Using whole-mount immunostainings we could demonstrate that an antibody against avian Na^+/K^+-ATPase (NKA) detects a protein at the luminal surface and apical cytoplasm of stomach and intestinal cells (Fig. 1e). NKA is an important enzyme in ion-regulatory epithelia of many marine organisms[16]. It creates an electrochemical gradient that can energize other secondary active transporters relevant for acid–base regulation[17]. Although it is tempting to speculate that NKA is mainly involved in gastric alkalization, this enzyme also contributes to nutrient and amino acid uptake in sea urchin larvae[18]. For example, the uptake of

[1]Department of Biological and Environmental Sciences, University of Gothenburg, The Sven Lovén Centre for Marine Sciences, Kristineberg 45178 Fiskebäckskil, Sweden, [2]Institute of Physiology, Christian Albrechts University Kiel, 24098 Kiel, Germany, [3]Helmholtz Centre for Ocean Research Kiel (GEOMAR), 24105 Kiel, Germany, [4]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Functional Ecology, 27515 Bremerhaven, Germany. †These authors contributed equally to this work. *e-mail: sam.dupont@bioenv.gu.se

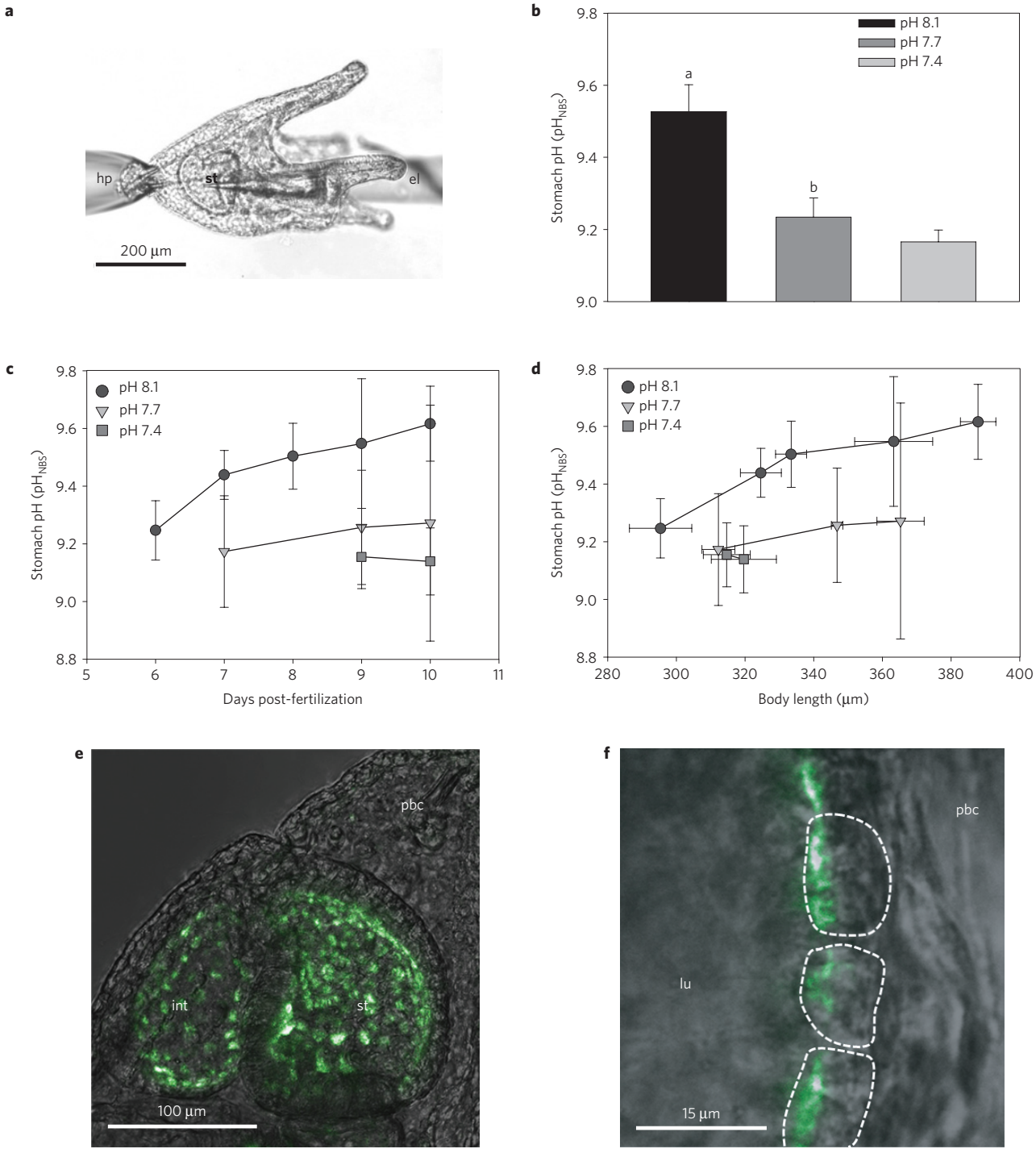


Figure 1 | Effects of acidified sea water on gastric pH homeostasis. a, *In vivo* measurements of larval stomach pH using ion-selective micro-electrodes. **b**,**c**, Gastric fluid pH average values (measured on the NBS scale) from 6 to 10 dpf (**b**) as well as pH over developmental time (**c**) revealed lower pH values in larvae reared in decreased pH. Letters denote significant differences among treatments. Values are presented as mean ± s.d. ($n = 3$). Within this time window two-way ANOVA results demonstrated no effect of time ($F(4, 30) = 2.38$, $p = 0.074$) but significant differences between control and CO_2-treated larvae ($F(2.30) = 12.86$, $p < 0.001$). **d**, The reduced gastric pH was still evident when pH values were corrected for developmental delay. **e**, Immunohistochemical detection of Na^+/K^+-ATPase (NKA)-rich cells in the stomach and intestinal epithelium. **f**, NKA is localized in luminal membranes. The dashed lines indicate the location of cells. For controls, see Supplementary Fig. S4. Microelectrode (el), stomach (st), holding pipette (hp), intestine (int), primary body cavity (pbc), lumen (lu).

neutral amino acids in pluteus larvae is Na^+-dependent and *in vivo* NKA activities increase in the presence of alanine in sea water[19,20].

The importance of NKA in sea urchin pluteus larvae is reflected in high (up to 77% of the total metabolic rate) metabolic requirements that can be attributed to energetic demands of this enzyme[20]. Increased expression of the NKA α-subunit in larvae of the purple sea urchin (*Strongylocentrotus purpuratus*) indicated a higher demand of this enzyme under

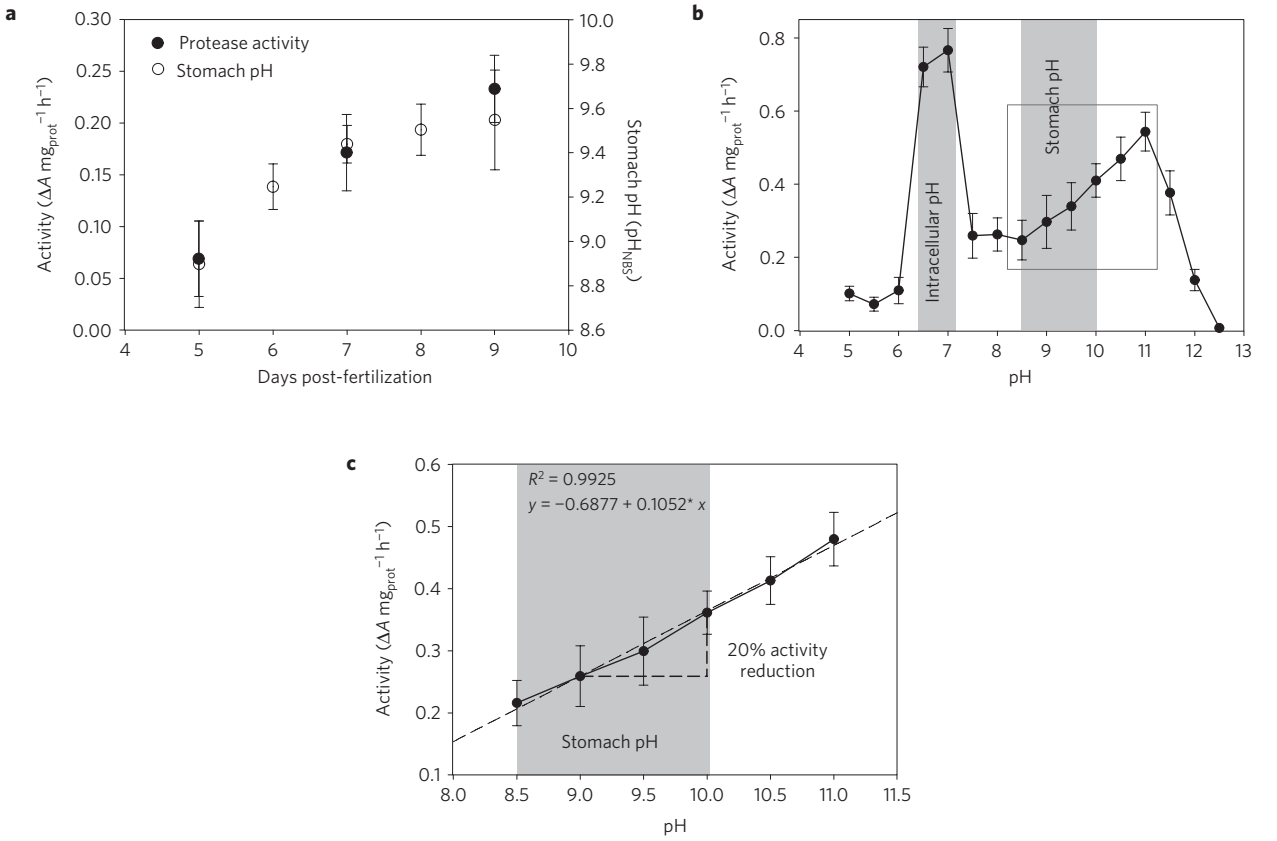


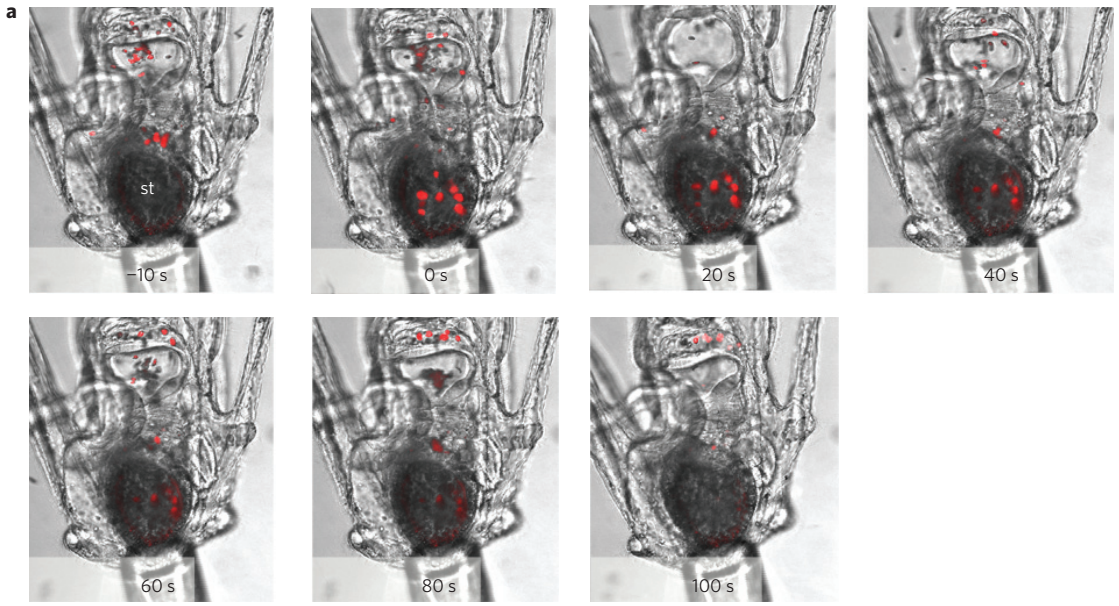
Figure 2 | Characterization of proteases in sea urchin larvae raised at pH 8.1. a, During early embryonic development total protease activity increased accompanied with an alkalization of gastric fluids. **b**, Proteases have one pH optimum at 6.5–7 and a second optimum at pH 11. **c**, The enzyme activity increased linearly between pH 8.5 and pH 11 by 20% of activity per pH unit. Values are presented as mean ± s.d. ($n = 3$).

acidified conditions[21]. The upregulation of NKA in this species is paralleled by an increase in metabolic rate, indicating a higher energy demand in response to CO_2-mediated seawater acidification[5]. Thus, additional costs for acid–base regulation to maintain alkaline digestive conditions or other energy-consuming compensatory digestive functions (for example, increased nutrient absorption) could significantly affect the organism's energy budget.

Proteolytic enzymes are important compounds of the digestive machinery of pluteus larvae to use dietary proteins and, thus, to meet nutritional nitrogen and amino acid requirements. Proteolytic activity as well as gastric pH increased in sea urchin larvae during ontogenesis (Fig. 2a), indicating a steadily rising digestive capacity.

Using *in vitro* enzyme assays, we found that sea urchin proteases are characterized by two pH-dependent maxima. The first maximum lies in a narrow pH range between pH 6.5 and pH 7 (Fig. 2b) and may be attributed to intracellular proteases that play an important role in specific post-translational processing, cell cycle, apoptosis mechanisms and digestive processes at intracellular pH conditions[22,23]. For example, type II cells of the pluteus larval stomach epithelium were described to phagocytose and digest whole algal cells[24]. This suggests the presence of intracellular digestion mechanisms, which specifically target phagocytosed algal cells. The second and wider maximum between pH 8.5 and pH 12 (Fig. 2b) can be attributed to alkaline proteases that have their maximum activity at pH 11. A very similar pH-dependent enzyme activity pattern with a peak in maximum activity between pH 8 and 10 was found for alkaline phosphatase in pluteus larvae of the sea urchin (*S. droebachiensis*)[25]. Alkaline phosphatase also is a very abundant enzyme in the digestive tract of *Strongylocentrotus* sea urchin larvae, particularly in stomach epithelia[26]. We found proteolytic activity to increase linearly by 20% per pH unit between pH 8.5 and pH 11 (Fig. 2c). Hence, a shift towards more acidic conditions in the stomach as we observed in the CO_2-treated larvae would reduce proteolytic activity. In sea urchin larvae exposed to acidified seawater of pH 7.5, this reduction would decrease protease activity by about 8%. Similarly, strong reductions (up to 40%) in alkaline phosphatase activity can be expected[25]. The degradation rate of ingested *Rhodomonas* cells, measured as chlorophyll *a*-dependent fluorescence intensity (Fig. 3a), was significantly (by 33%) reduced under acidified conditions (Fig. 3b), indicating a reduced digestive potential.

Larvae exposed to low pH (pH 7.2) had a delayed development and reduced somatic growth, which was also reflected by a delayed increase in proteolytic activity (Fig. 4a). However, *in vitro* determinations of maximum proteolytic activities (measured at the optimum pH of 11) at a body length of 350 µm (Fig. 4b) or at 10 dpf (Fig. 4b) did not result in differences between control and low-pH-treated larvae, suggesting that a loss in maximum proteolytic activity due to a shift in gastric pH is probably not compensated by increased enzyme synthesis. Nevertheless, at ecologically realistic food concentrations (6,000 cells ml^{-1}) CO_2-treated older larval stages (>400 µm body length) seemed to compensate for lower digestive capacities by feeding at higher rates (+11–33%, Fig. 4c,d). Our results suggest that decreased digestive efficiency seems to lead to compensatory feeding in sea urchin larvae. Compensatory feeding in response to lowered food quality and nutrient availability has been documented for terrestrial and marine invertebrates[27,28].



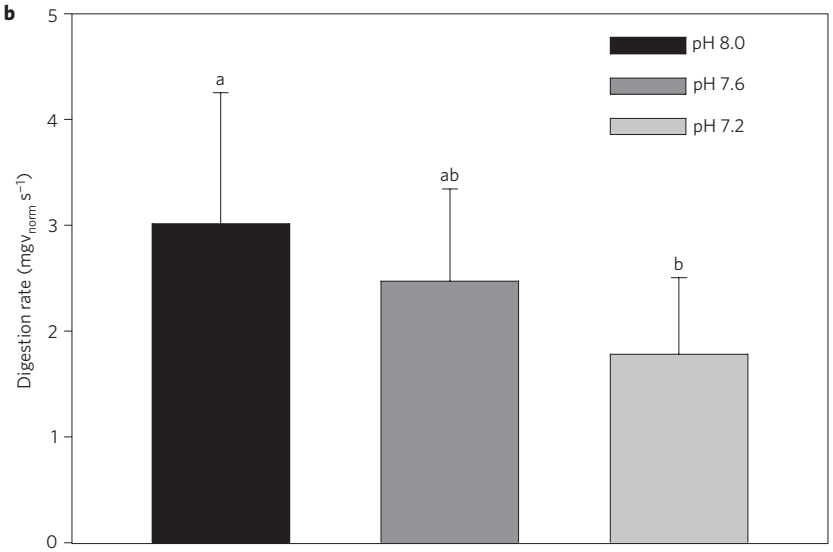


Figure 3 | *In vivo* digestion of sea urchin pluteus larvae exposed to different pH treatments. *In vivo* digestion rates of *Rhodomonas* cells were measured using confocal microscopy and are expressed as change in mean greyscale values (mgv) per time (Supplementary Fig. S5). **a**, Image series illustrating the *in vivo* digestion of *Rhodomonas* cells in a larva raised at pH 8.1. **b**, Digestion efficiency decreases in low-pH-treated larvae. Values are presented as mean ± s.d. ($n = 8$–9). For this experiment larvae between 8 and 16 dpf, corresponding to a body length range of 230–440 μm, were used. Different letters denote statistical differences among treatments (one-way ANOVA, $F(2,23) = 3.559$, $p = 0.045$); stomach (st).

However, compensatory feeding in response to decreased digestive abilities was not previously documented for larvae of marine invertebrate species. In this context, one has to keep in mind that the particle-feeding capacity in echinoid larvae is directly related to their morphological appearance, for example, ciliary band length on the larval arms[29]. As the allometric proportions of echinoid larvae are not changing in response to elevated p_{CO_2} (ref. 5), larvae from all treatments have the same maximum feeding potential and are thus limited in their capacity to compensate digestive inefficiency by increased feeding rates.

Elevated energetic costs for acid–base regulation to maintain calcification rates have been previously discussed as a critical factor for reduced development and growth in echinoid larvae[3]. The present work demonstrates that besides calcification alkalization of gastric pH constitutes another potential energy sink in sea urchin larvae facing ocean acidification. In addition, the shift of gastric pH towards more acidic conditions during ocean acidification decreases digestive efficiencies. Despite potential compensatory feeding mechanisms it is likely that the larval energy budget might be significantly affected by impaired food utilization accompanied with increased costs for gastric alkalization under acidified conditions.

Overall, the concept of energy limitation and reallocation in response to ocean acidification can provide an integrative approach for understanding the common principles of vulnerability of larval stages of key marine species. This will allow for a better understanding of the influence of climate change on recruitment success of marine keystone species and the ecosystems they shape.

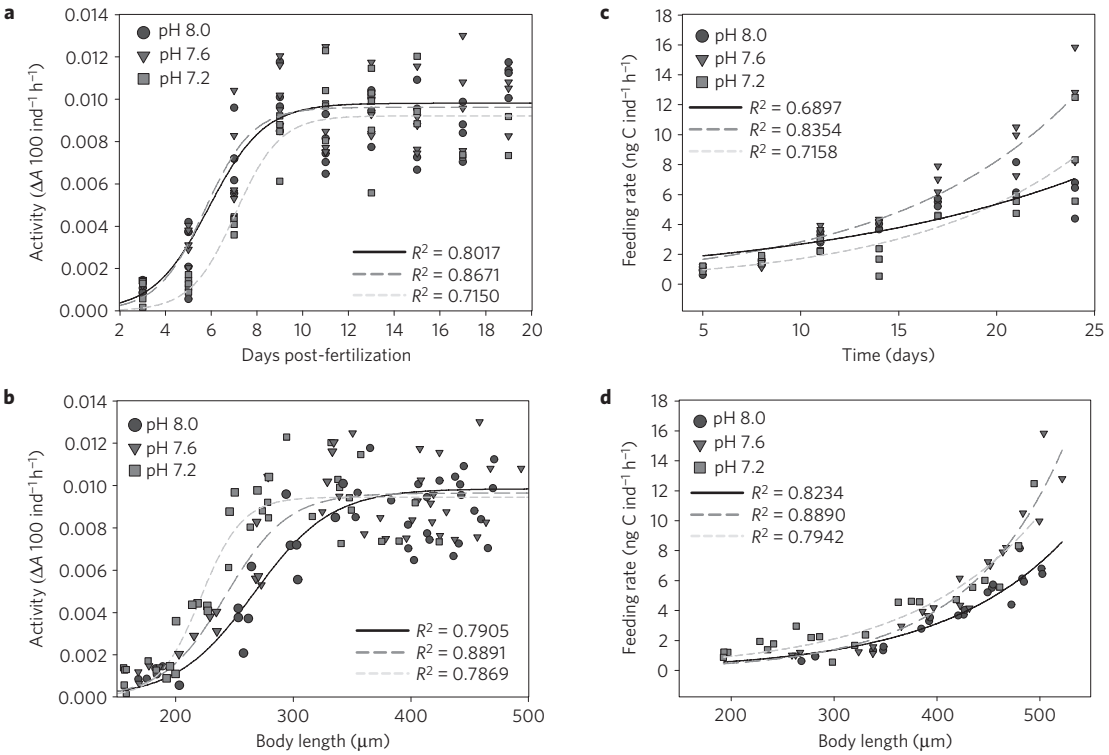


Figure 4 | Comparison of total protease activity in larvae cultured under different pH conditions. a, Larvae raised at pH 7.2 showed a delayed increase in protease activity compared with larvae raised at pH 8.0 and 7.6, but reached and maintain the same maximum activity after 11 days. **b**, Protease activity corrected to larval size. Total protease activity was fitted by a sigmoidal curve ($y = a/(1+e^{(-x-x0)/b)}$) with time and size as variables. **c**,**d**, Feeding rates as a function of time (**c**) and size (**d**). Feeding rates were fitted by an exponential function ($y = a*e^{(b*x)}$) and pH had a significant effect on feeding rate (analysis of co-variance, $F = 3.54$, $p = 0.029$ with time as a co-variable and $F = 14.90$, $p < 0.0001$ with body length as a co-variable). For statistical information on regression curves see Supplementary Table S2.

Methods

pH perturbation experiments. pH perturbation experiments for studies on the gastric pH in sea urchin larvae (*S. droebachiensis*) were carried out in 2011 at GEOMAR and CAU Kiel (Germany; exp 1; duration: 10 days). For enzyme assays and feeding experiments another pH perturbation experiment was conducted in spring 2012 at the Sven Lovén Centre for Marine Sciences (Kristineberg, Sweden; exp 2; duration: 24 days). Adult *S. droebachiensis* were collected in winter 2011/2012 in the Oslo Fjord (Dröbak, Norway; same population used in a previous study[3]) by scuba divers. Fertilization and CO_2 perturbation experiments were conducted as previously described[3,5] (also see Supplementary Information for more information).

Measurement of gastric H^+ concentrations. Ion-selective electrode measurements were performed as described previously[3] to measure H^+ concentrations in the stomach of sea urchin larvae. The linear regression between voltage output and $\log[H^+]$ values of artificial seawater solutions (pH 6, 7, 8 and 9) yielded a Nernstian slope of 49.8 ± 2.3 mV ($n = 12$) for 1 pH unit. The ion-selective probe was introduced into the stomach through the oesophagus (Fig. 1a). pH recordings were performed on pluteus larvae (5–10 dpf; pre-feeding day 5 omitted in Fig. 1) reared under control and hypercapnic conditions to address the effects of chronically elevated seawater p_{CO_2} (meaning continuous exposure starting after the first cell division) on gastric pH homeostasis.

Immunohistochemical staining. Whole-mount immunocytochemistry was performed as previously described[5]. Samples were incubated with a monoclonal antibody IgGα5 raised against the avian α subunit of NKA (Developmental Studies Hybridoma Bank, University of Iowa). After being rinsed with PBS, samples were further incubated in goat anti-rabbit IgG Alexa-Fluor 488 (dilution 1:100). Samples were examined with a Leica confocal microscope (SP5).

Protease activity measurements. Total protease activity was determined using azo-casein sodium salt (Sigma Aldrich) as the substrate. The assays were carried out with 2 µl of crude enzyme extracts that were added to 20 µl of universal buffer[30] adjusted to the required pH. After five minutes of pre-incubation, 5 µl of substrate (1% w/v in buffer) was added and incubated for 20 h at 25 °C. The reaction was stopped by the addition of 50 µl of ice-cold trichloroacetic acid solution (8% w/v in distilled water). The samples were centrifuged at 15,000*g* for 15 min at 4 °C. The absorbance (A) of the supernatant was read at 366 nm on a NanoDrop 1000 (Thermo Scientific) spectrophotometer. The absorbance of controls, to which crude extracts were added after stopping the reaction with trichloroacetic acid, was subtracted from the absorbance of the assay. Protein concentrations of the crude extracts were determined with a commercial protein assay kit (Pierce, Thermo Scientific) and bovine serum albumin as the standard. Enzyme activities are expressed as change in absorbance per milligram of protein and per hour ($\Delta A\ mg_{prot}^{-1}\ h^{-1}$).

***In vivo* digestion rates.** Larvae were kept in position using a holding pipette for 5 min before the start of the measurements inside the perfusion chamber (set to respective culture pH, that is, 8.0, 7.6, 7.2) on a Leica confocal microscope (SP5). Three min after the start of the measurements, *Rhodomonas* cells were added to the perfusate with a final density of 10,000 cells ml^{-1}. Ingested *Rhodomonas* cells were excited with 488 nm and the emission signal was recorded between 670 and 700 nm. Bleaching of cells was not observed (Supplementary Fig. S3 upper panel) and the settings resulted in a linear decrease in fluorescence intensity when algae were digested (Supplementary Fig. S5). Images were acquired every 10 s, and analysed using the open source program Image J.

Determination of feeding rates. To assess the impact of elevated seawater p_{CO_2} on feeding performance, feeding rates were determined as described previously[5]. Feeding rate was estimated as clearance rate by measuring the algal concentration (delta cells l^{-1}) at the end of the incubation (24 h) in control and experimental bottles with decreased pH levels. The rates were corrected for mortality and presented as rates per surviving animal in nanograms of carbon per individual per hour (ng C $ind^{-1}\ h^{-1}$).

Statistical analysis. Statistical analyses were performed using Sigma Stat 3.0 (Systat) and SAS software. Statistical differences between ion-selective electrode measurements and *in vivo* digestion rates were analysed by two-way and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Simple linear and logarithmic regression models were used to test the relationship type between the variables. Analysis of co-variance was used to test for pH effects on the growth and feeding rates using time post-fertilization (TPF) or body length as a co-variable. When logarithmic relationships were calculated, TPF and body length were logarithm transformed. Sigmoidal (three parameter) regression models were used

to fit the TPF versus protease activities and growth versus protease activities. Data sets were normally distributed (Kolmogorov–Smirnov test). Equal variance was tested using the Levene median test. The significance level was set to $p < 0.05$.

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Author contributions

M.S., M.H. and S.D. designed the study, conducted experiments, analysed the data and compiled the manuscript with the help of all other co-authors. R.S. contributed to enzyme characterizations. I.C. collected samples and performed the culture experiments including seawater chemistry analyses. M.B. and F.M. supported the planning operations for micro-electrode measurements and larval cultures.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.D.

Competing financial interests

The authors declare no competing financial interests.