Effects of low seawater pH on the marine polychaete *Platynereis dumerilii*

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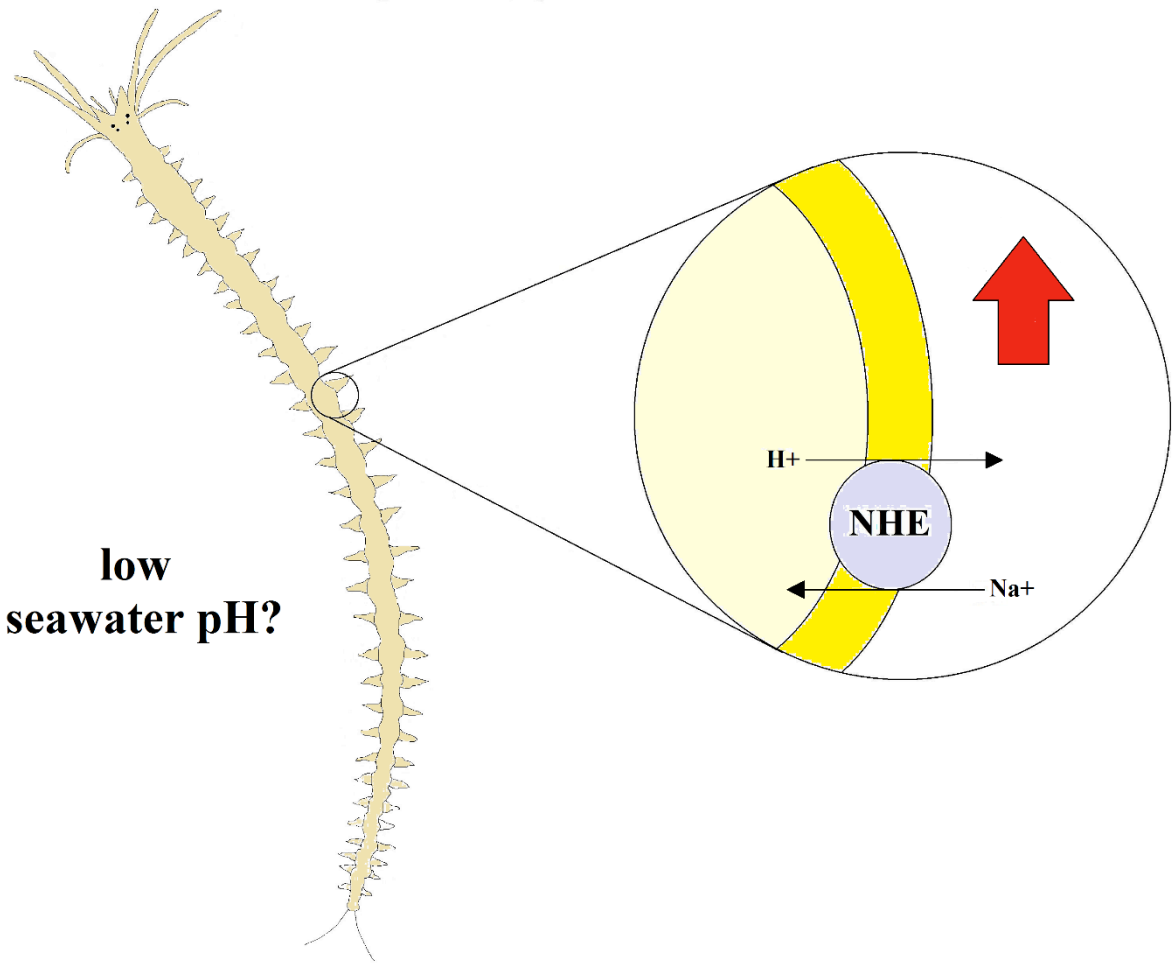

Abstract

An important priority for any organism is to maintain internal cellular homeostasis including acid-base balance. Yet, the molecular level impacts of changing environmental conditions, such as low pH, remain uncharacterised. Herein, we isolate partial Na^+/H^+ *exchangers* (*NHE)*, *carbonic anhydrase* (*CA)*, and *calmodulin* (*CaM)* genes from a polychaete, *Platynereis dumerilii* and investigate their relative expression in acidified seawater conditions. mRNA expression of *NHE* was significantly down-regulated after 1 h and up-regulated after 7 days under low pH treatment (pH 7.8), indicating changes in acid-base transport. Furthermore, the localisation of *NHE* expression was also altered. A trend of down regulation in *CA* after 1 h was also observed, suggesting a shift in the CO_2 and HCO_3^- balance. No change in *CaM* expression was detected after 7 days exposure to acidified seawater. This study provides insight into the molecular level changes taking place following exposure to acidified seawater in a non-calcifying, ubiquitous, organism.

Keywords**:** Acid-base regulation, low pH, gene expression, Polychaeta, *P. dumerilii*

Introduction

The regulation of the acid-base balance in cells is essential for cellular homeostasis. In order to maintain a normal pH range, hydrogen ions (H+) are regulated in the body fluids (Siggaard-Andersen, 2005). Most cellular processes have a small pH range within they will function, since protein conformations are altered by H^+, and therefore the biochemical activity may be modulated (Petsko and Ringe, 2004). The activity of an enzyme decreases outside the optimum pH range and greater changes can lead to denaturation (Ochei and Kolhatkar, 2000). There are other consequences of acid-base imbalance, such as metabolic suppression (Guppy and Withers, 1999), altered oxygen binding (Jensen, 2004), muscle malfunctions (Orchard and Kentish, 1990) and impaired synaptic transmission, neurotransmitter function and receptor binding (Sinning and Hübner, 2013; Ahn and Klinman, 1983; Ryu et al., 2003). Acid-base regulation also requires energy and can therefore cause changes in the energy budget depending on the function of the tissue (Pörtner et al., 2000).

Encountering a low pH environment can therefore potentially lead to acidification of bodily fluids unless cellular mechanisms, such as ion transporting or binding proteins, are able to maintain homeostasis (Michaelidis et al., 2005). To maintain the acid-base balance, ionic transporters are required to carry the excess ions out of the body (Walsh and Milligan, 1989), and these include the cell membrane Na^+/H^+ exchangers (NHEs) (Pörtner et al., 2000). NHEs are conserved throughout evolution, maintain pH and Na^+ homeostasis, and provide an adaptation mechanism to extreme pH environments (Hunte et al., 2005). Relevantly, NHE activities have been reported as inhibited at low pH (7.5) in the marine worm *Sipunculus nudus* relative to worms kept at pH 7.9 conditions (Pörtner et al., 2000).

Carbonic anhydrase (CA) encodes a zinc-containing enzyme that catalyses the reversible hydration of CO_2 to HCO_3^- ($CO_2+H_2O \rightleftharpoons HCO_3^-+H^+$) (Tripp et al., 2001), regulating H^+ and HCO_3^- levels in marine organisms such as the blue crab, *Callinectes sapidus* (Henry and Cameron, 1983).

The function of *CA* is well known in vertebrates, with roles in respiration, transport of CO_2/HCO_3^-, pH and inorganic carbon homeostasis and calcification (Bertucci et al., 2013). In invertebrate species, *CA* is known for its involvement in the biomineralisation process in coral, *Stylophora pistillata* (Moya et al., 2008), and is also highlighted as triggered by changing HCO_3^- concentrations and OA stress conditions (Moya et al., 2012). In contrast, calmodulin (CaM) is a multifunction Ca^{2+} binding protein, found in all eukaryotic cells, that triggers activation of more than 20 enzymes, and is essential in the regulation of cell proliferation and several stages of the cell cycle (Means et al., 1991). CaM is considered to have functions in the larval settlement and metamorphosis of the polychaete *Hydroides elegans* (Chen et al., 2012). Importantly, down regulation of *CaM* gene expression has been detected in oysters exposed to low pH seawater induced by CO_2 gas flow (Dineshram et al., 2012). The roles that each of these; NHE, CA, and CaM in turn, play in maintaining acid-base balance has yet to be determined in many marine invertebrates, including Polychaetes.

In this study we isolate partial *NHE, CA* and *CaM* cDNA sequences from a non-calcifying invertebrate, *P. dumerilii,* and investigate their gene expression in low pH (7.8) relative to control conditions (pH 8.2) following 1 h and 7 days experimental exposure. *P. dumerilii* is a model organism used for the study of molecular development, evolution, neurobiology, ecology and toxicology (Hardege, 1999; Hutchinson et al., 1995; Tessmar-Raible and Arendt, 2003) and, relevantly, can be found in, and perhaps adapted to, naturally occurring acidified habitats (Cigliano et al., 2010; Calosi et al., 2013). We report on significantly different gene expression profiles in worms maintained at low pH relative to those at normal conditions.

Materials and Methods

Animals and experimental exposure.

P. dumerilii (mean mass ± SEM: pH 8.2 worms 1h: 13.18 ± 1.63 mg, n=10; pH 7.8 worms 1h 11.32 ± 2.06 mg, n=10; pH 8.2 worms 1 week: 12.68 ± 1.65 mg, n=10; pH 7.8 worms 1 week:

10.47 ± 1.89 mg, n=10) were used from the laboratory culture from the EMBL Heidelberg (Germany). The gene isolation and temporal expression experiments were conducted with adult, but sexually immature atokus worms to reduce natural variation resulting from metabolic processes linked to reproduction and metamorphosis. The spatial gene expression experiments were conducted using larvae. Before the experiment, all specimens were kept in filtered natural seawater (~pH 8.2) at a light regime of 16 hrs light/ 8 hrs dark in a temperature controlled room at 18 °C. 20 worms were transferred into closed plastic containers (2000 cm^3) with approximately 800 ml filtered natural seawater (salinity 35ppt) of pH 8.2 (control) and another 20 worms were transferred into closed plastic containers (2000 cm^3) with approximately 800 ml filtered natural seawater of pH 7.8 (treatment) kept at the same light regime and temperature. The pH was adjusted using hydrochloric acid (1M) and sodium hydroxide (1M), and the water was changed every 24 h to ensure that the desired pH was maintained. After 1 h, 10 individuals from each treatment were transferred into RNALater solution (Sigma-Aldrich Company Ltd., Gillingham, U.K.). The remaining worms were kept for 7 days in the different pH seawater and then transferred into RNALater. Samples were stored at -80 °C until further processing. The two time points were selected to represent the initial stress response (1 h) and the acclimation response (at 7 days).

For the *in situ* hybridisation investigation of gene expression localisation, 1 dpf *P. dumerilii* larvae were separated from their jelly and transferred into closed plastic containers (100 cm^3) with approximately 80 ml filtered natural seawater at a pH of 7.8 or 8.2 for 7 days at 18 °C. The light regime, pH maintenance, and water change were the same as described for the adults. *P. dumerilii* larvae were then fixed in 4 % PFA for 2 h and stored in 100% methanol at -20 ˚C until hybridisation.

Target gene isolation and characterisation.

Nucleotide sequences for *18S ribosomal RNA* (*18S rRNA*), *alpha-tubulin* (*α-TUB*), *CA, NHE*, and *CaM* were obtained from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Blasted against the

worm database PLATYpopsys (http://hydra.cos.uni-heidelberg.de/pps/styled-2/) from the EMBL, Heidelberg. Total RNA was extracted from whole worms using High Pure RNA Tissue reagents (Roche Diagnostics Ltd., Burgess Hill, U.K.), according to the manufacturer's protocol. The integrity of total RNA was tested on a denaturing TAE agarose gel stained with ethidium bromide (Life Technologies, Paisley, U.K.). cDNA was synthesised using SuperScript VILO cDNA Synthesis reagents (Life Technologies, U.K.), with 14 µl of total RNA (pH 8.2 1 h: 0.52 ± 0.077 µg, pH 7.8 1 h: 0.51 ± 0.077 µg; pH 8.2 1 week: 0.46 ± 0.069 µg, pH 7.8 1 week: 0.41 ± 0.060 µg) and following the manufacturer's protocol. To degrade remaining RNA template the enzyme RNase H was used with its corresponding buffer (Thermo Fisher Scientific, Loughborough, U.K.) for a 45 min, 37 °C incubation. For the generation of *CA, NHE* and *CaM* PCR products, 1 µl of cDNA was combined with 0.5 µl of 10 pmol/ µl forward and reverse primer (Table 1), 0.25 µl of Herculase cDNA polymerase (Agilent Technologies, Wokingham, U.K.), 5 µl of 5× PCR buffer (Agilent Technologies, U.K.), 0.5 µL 40 mM dNTP mix (Thermo Fisher Scientific, U.K.), 0.5 µL DMSO (Agilent Technologies, U.K.), 0.5 µL 25 mM $MgCl_2$ (Thermo Fisher Scientific) and 16.25 µL sterile nuclease-free water (Fisher Scientific, U.K.) to prepare a total reaction volume of 25 µL. For the PCR conditions an initial denaturation at 94 °C for 30 sec was used, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec, finishing with a final extension of 72 °C for 2 min. The PCR products were analysed on a 1% agarose gel, stained with SYBR Safe DNA gel stain (Life Technologies, Paisley, U.K.), to confirm the presence of a single, correctly-sized band, and the PCR products were then sequenced directly (Macrogen Europe, Amsterdam, the Netherlands). Sequence identities were verified using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to complete nucleotide comparisons (blastn), and to compare the translated nucleotide sequence against the protein database (blastx). Additionally, the sequences were investigated on PLATYpopsys using nucleotide sequence comparison (blastn) and comparison of the translated nucleotide sequence against the protein database (blastx). Sequences were aligned using Jalview2.8.0b1 (http://www.jalview.org/). For phylogenetic analysis of the

partial amino acid sequence, MEGA6 was used. A Maximum Likelihood Analysis with the Nearest Neighbor Interchange method (1000 bootstrap replicates) was performed.

To determine the localisation of a selected gene expression (*NHE*), an *in situ* hybridisation experiment was conducted in parallel. For the generation of the NHE_{Probe} PCR product, 1 µl of cDNA (representing a mixture of different stages of *P. dumerilii*) was combined with 1 µl of 10 pmol/µl forward and reverse primer (Table 1), 0.12 µl of HotStar Taq Polymerase (Qiagen, Hilden, Germany), 1.2 µl 10× PCR buffer (Qiagen, Hilden, Germany), 0.5 µl 40 mM dNTP mix (Thermo Fisher Scientific, Schwerte, Germany), 0.5 µl 25 mM $MgCl_2$ (Thermo Fisher Scientific, Schwerte, Germany) and 17.68 µl of sterile nuclease-free water (Thermo Fisher Scientific, Schwerte, Germany) with a total reaction volume of 25 µl. The following PCR conditions were used: initial denaturation at 95 °C for 15 sec, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min, finishing with a final extension of 72 °C for 10 min. The PCR product was analysed on a 1% agarose gel followed by a purification step using QIAquick PCR Purification reagents (Qiagen, Hilden, Germany) and according to the manufacturer's protocol. The desired PCR product was subsequently cloned using TOPO® TA Cloning® reagents following the manufacturer's protocol (Invitrogen, Life Technologies, Darmstadt, Germany). Plasmids containing the desired PCR product were purified using a standard protocol (Qiagen). The identity of the desired *NHE* PCR product was confirmed by sequencing (GATC Biotech, Konstanz, Germany). The vector (20 µl, 14.12ng of DNA) was restriction digested with 4 µl of *Not*I enzyme (5u/µg), 5 µl of the corresponding buffer and 11 µl H_2O, with an overnight incubation at 37°C. The vector digest was cleaned with QIAquick® Nucleotide Removal reagents (Qiagen) and following the manufacturer's instructions. 1 µg of DNA was mixed with 2 µl 100 mM dithiothreitol (DTT), 1.3 µl NTP mixture (15.4 mM each ATP, CTP, GTP, and 10 mM UTP), 0.7 µl 10 mM DIG-UTP, 0.5 µl RNase inhibitor (4u/µl), 1 µl of Polymerase T7/Sp6 (20u/ µl) and 2 µl corresponding 10× transcription buffer. The total volume was adjusted to 20 µl with sterile nuclease-free water and then incubated at 37 ˚C for 6 h. After the incubation, 1 µl DNase I (1u/µl)

was added and a further incubation at 37 ˚C for 1 h conducted. Purification was then completed with the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol and using 40 µl for the final elution step.

***In situ* hybridization.**

The larvae were rehydrated as follows: 5 min 75 % methanol/25 % 1×PBS+0.1% Tween20 (PTW), 5 min 50 % methanol/ 50 % PTW, 5 min 25 % methanol/ 75 % PTW, and two rinses in PTW. This was followed by a Proteinase K (100 µg/ml) digestion in PTW for 2 min, two rinses in 2 ml/ml of glycine in PTW, a 5 min wash with 1 % triethanolamine, a 5 min wash with 1 % triethanolamine with 0.5% acetic anhydride, two 5 min washes with PTW, a post-fixation step in 4 % PFA and five 5 min washes in PTW. The animals were then prehybridized for 1 h in hybmix (50% formamide, 5× SSC, 50 µg/ml heparin, 0.1 % Tween20, 5 mg/ml Torula RNA) at 64 ˚C. Afterwards, the probe was denatured at 80 ˚C for 10 min and hybridised at 65 ˚C overnight. This was followed by two 30 min washes in 50 % hybmix/ 2× SSCT at 64 ˚C, a 15 min wash in 2× SSCT at 65 ˚C, two 30 min washes in 0.2× SSCT at 64 ˚C and blocked for 1 h in 5 % sheep serum in PTW. After adding pre-absorbed anti-DIG-AP Fab antibody (in 5 % Sheep Serum/PTW) (Roche) at a 1:2000 dilution and anti-acetylated tubulin at 1:250 dilution, the specimens were incubated at 4°C overnight. Specimens were washed six times, with shaking, in PTW for 10 min and equilibrated twice for 5 min in staining buffer (100mM TrisCl, pH 9.5, 100 mM NaCl, 50mM $MgCl_2$, 0.1% Tween20) while shaking. Larvae were transferred into a well plate and NBT/BCIP (337.5 and 175 µg/ml of NBT and BCIP) staining buffer was added. The staining was carried out in the dark and the staining process was monitored frequently under a microscope. Finally, the specimens were washed in PTW three times for 5 min.

qRT-PCR analysis of gene expression.

Total RNA was isolated and approximately 0.5 μg used to generate cDNA as described above. This was followed by a 45 min incubation at 37 °C with 1 μl RNase H enzyme (5u/μl) and 10× buffer (Fisher Scientific, U.K.). For the qRT-PCR reactions a final volume of 20 μl containing 10 μl of qPCR Fast Start SYBR Green Master Rox (Roche, U.K.), 1 μl of cDNA and 2 μl of 100 nM primers (Table 1) and 7 μl molecular grade water (Fisher Scientific, U.S.A.) was used. To determine the target cDNA amplification specificity, a control lacking cDNA template was included in the qRT-PCR analysis. A CFX96 Real Time PCR Detection System (Bio-Rad, Hemel Hempstead, U.K.) was used to detect amplification. The following cycling conditions were used: after 2 min at 95˚C, 45 cycles at 95˚C for 10 sec, 60˚C for 1 min and 72˚C for 1 min were used. To generate a melt curve, a heating step of 5 sec at 60˚C and 5 sec at 95˚C was added to the end of the PCR run. Two established reference genes, *18S rRNA* and *α-TUB,* were used (Zheng et al., 2011; Won et al., 2011) and validated: an analysis of variance (ANOVA) of the Cq values for the *18S rRNA* and *α-TUB* amplifications showed no significant difference (p=0.4341) between the pH treatment and/or time points. For the verification of the *target* mRNAs, melting curves and gel pictures were analysed to check for amplification specificity and absence of primer dimers. The amplification efficiency of each primer pair was calculated using a serial dilution of cDNA. The efficiency for the *target* genes ranged from 90.4% to 98.9%. For normalisation in pH 7.8 and pH 8.2 tissue of *P. dumerilii,* the reference mRNAs *18S rRNA* and *α-TUB* were used. To calculate relative expression levels of the target gene the $2^{-\Delta\Delta}Ct$ method was applied (Livak and Schmittgen, 2001).

Statistical analyses were conducted using GraphPad InStat v3 (GraphPad Software Inc., La Jolla, U.S.A.). Significance for relative gene expression was tested using an unpaired *t*-test. Outliers were identified, and removed, if they differed by more than twice the standard deviation of the mean. For each *NHE* (pH 7.8; 1 h) and for *CA* (pH 8.2; 1 h) one value was identified as an outlier and

excluded from the statistical analysis. For all analyses, statistical significance was accepted at $p<0.05$. Values are presented as means ± SE.

Results

Isolation of a partial *NHE-like* cDNA sequence from *P. dumerilii*. A 110 bp PCR product was isolated matching an *NHE2* from *Physeter catodon* **(XM_007108520.1)** and *Rattus norvegicus* **(NM_012653.2),** and an *NHE* from *Rattus* sp. **(L11236.1)** with 78% similarity in each case. The phylogenetic analysis divides the sequences into two main branches, whereby the *P. dumerilii* sequence clusters with *Saccoglossus kowalevskii* and two mammalian sequences (Supplementary Information Fig. 2a).

Isolation of a partial *CA* cDNA sequence from *P. dumerilii*. A partial sequence for *CA* gene (113 bp) was obtained by PCR from *P. dumerilii* cDNA. The Blastx search showed 76% and 62% similarity of the translated nucleotide sequence with *carbonic anhydrase 9-like* sequence and *carbonic anhydrase 1-like* sequence, both from *S. kowalevskii* **(XP_006811134.1** and **XP_006822732.1** respectively). In terms of conserved features, partial coverage of the alpha-CA domain was identified (Supplementary Information Fig. 1b) and phylogenetic analysis showed clustering of the sequence with other alpha-CA sequences (Supplementary Information Fig. 2b).

Isolation of a partial *CaM* cDNA sequence from *P. dumerilii*. A 57 bp *CaM* PCR product was sequenced from *P. dumerilii*, showing highest similarity to calmodulins from *S. purpuratus* (**P05934.1**), *Hydractinia symbiolongicarpus* (**AGB14582.1**) and *Clytia gracilis* (**AAZ23122.1**). Blastn searches revealed the highest nucleotide sequence similarity (93%) with calmodulin-1 of *Trichinella spiralis* (**XM_003379512.1**), followed by the calmodulin of *Spodoptera littoralis* (**HM445737.1**) (91%) and GM21351 of *Drosophila sechellia* (**XM_002033477.1**) (91%), all suggesting a partial *CaM* sequence. In terms of conserved features, two EF-hand domains with Ca^{2+} binding sites were identified (Supplementary Information Fig. 1c) and phylogenetic analysis

showed that *P.dumerilii* clusters with other invertebrate *CaM* sequences (Supplementary Information Fig. 2c).

Quantitative real-time PCR analysis of *NHE, CA* and *CaM* mRNA expression in *P. dumerilii*. The expression level of each target mRNA was analysed in worms maintained in normal or low pH conditions using qRT-PCR (Fig. 1). *NHE* was significantly ($p=<0.05$) down-regulated after 1 h ($p=0.0459$), but subsequently up-regulated after 7 days ($p=0.0480$) of treatment (Fig. 1a). *CA* mRNA expression showed a down-regulated trend ($p=0.0966$) after 1 h treatment, yet no clear trend was detected after 7 days of treatment (Fig. 1b). *CaM* gene expression showed no statistically significant difference between pH treatments at any of the two time-points (Fig. 1c).

***In situ* hybridization of *NHE*.** The *NHE* transcript was localised via *in situ* hybridization in 7 dpf old larvae kept at pH 8.2 and pH 7.8 (Fig. 2). Larvae kept at pH 8.2 (Fig. 2a and b) displayed NHE expression in the parapodia, as well as areas of the antennae and palpi. Worms maintained at pH 7.8, similarly displayed NHE expression in the parapodia, antennae and palpi, yet there was an additional expression detected in the area of the protodeum (Fig. 2c and d).

Discussion

The present study examined selected molecular-level biological effects of acidified seawater (pH 7.8) on adult, atokus marine polychaete, *P. dumerilii*. *NHE*, *CA,* and *CaM* partial sequences were isolated and their mRNA expression analysed in worms kept at acidified and normal pH seawater. These *targeted* genes represent several of the four general coping mechanisms for changing CO_2 and H^+ concentrations: namely passive intra- and extracellular fluids buffering; ion exchange and transport; transport of CO_2 in the blood if respiratory pigments exist; and change to a stage of metabolic suppression lasting until normal environmental conditions are restored (Fabry et al., 2008). Each of the partial sequences isolated from *P. dumerilii* (see: PLATYpopsys database:

http://hydra.cos.uni-heidelberg.de/pps/) showed similarity to a range of other species counterparts, as well as conserved domains, confirming their likely identities.

The expression of each gene was investigated in *P. dumerilii* kept at normal (8.2) and low (7.8) pH, at two times points of 1 h and 7 days. The seawater low pH treatment was achieved using acid adjustment, to gain a better general understanding of mechanisms involved in internal pH regulation. Following this simulated low pH exposure regime, *NHE* expression showed a significant initial down-regulation at 1 h, followed by a subsequent up-regulation at 7 days. *CaM* and *CA* showed no statistically significant changes at either time point relative to the control pH samples.

The significant up regulation of the NHE transport protein after 7 days suggests that the activity of existing protein levels were insufficient to regulate the pH under low pH conditions, and that an increase in *NHE* was required to maintain the acid-base balance. Stumpp et al. (2011) report *NHE3* expression level changes in sea urchin, *S. purpuratus*, pluteus larvae, kept at pH 7.7 for 2, 4 and 7 days post-fertilization (Stumpp et al., 2011). After 2 days an up-regulation in *NHE3* was detected followed by a down regulation of 45 % after 4 days (Stumpp et al., 2011). In contrast, a different study conducted on the same species showed no change in NHE expression under acidified conditions (pH 7.88-7.96) within <72 h (Todgham and Hofmann, 2009). In line with our result, the gene expression of *NHE* in the Japanese medaka fish, *Oryzias latipes*, showed an up-regulation in different ontogenetic stages (embryos, hatchlings, adults) as well as different tissue (gill, intestine) (pH 7.1-7.6) (Tseng et al., 2013). Each of these *NHE* expression studies discussed were, however, conducted using CO_2 modulated pH and the molecular level changes observed may have been triggered through different acid-base balance mechanisms compared with the acid modulated pH change used in the present study.

Perhaps surprisingly, neither gene expression of *CaM* nor *CA* changed significantly at altered pH in the worms studied, although *CA* mRNA expression did show an initial down-regulated trend ($p=0.0966$) after 1 h treatment (Fig. 1c). This agrees with a study using the coral *A. millepora*, using CO_2 altered pH conditions, where membrane associated and secreted *CA* were expressed at lower

levels in elevated pCO_2 conditions after 3 days (Moya et al., 2012). In contrast, *CA* expression in a second coral species, *P. damicornis*, was found to be up-regulated at pH 7.8 and 7.4 after a significantly longer exposure time of 3 weeks using CO_2 driven pH changes (Vidal-Dupiol et al., 2013).

That no significant changes in *CaM* expression were detected is in contrast to a number of other studies using calcifying organisms, such as oyster and coral. *CaM* expression has been previously reported as down-regulated in Pacific oyster (*C. gigas)* larvae and coral (*A. millepora*), in response to low pH seawater exposure induced using CO_2 (*C. gigas*: pH ~7.5 for 6 days; *A. millepora*: pH 7.8-7.9 and 7.6-7.77 for 10 days) (Dineshram et al., 2012; Kaniewska et al., 2012). In the commercial oyster, *C. hongkongensis*, CaM was found to be significantly down regulated at moderate low pH (7.9) and slightly up-regulated at low pH (7.6) (Dineshram et al., 2013). The comparison between the calcifying species, *C. gigas*, *A. millepora* and *C. hongkongensis* and *P. dumerilii*, a non calcifying species, may suggest that the expression of *CaM* under low pH conditions is more important for calcification / biomineralisation processes rather than any other stages in the cell cycle. Alternatively, the CO_2 driven pH change may trigger a change in *CaM* expression, via hypercapnia induced cellular processes, that are not responsive to the acid manipulated pH changes used in this study.

Possible explanations for a lack of significant changes in *CaM* or *CA* expression herein may relate to tolerance towards exposure to a relatively acidified environment and relative ion regulatory capacity or isoform differences. There is some evidence to indicate that *P. dumerilii* is more tolerant to low pH than calcifying species based on its occurrence in CO_2 vents in the Tyrrhenian Sea (Cigliano et al., 2010; Calosi et al., 2013). It has been shown that the species reside in naturally low pH environments, although it remains unclear whether they spend their entire life cycle in these areas or just inhabit them for a period of time. Another relevant factor is that adaptability towards environmental changes may vary according to different life stages, whereby many studies focus on early life history stage (O'Donnell et al., 2010; Wong et al., 2011; Zippay and Hofmann, 2010). In

cuttlefish *Sepia officinalis,* early life stages were affected by elevated pCO_2 and Hu et al. (2011) hypothesize that 'hypercapnia' causes metabolic depression, diverting energy towards ion regulation processes, and diverting it from embryonic growth (Hu et al., 2011). Thus, it is possible that *P. dumerilii* larvae may respond differently to low pH compared with adults, but atokous stages and further studies are needed to clarify such questions. A field investigation would also clarify whether acid-base impacts observed in the aquaria-reared worms are similar in worms sampled directly from the environment. Further studies would also be required to establish whether different isoforms of each gene exist in this, and other species, and any subsequent differing activities.

The localization of transcripts via *in situ* hybridization contributes to a better understanding of their potential molecular mechanisms of action (Dale et al., 2012; Moya et al., 2012). This study examined the expression patterns of *NHE* in 7 dpf old larvae using the same pH treatment (pH 8.2 and pH 7.8) regime, indicating differences in the *NHE* expression localization (Fig. 2). A pH-induced localization change, of glutamate decarboxylase, has previously been reported in the bacterium *Escherichia coli* (Capitani et al., 2003). The novel *NHE* expression detected in larvae kept at low pH near the anus area (Fig. 2d) is interesting since the anal papillae from *Aëdes aegypti* larvae take up Na^+ and Cl^- ions by Na^+/H^+ and Cl^-/HCO_3^- exchange (Stobbart, 1971). Studies using the teleost fish (*Opsanus beta*) showed that the intestinal epithelium plays a major role in acid-base relevant ion transport (Wilson et al., 2009; Grosell and Genz, 2006; Grosell et al., 2009a, 2009b). It thus appears that such additional changes take place around the protodeum area in *P. dumerilii*.

The molecular underpinning mechanisms of internal pH regulation are largely uncharacterised in many marine organisms. Herein, we have isolated *NHE*, *CA* and *CaM* partial cDNA sequences from *P. dumerilii* and examined their expression in worms kept at normal and low seawater pH. After 7 days, *NHE* expression was higher, and localized in different tissues, in worms kept at low pH levels compared to worms at normal pH levels. This indicates that under low pH conditions at least one active proton-ion transport mechanism is affected presumably in order to cope with the environmental changes taking place. This study provides a first insight into the

molecular mechanisms of action involved in seawater acidification in the non-calcifying marine model organism *P. dumerilii*.

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Figure and Table Legends

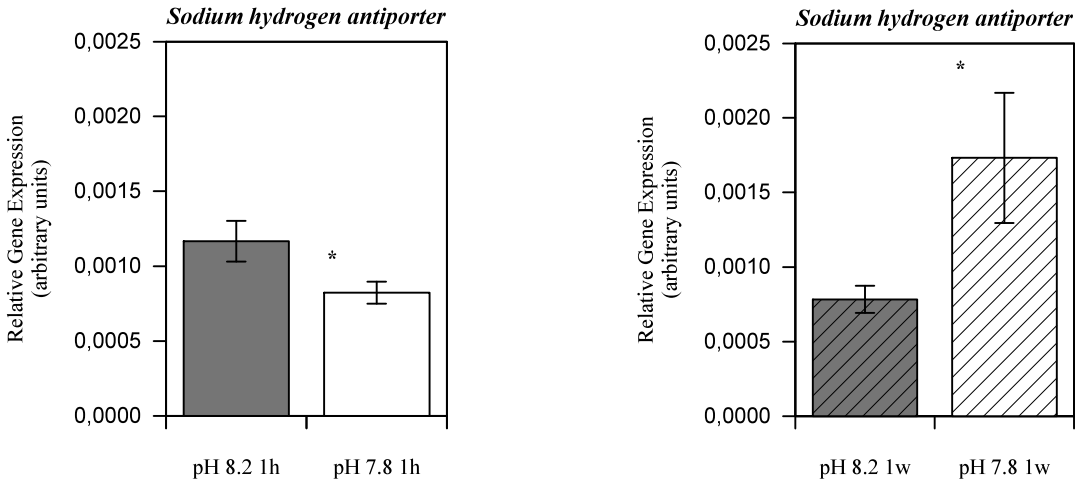
Figure 1. Normalised average relative mRNA transcription ± standard error of the mean in *P. dumerilii* for (a) *NHE* (b) *CA* and (c) *CaM* after 1 h and after 1 week in sea water with pH 8.2 and pH 7.8. Analysis was performed by unpaired *t*-test.* indicates significant differences ($p < 0.05$) of mRNA transcription between pH 8.2 and pH 7.8.

Figure 2. Localized *NHE* expression in 7dpf larvae maintained at pH 8.2 (a and b) and 7.8 (c and d). Bright-field images were taken on a Zeiss Axiophot microscope with a 200x magnification (Carl Zeiss, Jena, Germany).

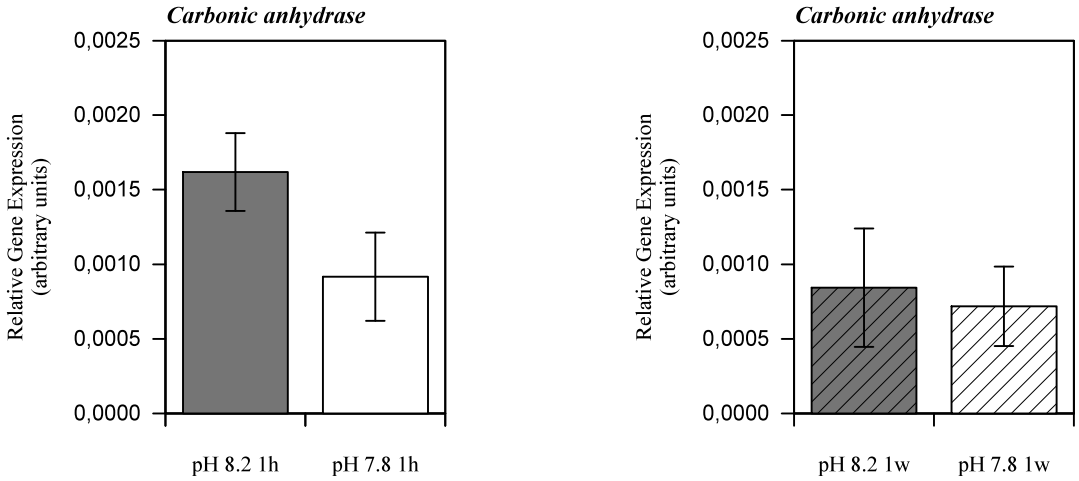
Table 1. Primers designed for isolation, qRT-PCR and hybridization analysis, and their expected amplicon size (bp).

Figure 1.

a)NHE



b)CA



c)CaM

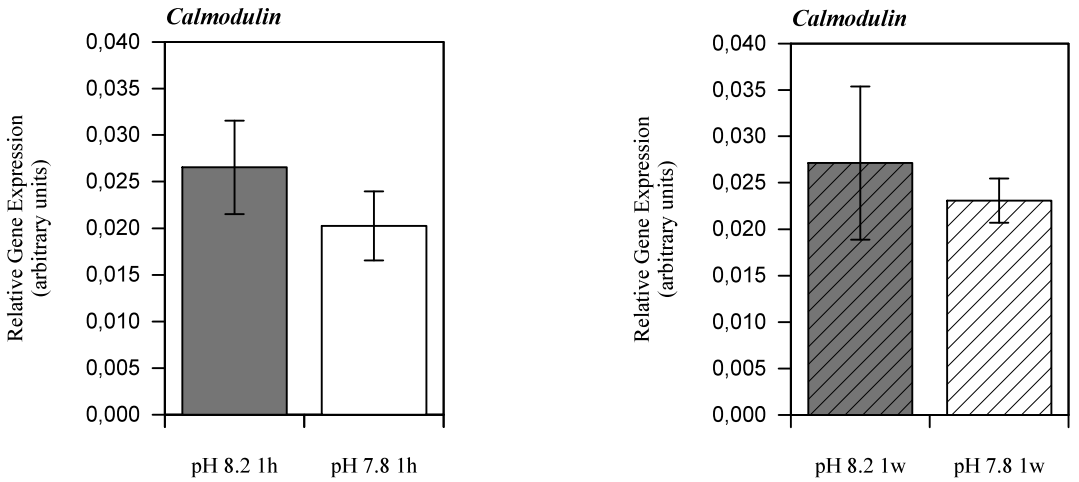


Figure 2.

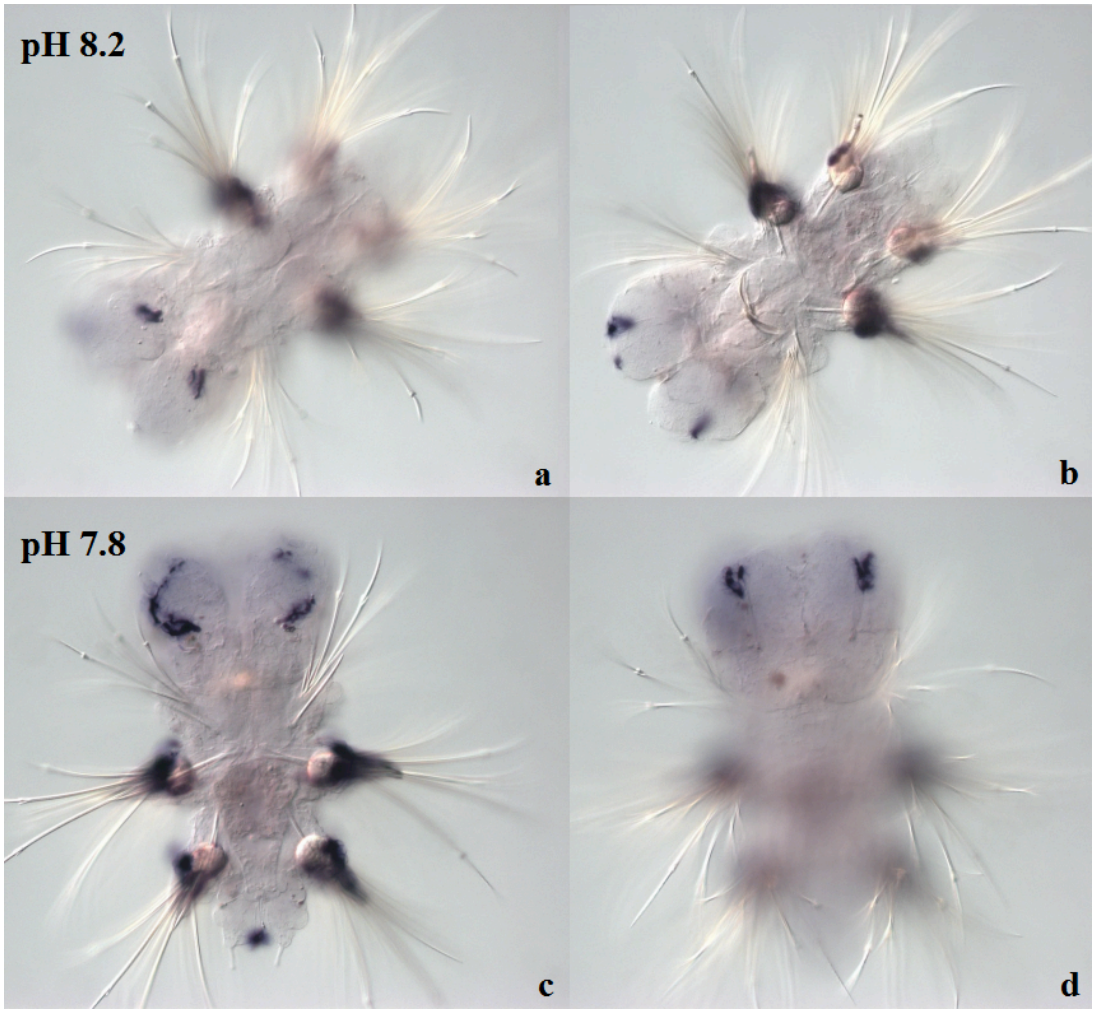


Table 1

Target gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**
18S rRNA	GCGCATTTATCAGCACAAGA	CTTGGATGTGGTAGCCGTTT	239
α-TUB	CTTCAAGGTCGGCATCAACT	TGGCAGTGGTATTGCTCAAC	101
NHE	CGCTCTGTTGCTGTCTTGAG	TGGCTACTAAGGCGAATGCT	130
CA	TAACCACCTCAACCGGAGAC	ATGGTGTGCTCTGAGCCTTT	118
CaM	AAGCTTTCCGAGTGTTCGAC	CCTCTTCGTCCGTCAATTTC	102
NHE_{Probe}	TCATGACAGCCATGGTCCTA	ACGTCAGGTATCCGAAGGTG	803

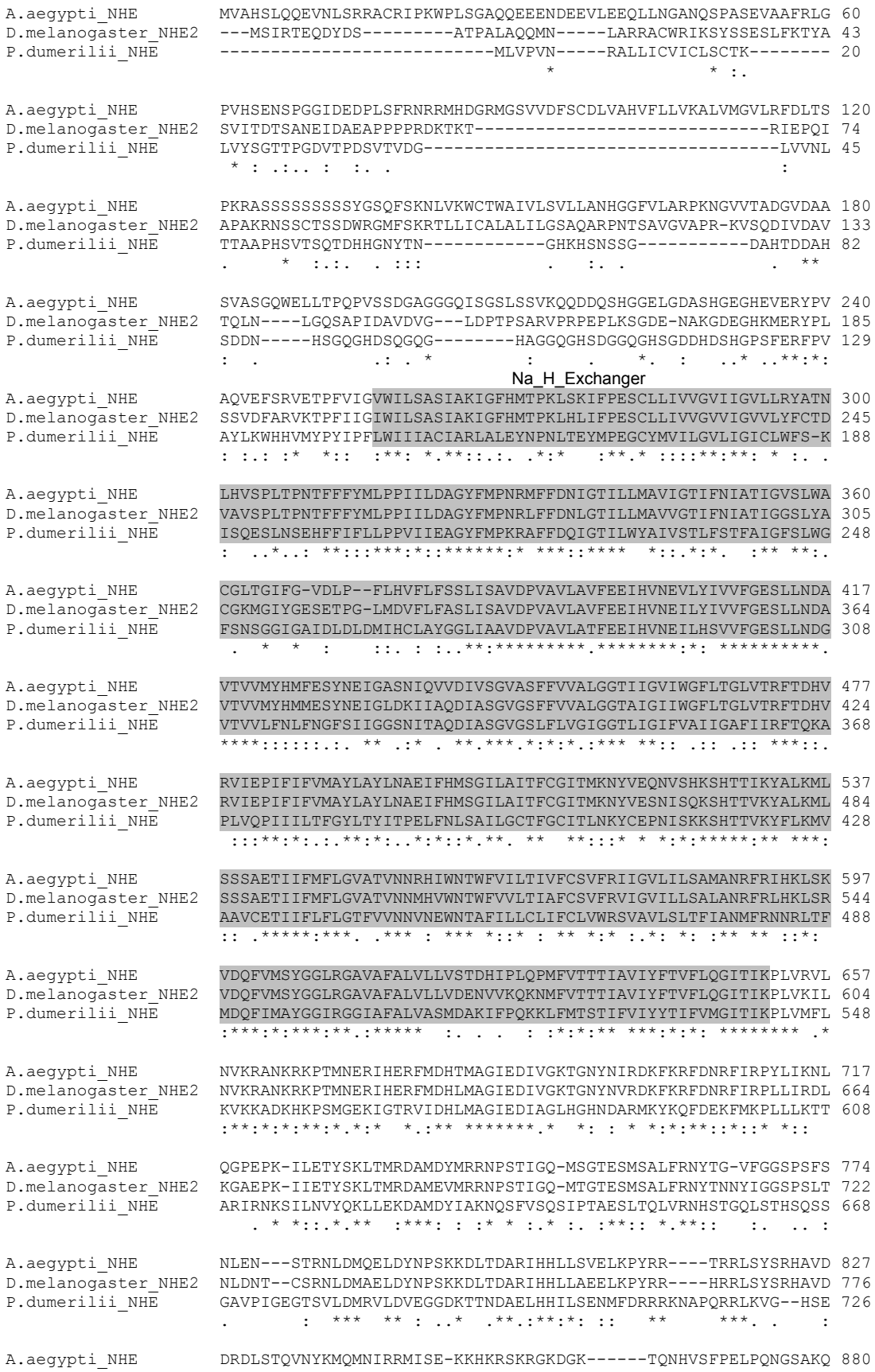
Supplementary Information

Figure 1. a) Alignment of partial *P. dumerilii*, *Aedes aegypti NHE* **(AAF80554.1)** and *Drosophila melanogaster NHE2* **(NP_001137847.1)** amino acid sequences. Dashes represent alignment gaps and asterisks represent homology. Colons represent sites with conserved amino acid substitutions and dots represent semi-conserved amino acid substitutions (similar shape). Light grey shaded regions represent functional protein domains. b) Alignment of partial *P. dumerilii CA*, *Patella vulgata CA* **(CCJ09594.1)** and *Nematostella vectensis CA1* **(DAA06053.1)** amino acid sequences. Dashes represent alignment gaps and asterisks represent homology. Colons represent sites with conserved amino acid substitutions and dots represent semi-conserved amino acid substitutions (similar shape). Light grey shaded regions represent functional protein domains. Dark grey shaded regions represent a zinc binding site conserved domain. c) Alignment of partial *P. dumerilii*, *Litopenaeus vannamei* **(AEK21539.1)** and *Bos taurus CaM* **(NP_001159980.1)** amino acid sequences. Dashes represent alignment gaps and asterisks represent homology. Colons represent sites with conserved amino acid substitutions and dots represent semi-conserved amino acid substitutions (similar shape). Light grey shaded regions represent functional protein domains. Dark grey shaded regions represent Ca^{2+} binding site on conserved domain.

Figure 2. Phylogenies of partial amino acid sequences for a) *NHE,* b) *CA* and c) *CaM,* using amino acid sequence with gaps in each case. Sequences were aligned and edited in Jalview and for the Maximum likelihood analysis Mega6 was used. The Jones-TaylorThornton (JTT) model was applied and for the heuristic search the Nearest Neighbour Interchange (NNI) method was performed. The numbers in the nodes represent bootstrap probabilities with 1000 replicates.

Figure 1.

a)NHE

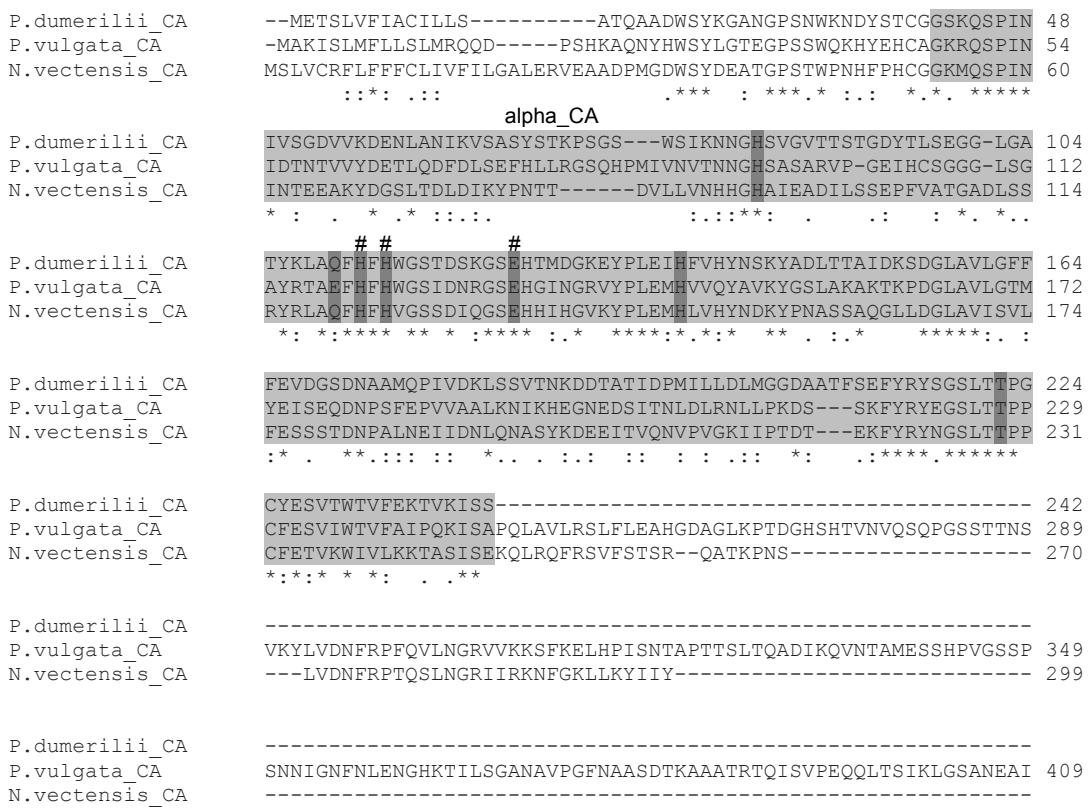


```
P.dumerilii_NHE      DAQQDSIIRHKAKLHLRSLAKQKRPSGMRKR--------------HSNMKNAGKKHDKKH 772
                     * : .: :.:* ::::* : .: :     **              * .: :  ::   *:

A.aegypti_NHE        FANDYINEVLHEDNEDEDKLPTAAGDDWDGGGLTFTAKSSLDEKSEQPKNDKRMSKDLRD 940
D.melanogaster_NHE2  LTN--AEECQQNPNEIN---VVGPSDDWDDG-LTFTAKSSLAEHP-IPEEDRNLSRES-- 887
P.dumerilii_NHE      KSG-------HHSDKHDDDKLSVASSGSDGGIVFYVPQNEEEEDS------KGKKKEP-- 817
                      :.       :. :: :      .... *.* : :..:..  *..      :  .::

A.aegypti_NHE        LEAAENRVTTPTAIEAVLPWKRVDEDDENGAIKQNEFPSWASNKEYLAYNSPSATFLGGL 1000
D.melanogaster_NHE2  --DGERRVATPTATESQLPWKRQG-DECTDAVQQNEFPAWASNKEYLAYNSPSATFLGGI 944
P.dumerilii_NHE      ----PEQIEEEPSATQTLPWRRET---VQEPESRASWPTWWRKTNHKSH----------- 859
                          .::   .:    ***:*        . .: .:*:*  :.:: ::
```

b)CA



c)CaM

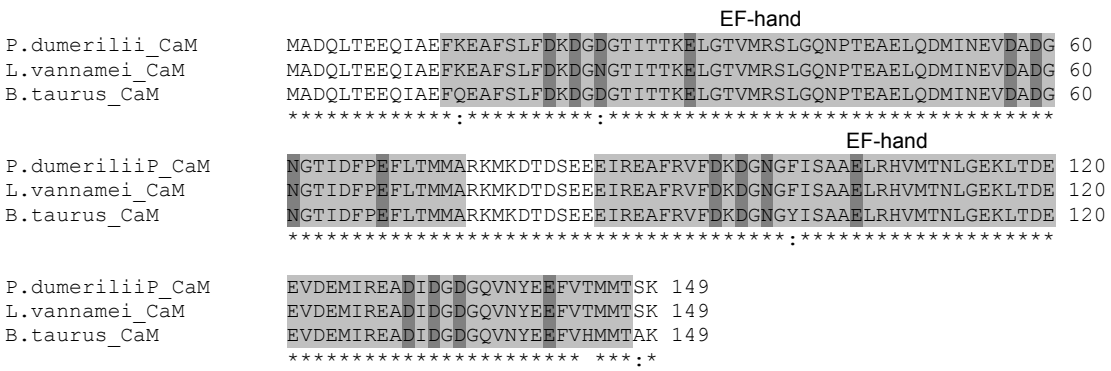
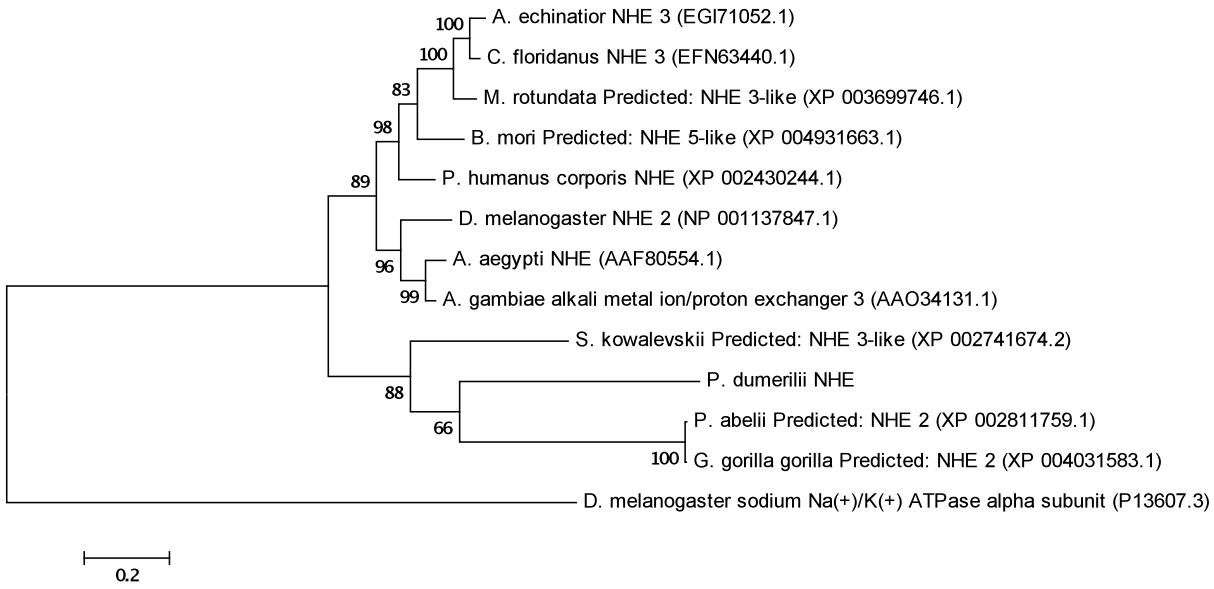
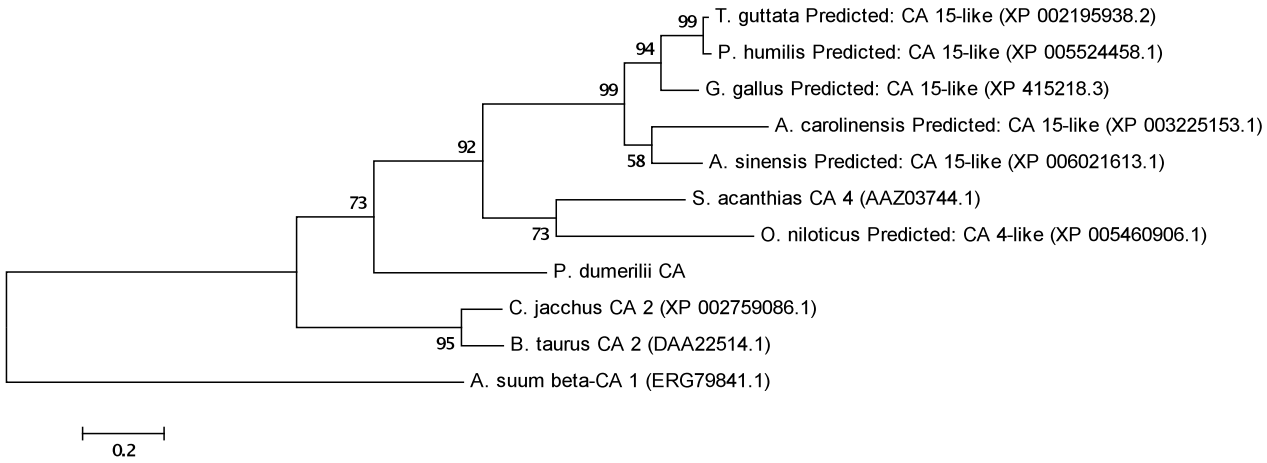


Figure 2.

a)NHE



b)CA



c)CaM