This paper shows that Anachis misera shells get more globular and are more eroded at sulphurous volcanic vents off Taiwan where the pH can fall to 7.22, and that protein expression co-varies with pH.

The first paragraph of the introduction has issues that are symptomatic of those that could be improved here and in the rest of the paper. The ocean acidification literature should be updated from Fabry et al. 2008 using more comprehensive meta-analyses such as that of Kroeker et al. 2013. The suggestion that field evidence on the effects of OA on gastropods is limited misses some key works e.g. Cigliano et al. 2010 who looked at juvenile gastropod settlement along CO2 gradients, Marshall et al. 2008 and others who have looked at gastropod shell parameters along coastal pH gradients an work on gastropods at Ischia and Vulcano using locations with elevated CO2 levels but without the confounding effects that are clearly present off Kueishan (Rodolfo-Metalpa et al. 2012; Milazzo et al. 2014; Langer et al. 2014).

Reply: Thanks for the comments. We already updated the references and discussion in the revised version as suggested.

The authors might be better playing down the link with ocean acidification but instead concentrating on effects of extreme hydrothermal environments on mollusc shell growth and relating this to how extreme events in the past may have altered mollusc shell morphology or calcification mineralogy in general.

Reply: We added one different dove snail, *Euplica* sp. for comparison with the vent snails and focus our findings on the shallow vent environments.

This paper lacks the level of carbonate chemistry detail required for a field-based examination of ocean acidification. The lack of geochemical data, lack of control or reference site data with normal pH values (ca 8.1) and some poor Figures (e.g. Fig 2) means that the paper has several points that could be improved.

Reply: We could not find *Anachis misera* in any other coastal environments in Taiwan. In the revised version, for the comparative purpose, we analyzed two populations of *Euplica* sp. collected in northeastern Taiwan where is in the west of Kueishan Islet about 10km apart.

Additional environmental information of the vents was listed in Table and the quality of Fig. 2 was improved too.

I have not reviewed the proteomics part of the paper as this is outside my expertise, but I would like a new version of the manuscript to review why and how protein expression typically alters in marine molluscs under environmental stress.

Reply: We added on paragraph to explain the proteomic-based method in “Introduction”. Our *Anachis* snails collected from 5 vent sites were classified to two
groups, i.e. V-South (pH 7.78-7.82) and V-Rest (pH 7.31-7.83) based on the analyses of protein expression profiles. Then the effects of naturally acidified seawater on shell traits of *A. misera* were quantified following. Application of proteomic approach was also discussed in the section of “Discussion”.

Interactive comment on *Biogeosciences Discuss.*, 11, 17207, 2014.

**K. S. Tan (Referee)**

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Received and published: 30 December 2014

This interesting paper examines shell characteristics and gross protein expression in populations of subtidal columbellid gastropods living in habitats influenced by shallow hydrothermal vent discharge. My main concern is that the authors have not identified a control site at the onset with a population that is not affected (at least relatively speaking) by the vent discharge. If we do not know how the population parameter varies at a control site, it would be difficult to determine if differences seen of the same parameter measured in another population at another site are caused by the conditions there. Based on pH data in Table 1, the two sites S and SW might be suitable control sites, and the two other affected sites should then be compared with the control sites. The authors in fact might want to consider pooling the data from S and SW.

Reply: Thanks for the comments. We could not find *Anachis misera* in any other coastal environments in Taiwan. In the revised version, for the comparative purpose, we analyzed two populations of *Euplica* sp. collected in northeastern Taiwan where is in the west of Kueishan Islet about 10km apart. Our *Anachis* snails collected from 5 vent sites were classified to two groups, i.e. V-South (pH 7.78-7.82) and V-Rest (pH 7.31-7.83) based on the analyses of protein expression profiles. Then the effects of naturally acidified seawater on shell traits of *A. misera* were quantified following.

Other issues:

1. Figure 1 is lacking in detail and would suggest the collecting localities (N, E, S, SW and NW as stated in the M & M section) and the locations of white and yellow vents be indicated clearly on a large-scale map of Kueishan Island. It would also help to indicate the prevalent current (Kuroshio) direction affecting the snail populations. What does the asterisk shown in the inset represent?

Reply: Fig. 1 was revised as suggested.

2. It is not stated how the pH of the environment given in Table 1 was measured at the different localities. How many readings were taken for each location over how long a time? Provide the sample size and period of sampling.

Reply: The description on variable measurements was added in “Materials and Methods”.

3. The shapes of the two shells shown in Figure 2 appear to be sufficiently different to
represent different species. How did the authors confirm that they are indeed one species?

Reply: Species identification on the dove snails (*A. misera* and *Euplica* sp.1) have been confirmed by COI and 16S rRNA genes as shown in the following figures. The snails collected in the Kueishan Islet belong to the same species.

![Maximum Likelihood tree of Columbellidae using GTR+G model based on the COI gene sequence.](image1)

![Maximum Likelihood tree of Columbellidae using GTR+G model based on the 16S gene sequence.](image2)

4. The authors seem to refer to the extent of erosion of the shell apex as a criterion (Results section, section 3.1 2nd paragraph) but it is not explicitly stated how this was ascertained. The number of individuals whose apices of the shells were eroded and the extent of erosion of the apex could be another parameter used to determine the effect of pH on the columbellid population.

Reply: It is subjective to determine the eroded condition. So, we revised the MS only report some eroded snails from East and Northwest sites (shown in Fig. 2).

5. Figure 6: what does the letters A and B represent? I would suggest that the authors compare the thickness and total animal weight of the shells of each size class separately, instead of standardizing the data, which has the effect of compressing the differences. Given that the mean shell lengths are between 9.0 and 9.2, comparing all shells between 8 and 10 mm in length might provide a cleaner conclusion.

Reply: The data were re-analyzed completely. So, the part was deleted already.

6. The Discussion section would benefit from re-organization of the paragraphs (which I find...
to be rather disconnected from each other), focusing on the implications of the results obtained, i.e., the change in shell morphometry caused by low pH, and the possible reasons behind the protein patterns obtained (this is presently not addressed in the Discussion), in relation to the known ecology of Kueishan Island. The discussion can then be extended to the greater realm of ocean acidification and how the results here contribute towards the existing understanding of this phenomenon. The paragraphs in the Discussion are rather unconnected in thought and does not read well. For instance, in section 4.1, the first paragraph reviews the evidence for reduction in shell growth in acidic conditions, but the results of the Kueishan Island study are not discussed. In the second paragraph, shell shape is suddenly brought into the picture, and the authors assert that a rounded shell shape is less vulnerable to crab predation, but it is unclear how this is related to the authors’ results, when there is no data presented on crab predation of Anachis at the study site. The next paragraph jumps to conditions in the deep sea, and the last paragraph finally refers to the results from this paper, which should really be moved to the first paragraph. Similarly, the second section (comparison with other Anachis studies) would be more appropriately positioned earlier in the Discussion section.

Reply: The discussion was extensively revised as suggested. We separated it to 3 parts, including 1. Application of proteomic-based approach to shallow vent snails; 2. Comparison with other dove snail studies; 3. Comparison with other ocean acidification studies.

7. The authors should also address the question of whether columbellids are particularly adapted to acidic environments compared to other gastropods or mollusks in the Discussion.

Reply: The discussion was added but adaptation mechanism is uncertain.

Interactive comment on Biogeosciences Discuss., 11, 17207, 2014.
Effects of low pH stress on shell traits of the dove snail, *Anachis misera* inhabiting in shallow vent environments off Kueishan Islet, Taiwan

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Abstract
The effects of naturally acidified seawater on shell traits were quantified through the comparison of dove snails (Family: Columbellidae) *Anachis misera* from vent environments and *Euplica* sp. from non-vent sites in northeastern Taiwan. Samples of *A. misera* were collected around the shallow vent (24.8341°N, 121.96191°E), including the East, South, Southwest and Northwest sites. An absence of *Anachis* snails was found in the most acidic North site (pH 7.19-7.25). Based on the similarities of protein expression profiles, the *Anachis* snails were classified to two groups, i.e. V-South (pH 7.78-7.82) and V-Rest (pH 7.31-7.83). Comparing their shell traits to the non-vent *Euplica* sp. from Da-xi (DX) and Geng-fang (GF) (pH 8.1-8.2), difference in shell shape (shell width:shell length) as vent populations being more globular than that of non-vent ones was found. The means of shell width were significantly different among sites (p<0.01), with a descending order of GF > DX > V-South, V-Rest. The relationships of shell length to total weight were curvilinear for both *Anachis* and *Euplica* snails. The logarithmic transformed slopes differed significantly among sites and the mean body weight of GF population was greater than others (p<0.01). Positive correlations between shell length and shell thickness of body whorl (T1) and penultimate whorl (T2) were only observed in non-vent GF and DX populations. *Anachis* snails from vent sites were thinner in T1 and T2 compared to the *Euplica* snails from non-vent sites (p<0.05). Within each vent group, shell thickness between T1 and T2 was insignificantly different. Between vent groups, T1 and T2 from V-Rest showed a decrease of 10.6% and 10.2%, respectively, compared to V-South ones. The decrease of T1 and T2 between vent *Anachis* snails and non-vent *Euplica* snails was as great as 55.6% and 29.0%, respectively. This was the first study to compare snail’s morphological traits under varying shallow vent stresses with populations prior classified by biochemical responses. As a whole, the shallow vent-based findings provide additional information from subtropics on the effects of acidified seawater on gastropod snails in natural environments.
1 Introduction

Although current evidence indicates that organisms with a CaCO₃ skeleton, e.g., mollusks, echinoderms and corals, are likely to be among the most susceptible to ocean acidification (such as in Fabry et al., 2008; Sokolov et al., 2009), specific information obtained from field investigations has been limited, particularly in gastropod snails (Gazeau et al., 2013). Thus, the current study was performed to address this issue within an extreme hydrothermal environment.

The shallow hydrothermal vents locate east of Kueishan (KS) Islet, Taiwan, near the southern end of the Okinawa Trough (Fig. 1). The vents emit yellow or white plumes with temperature and pH varying in the ranges of 78-116 °C and 1.52-6.32 vs. 30-65 °C and 1.84-6.96, respectively. The gas bubbles are comprised of 90–99% CO₂, 0.8–8.4% H₂S, <0.03% SO₂, and <50ppm HCl (Chen et al., 2005). The diffusive plumes are affected by the wind, sea waves, and tides (Chen et al., 2005; Han et al., 2014). Based on the observed data, the emitted fluids diffused mainly from north to south due to ebb tide and moved from southeast to northwest during the spring tide. In addition, the fluids are also directed by the Kuroshio current flowing along the coast of Kueishan Islet to the north throughout the year. Because the diffusion is closely correlated with diurnal tides, benthic organisms would face the lowest pH twice per day but for no more than four hours each time.

Near the yellow vents, the crab *Xenograpsus testudinatus* is the only benthic macrofauna (Jeng et al., 2004). In contrast, around the white vents, benthic invertebrates include the crab *X. testudinatus*, two sea anemones, hexacoral *Tubastrea aurea*, serpulid polychaete, a chiton, snail *Nassarius* sp., and the dove snail *Anachis misera*. These vent organisms naturally inhabit acidic and toxic environments. High concentrations of trace metals in various tissues of the crab *X. testudinatus* are reported and the levels are not beyond other crabs collected from different habitats (Peng et al., 2011).

We herein test the hypothesis that populations of *A. misera* distributed around vents exposed to varying degrees of plumes would exhibit different ecophysiological performance compared to the non-vent dove snail, *Euplica* sp., a common species in coastal waters of northeastern Taiwan.

A proteomic-based method was used to classify the samples of *A. misera* collected around the vent-based environments. This approach involves measuring changes in many proteins. Through the comparison of protein expression profile of each snail by cluster analysis, similarities among samples can be determined and classified. This method has been applied to laboratory and field pollution studies, such as blue mussels exposed to polyaromatic hydrocarbons and heavy metals (Knigge et al., 2004), and Sydney rock oysters inhabiting in acid sulfate runoff estuary (Amaral et al., 2011).
2 Materials and methods

2.1 Sampling sites and collection of snails

Anachis misera was collected around a shallow-water vent in Kueishan Islet, Taiwan (Fig. 1), including the north (N), east (E), south (S), southwest (SW), and northwest (NW) sites during the period of June 28 to July 1, 2011. The sampling vent emitted white plumes where another vent with yellow plumes was nearby northeasterly. The distance of the collection sites to the vent center was 10-16m, and the water depth was in the range of 14.5-17.5m. Snails of Euplica sp. were sampled from Da-xi (DX) and Geng-fang (GF), northeastern Taiwan, between July and Sept. 2012. Sampling locations were identified by SCUBA divers equipped with GPS. Temperature was determined by a thermometer inserted into the seawater samples. Flow rate was measured by a hydrobios digital flow meter (Model 438 110). The pH was measured by a radiometer PHM 85 system. Each environmental parameter was determined with one or three replicates and the results were shown in Table 1. The collected snails were preserved in dry ice in the field. Upon returning to the laboratory, they were deep-frozen at -70°C for later use.

2.2 Measurements on snail morphological traits

Shell traits, i.e., shell length and width, shell thickness of body whorl (T1) and penultimate whorl (T2), as well as total weight of the intact individual, were measured (Fig. 2). Shell thickness was determined through enlarged X-ray radiographs which were produced by exposing snail shells to X-ray with the settings of 80kVp and 1mA for 116.7ms. The distance between the X-ray source and the objects was 50cm. The shell images were further drawn outlines using GIMP version 2.8 which is an open source imaging system (http://www.gimp.org/).

For statistical analysis, an ANCOVA (analysis of covariance) was used to compare the least square means (LSM) for each variable (i.e., shell width, total weight, shell thickness T1 and T2) among sites with shell length as the covariate. The relationships of shell length to shell width, shell thickness of T1 and T2 were calculated using linear regression analysis. If the relationship of total weight and shell length was curvilinear, linear regression slopes were obtained and compared after data are logarithmic transformed.

2.3 Proteomic study

The protein expression profiles of Anachis snails were determined by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1-D SDS-PAGE). The foot tissue was taken and homogenized with lysis buffer (0.5 M Tris-HCl, pH 7.4, 10% SDS, 0.5 M DTT) for proteomic analysis. Homogenates
were centrifuged at 13000g for 10 min at 4°C. The homogenous supernatant was collected, and the protein concentration was determined by Bradford assay, using bovine serum albumin as the standard.

The stacking and resolving gels were prepared in the percentages of 5% and 12% (Hoefer SEM 260 system, Amersham Pharmacia). After loading 25ug protein in each sample lane, electrophoresis was run for 30min at 120V then 4h at 180V. The gels were stained with Coomassie blue G-250 (Candiano et al., 2004).

Stained gels were scanned and transformed into digitalized images using Image Scanner (Amersham Pharmacia). The Multi Gauge software v2.2 (Fujifilm) was used for protein quantification. The protein bands were assigned band numbers, and their intensity levels were calculated as their relative area to the total protein area on the gel. A cluster analysis of the Bray-Curtis Similarity (BCs) Indices (Primer 6.0) was employed to compare the expression of overall protein patterns among snail individuals (Clarke and Warwick, 2001). In addition, the contribution of each protein band was further estimated by principal component analysis (PCA).

3 Results

3.1 Morphological traits of Anachis snails from vent sites

Temperature ranges of the sampling sites were from 26 to 27°C (Table 2). Spatial variability in pH among sites was clearly observed, and the lowest one was 7.22±0.03 at the North site (p<0.01). Anachis snails were found around the vent, except for the most acidic North site. Several snails with eroded apex were observed in the East and Northwest sites (Fig. 2).

3.2 Protein expression profiles of Anachis snails from vent sites

Based on the protein expression results, 16 protein bands were selected for further Bray-Curtis Similarity (BCs) analysis (Fig. 3). And the classification of snails fell into three clusters (Fig. 4). Snails from the high pH South were all within one cluster. In contrast, snails from the remaining sites were indistinguishable in other clusters. With further determination on the contribution of each protein variable, the data were characterized by principle component analysis (PCA). The first to the fifth principal components accounted for 35.4, 28.5, 13.2, 8.8, and 4.2% of the total variance, respectively. The separation was mainly contributed by the first (i.e., bands 8, 1, 15, and 12) and second (i.e., bands 15, 13, 12, 1, and 11) principal-components.

Based on the cluster results, the Anachis snails were classified into groups of V-South (pH 7.78-7.82) and V-Rest (pH 7.31-7.83). Their shell traits were compared to non-vent Euplica snails (pH 8.10-8.20) subsequently.
3.3 Comparison of shell traits of dove snails among vent and non-vent sites

Shell traits of the *Anachis* and *Euplica* snails were listed in Table 3. A positive correlation between shell length and shell width was observed in all populations. Difference in shell shape (shell width:shell length), with vent populations being more globular, was also found, as shown by the significant difference in regressions’ slopes. By ANCOVA with shell length as the covariate, the mean values of shell width were significantly different among sites (p<0.01), with a descending order of GF > DX > V-South, V-Rest (Table 3).

The relationships of shell length to total weight were curvilinear for both *Anachis* and *Euplica* snails (Fig. 6). The slopes among sites were significantly different and the mean body weight of GF population was significantly greater than others (Table 3 & Fig. 5).

Positive correlations between shell length and shell thickness of body whorl (T1) and penultimate whorl (T2) were only observed in non-vent GF and DX populations. Their slopes were significantly different for T1 only (Fig. 7). The mean shell thickness of T1 and T2 varied among sites (Table 3). *Anachis* snails from vent sites were thinner in T1 compared to the non-vent *Euplica* snails (p<0.001), with a descending order of GF > DX > V-South > V-Rest. A similar trend was also found in T2. Within each vent site, shell thickness between T1 and T2 was insignificantly different (paired t-test, p>0.05). By comparison, T1 and T2 of the snails from V-Rest were 89.4% and 89.8%, respectively, of the V-South ones. With the comparison of vent and non-vent sites, T1 and T2 of the *Anachis* snails from V-Rest showed a 55.6% and 29.0% decrease in T1 and T2, respectively, of the *Euplica* snails from GF. Clearly, both measurements on shell thickness decreased under acidic environments.

4 Discussion

This was the first study to compare morphological traits of snails under varying shallow vent stresses with populations prior classified by biochemical responses. Difference in shell shape (shell width:shell length), with vent populations being more globular was found. Snails from V-Rest (pH 7.31-7.83) exhibited a 10.6% and 10.2% decrease in shell thickness of body whorl (T1) and penultimate whorl (T2), respectively, compared to snails from the V-South (pH 7.78-7.82). Comparing to non-vent sites (pH 8.10-8.20), T1 and T2 of the *Anachis* snails from V-Rest showed a 55.6% and 29.0% decrease in T1 and T2, respectively, to *Euplica* snails from GF. Our shallow vent-based results were, in general, consistent with laboratory, controlled and deep-sea vent studies, i.e., shell-organisms are susceptible to acidic environments.

4.1 Application of proteomic-based approach to shallow vent snails

Proteomic-based method has been used in environmental toxicology to characterized...
organism’s responses to specific treatments with various gradients (Bradley et al., 2002; Jackson et al., 2002). It has been applied to laboratory and field studies, such as blue mussel Mytilus edulis exposed to polyaromatic hydrocarbons and heavy metals (Knigge et al., 2004), to crude oil (Mi et al., 2006), to PCBS and PAHs extracted from Baltic Sea sediments (Olsson et al., 2004) and mussel Mytilus galloprovincialis exposed to a tributyltin-polluted area (Magi et al., 2008).

Application of proteomic approach to vent mussel Bathymodiolus azoricus has been conducted with samples collected from three distinct hydrothermal vent fields in the Mid-Atlantic Ridge (Companysa et al., 2011). The expression profiles of 35 proteins from the gill revealed clear separation among sites, which indicates that specific adaptations of B. azoricus depend on local conditions.

It is known that large spatial and temporal variations in environmental parameters are detected around vent environments, such as temperature, pH and hydrothermal fluid composition in terms of dissolved oxygen, methane and sulphide concentrations etc. The pH of the hydrothermal fluids within our sampling vent and surrounding seawater had been determined on 31 May, 2011, the pH ranges from 2.29 to 5.11 and 5.51 to 6.15, respectively (Zeng et al., 2013). The diffusion activities of vent plumes were also evaluated through environmental factors of temperature, pH and Eh (Han et al., 2014). The diffusive plume is mainly affected by the wind, sea waves and tides.

If ocean currents in the east-west direction are not considered, sea currents around vents are from north to south during ebb tide, while in flood tide the opposite direction dominates. Our proteomic results indicated that snails from the South were distinguished from the rest sites which are consistent with the diffusion activities of local vent fluids.

4.2 Comparison with other dove snail studies

Among the dove snails (Family: Columbellidae), Anachis avara is a common one living on the coast of the eastern United States (Scheltema, 1968; Hatfield, 1980). At Bear Cut, FL, the population of A. avara showed seasonal fluctuation in its size structure (Hatfield, 1980). It reached a mean terminal size of 10.50 mm (8.00-13.29 mm) and matured quickly at the age of six to seven months. The estimated life span was less than two years. It is suggested that the fluctuation in size structure was primarily the result of seasonal recruitment, and the abundance was probably determined by predation.

In Anachis fluctuata, the regression equation of shell length (mm) and dry tissue weight or shell weight (g) had been reported, i.e., \( Y = -0.025 + 0.003SL \) \((R^2=0.88; N=26)\) and \( Y = -2.39 + 1.04\ln SL \) \((R^2=0.92)\), respectively (Bertness and Cunningham, 1981). By comparison, in this study, shell lengths of A. misera and Euplica sp. were from 6.88 to 11.01 mm and 3.40 to 7.56 mm, respectively (Tables 2 & 3). Variations
Positive correlations between shell length and shell width or total weight in both dove snails was present, but the $R^2$ of the equations were low in *A. misera* (0.07-0.28) compared to *Euplica* sp. (0.94-0.98) (Figs. 5 and 6). Positive correlations between shell length and shell thickness of body whorl (T1) and penultimate whorl (T2) were only found in non-vent populations with the $R^2$ of 0.43-0.64 (Fig. 7). Although differential recruitment and acidic stress are potential factors to account for low or even no correlation between the above shell traits in vent *A. misera*, further study is needed to address this question.

### 4.3 Comparison with other ocean acidification studies

To date, ocean acidification studies have been conducted mostly in the laboratory or controlled environments for a short period of time. The results indicate exposures to future global change scenarios (Caldeira and Wickett, 2003; Sokolov et al., 2009) may alter the tolerance of calcifying species and, ultimately, their fitness and survival through complex physiological and ecological pathways. Based on literatures’ data, it is concluded that effects of acidified seawater on species growth were at higher pH than those on species reproduction (mean $pH_{10}$ was 7.73 vs 7.63 and mean $pH_{50}$ was 7.28 vs 7.11, respectively) (Azevedo et al., 2015).

Studies conducted in the natural vent system at Ischia, Italy indicated that the settlement and colonization of mollusks and microfauna showed highly reductions in recruitment in the acidified stations (Cigliano et al. 2010; Ricevuto et al., 2012). In the experiments of juvenile pen shell *Pinna nobilis* transplanted to Ischia for 45 days, decreases in survival, growth, and oxygen consumption were found. A 22% decrease in survival rate for specimens transplanted at pH 7.7 compared to those at pH 8.1 was reported (Basso et al., 2015). Studies on the limpet *Patella caerulea* within and outside the Ischia vent, shell formation and dissolution are both observed in low pH site where enhanced shell production counteracts shell dissolution (Hall-Spencer et al., 2008; Rodolfo-Metalpa et al., 2011; Langer et al., 2014). In contrast, shell dissolution is absence in normal pH site. Compared with deep-sea vent studies, in the northwest Eifuku volcano, Mariana arc, the vent mussel, *Bathymodiolus brevior* inhabiting low pH environments (pH 5.36-7.29), exhibited shell thickness and daily growth increments in shells of only about half of the ones with pH>7.8 (Tunnicliffe et al., 2009).

Under low pH (7.7 vs. 8.0), periwinkle *Littorina littorea* increased less in weight and were shorter than snails grown in current conditions (Melatunan et al., 2013). Similar results have been obtained for other calcifying organisms, e.g., the reduction in shell growth of the oysters *Crassostrea gigas* (Lannig et al., 2010) and *Crassostrea virginica* (Beniash et al., 2010), larvae of the Mediterranean pteropods *Cavolinia inflexa* (Comeau et al., 2010), and the mussels *Mytilus edulis* (Gazeau et al., 2010).
and *Mytilus californianus* (Gaylord et al., 2011). Along a gradient of pH (5.78-8.30) and salinity (3.58-31.2 psu) in the Sungai Brunei estuary, Malaysia, whelk *Thais gradata* exposed to acidified sites possessed heavier shells and the degrees of erosion was negatively related to water pH and calcium concentration (Marshall et al., 2008).

At low pH (7.7), a 2.45% change in shell shape (shell width:shell length) towards more globular and a decrease in the outer lip shell thickness of up to 27% in *Littorina littorea* were observed (Melatunan et al., 2013).

In this study, comparison of *A. misera* between V-South (pH 7.78-7.82) and V-Rest (pH 7.31-7.83), the change of shell ratio was 3.4%, to more rounded in the V-Rest group. In addition, snails of V-Rest exhibited a 10.6% and 10.2% decrease in shell thickness of body whorl (T1) and penultimate whorl (T2), respectively, compared to the V-South snails. With the comparison of vent and non-vent sites, T1 and T2 of the *Anachis* snails from V-Rest were 44.4% and 71.0%, respectively, of the *Euplica* snails from GF (pH 8.1-8.2). Our shallow vent-based results were, in general, consistent with other laboratory, controlled and field studies, i.e., shell-organisms are susceptible to acidic environments.

It is known that vent systems are not entirely representative of future ocean changes because of not only the temporal variability in pH, but also the existence of other toxic elements. However, vents’ acidifying environments are sufficiently large in spatial and temporal scales. Still, it is a naturally applicable system to assess the effects of ocean acidification on the whole life cycle and across multiple generations of target organisms.

**Acknowledgments.** We are grateful to the anonymous reviewers for their constructive comments which have substantially improved the manuscript. We thank Mr. Siou-Yan Lin, Mr.. Chiu-Yeh Liao, and Ms. Yalan Chou, Dun-Ru Kang for conducting experiments. We also thank Dr. Kotaro Tsuchiya for species identification on dove shells and Dr. Hui-Ling Lin for using the X-ray machine. This study was supported by the Ministry of Science and Technology, Republic of China (NSC99-2321-B-110-006; NSC 102-3113-P-005 -005 -002; MOST 103-3113-M-005-001), the Asia-Pacific Ocean Research Center, and the Center for Emerging Contaminants Research, National Sun Yat-sen University, supported by the Ministry of Education, Taiwan.

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Table 1. Sampling locations and environmental parameters of vent sites in the Kueishan Islet and northeastern Taiwan. Data are shown as Mean±SD and ranges.

<table>
<thead>
<tr>
<th>Site</th>
<th>White vent</th>
<th>Yellow vent</th>
<th>Da-xi (DX)</th>
<th>Geng-fang (GF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude</td>
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<td>24.941°N</td>
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<tr>
<td>Longitude</td>
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<td>121.963°E</td>
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<td>Depth (m)</td>
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</tr>
<tr>
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<td>21.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>55.0</td>
<td>115.0</td>
<td>27.2 ± 0.2 (27.0 - 27.4)</td>
<td>27.4 ± 0.5 (27.0 - 27.9)</td>
</tr>
<tr>
<td>pH</td>
<td>4.0</td>
<td>2.3</td>
<td>8.13 ± 0.06 (8.10-8.20)</td>
<td>8.13 ± 0.06 (8.10-8.20)</td>
</tr>
</tbody>
</table>
Table 2. Environmental parameters and shell traits of *Anachis misera* around the vent off Kueishan Islet. Data are shown as Mean±SD and ranges. SH: shell length; SW: shell width; TW: total weight; T1: thickness of body whorl; T2: thickness of penultimate whorl; Means that differ significantly from each other are indicated by different letters.

<table>
<thead>
<tr>
<th>Site</th>
<th>North (N)</th>
<th>East (E)</th>
<th>South (S)</th>
<th>Southwest (SW)</th>
<th>Northwest (NW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plume distance (m)</td>
<td>15.6</td>
<td>18.0</td>
<td>10.5</td>
<td>12.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Depth (m)</td>
<td>15.0</td>
<td>14.5</td>
<td>14.2</td>
<td>15.7</td>
<td>17.4</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>pH</td>
<td>7.22 ± 0.03 (7.19 - 7.25)</td>
<td>7.66 ± 0.06 (7.59 - 7.75)</td>
<td>7.88 ± 0.02 (7.78 - 7.82)</td>
<td>7.80 ± 0.03 (7.78 - 7.83)</td>
<td>7.33 ± 0.02 (7.31 - 7.35)</td>
</tr>
<tr>
<td>SL (mm)</td>
<td>-</td>
<td>9.23 ± 0.63 (8.33 - 9.97)</td>
<td>9.01 ± 0.39 (8.68 - 11.01)</td>
<td>9.14 ± 1.11 (6.93 - 10.84)</td>
<td>9.13 ± 0.56 (7.81 - 10.40)</td>
</tr>
<tr>
<td>SW (mm)</td>
<td>-</td>
<td>4.54 ± 0.32 (4.46 - 4.60)</td>
<td>4.42 ± 0.29 (3.65 - 4.98)</td>
<td>4.41 ± 0.30 (3.71 - 5.16)</td>
<td>4.30 ± 0.72 (3.86 - 4.93)</td>
</tr>
<tr>
<td>TW (mg)</td>
<td>-</td>
<td>135 ± 18 (104 - 152)</td>
<td>121 ± 22 (97 - 180)</td>
<td>137 ± 23 (91 - 213)</td>
<td>113 ± 29 (75 - 153)</td>
</tr>
<tr>
<td>T1 (um)</td>
<td>-</td>
<td>199 ± 56 (136 - 285)</td>
<td>225 ± 69 (109 - 481)</td>
<td>200 ± 56 (118 - 290)</td>
<td>168 ± 49 (79 - 276)</td>
</tr>
<tr>
<td>T2 (um)</td>
<td>-</td>
<td>188 ± 44 (109 - 248)</td>
<td>240 ± 51 (112 - 328)</td>
<td>265 ± 55 (117 - 354)</td>
<td>180 ± 55 (79 - 325)</td>
</tr>
</tbody>
</table>
Table 3. Shell traits of *Anachis misera* around the vent off Kueishan Islet and *Euplica* sp. from non-vent control sites of Da-xi and Geng-fang. Data are shown as Mean ± SD and ranges. SH: shell length; SW: shell width; TW: total weight; T1: thickness of body whorl; T2: thickness of penultimate whorl; Least square (LS) means that differ significantly from each other are indicated by different letters.

<table>
<thead>
<tr>
<th>Site</th>
<th>V-South (S)</th>
<th>V-Rest (R)</th>
<th>Da-xi (DX)</th>
<th>Geng-fang (GF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail sp.</td>
<td><em>Anachis misera</em></td>
<td><em>Anachis misera</em></td>
<td><em>Euplica</em> sp.</td>
<td><em>Euplica</em> sp.</td>
</tr>
<tr>
<td>No. snails (N)</td>
<td>65</td>
<td>76</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>SL (mm)</td>
<td>9.01 ± 0.89 (6.88 - 11.01)</td>
<td>9.14 ± 0.84 (6.93 - 10.84)</td>
<td>7.33 ± 1.34 (5.92 - 10.58)</td>
<td>9.61 ± 1.75 (6.74 - 13.19)</td>
</tr>
<tr>
<td>SW (mm)</td>
<td>4.42 ± 0.29 (3.65 - 4.96)</td>
<td>4.37 ± 0.29 (3.71 - 5.16)</td>
<td>4.14 ± 0.87 (3.40 - 6.34)</td>
<td>5.50 ± 1.01 (3.62 - 7.56)</td>
</tr>
<tr>
<td>LSMean of SW (mm)</td>
<td>4.42 ± 0.32 c</td>
<td>4.33 ± 0.35 c</td>
<td>4.77 ± 0.40 b</td>
<td>5.27 ± 0.38 a</td>
</tr>
<tr>
<td>TW (mg)</td>
<td>121 ± 22 (67 - 188)</td>
<td>124 ± 24 (75 - 213)</td>
<td>84 ± 70 (39.3 - 294.3)</td>
<td>195 ± 105 (42 - 436)</td>
</tr>
<tr>
<td>LSMean of TW (mg)</td>
<td>118.07 ± 8.30 b</td>
<td>117.59 ± 8.98 b</td>
<td>105.94 ± 4.28 b</td>
<td>149.66 ± 5.70 a</td>
</tr>
<tr>
<td>T1 (um)</td>
<td>225 ± 69 (109 - 481)</td>
<td>232 ± 31 (141 - 299)</td>
<td>385 ± 113 (243 - 653)</td>
<td>536 ± 171 (201 - 852)</td>
</tr>
<tr>
<td>LSMean of T1 (um)</td>
<td>255 ± 73 c</td>
<td>228 ± 70 d</td>
<td>446 ± 76 b</td>
<td>514 ± 71 a</td>
</tr>
<tr>
<td>T2 (um)</td>
<td>200 ± 51 (112 - 328)</td>
<td>234 ± 36 (148 - 304)</td>
<td>241 ± 104 (147 - 588)</td>
<td>343 ± 124 (157 - 702)</td>
</tr>
<tr>
<td>LSMean of T2 (um)</td>
<td>256 ± 56 b</td>
<td>230 ± 52 c</td>
<td>295 ± 60 a</td>
<td>324 ± 55 a</td>
</tr>
</tbody>
</table>
Figure 1. Map showing the sampling sites. *DX: *Euplica* sp. from Da-xi (24.9413° N, 121.9039° E); *GF: *Euplica* sp. from Geng-fang (24.9046° N, 121.8720° E); \( \square \); *Anachis misera* from the white vent (24.8341° N, 121.9619° E); \#Y; yellow vent (24.8355° N, 121.9637° E); N: north; E: east; S: south; SW: southwest; NW: northwest (Source: Google map).
Figure 2. Shell morphology and x-ray photos of *Anachis misera* around the vent off Kueishan Islet and *Euplica* sp. from non-vent control sites of Da-xi and Geng-fang. (A) *A. misera* from the East; (B) *A. misera* from the South; (C) *A. misera* from the Southwest; (D) *A. misera* from the Northwest; (E) *Euplica* sp. from Da-xi; (F) *Euplica* sp. from Geng-fang; (G) x-ray photos of *A. misera* from the South (left) and the Northwest (right). Scale bar: 5mm; SL: shell length; SW: shell width; T1: thickness of body whorl; T2: thickness of penultimate whorl.
Figure 3. Gel electropherogram with molecular markers of *Anachis misera*. Number: protein band serial number.
Figure 4. Results from the combined principal component analysis (PCA) and cluster analysis. The cluster analysis of Bray–Curtis similarity (BCs) indices using standardized overall protein expressions of *Anachis* snails from different sampling sites. E: east, S: south; SW: southwest; NW: northwest; 1-16: protein spot variable.

Relationship between shell length and shell width of *Anachis misera* from different sites. S: standard error of the regression; Different letters indicate that the regression lines differ significantly (*p*<0.05).
Figure 5. Relationship between shell length and shell width of *Anachis* and *Euplica* snails from different sites. Different letters indicate that the regression lines differ significantly ($p<0.05$).

- **DX** $y=0.646x-0.59$, $N=16$; $R^2=0.982$; $p<0.0001$  
- **GF** $y=0.058+0.566x$, $N=30$; $R^2=0.960$; $p<0.0001$  
- **V-South** $y=2.882+0.17x$, $N=65$; $R^2=0.281$; $p<0.0001$  
- **V-Rest** $y=3.463+0.10x$, $N=75$; $R^2=0.068$; $p=0.01$
Figure 6. Relationship between shell length and total weight of *Anachis* and *Euplica* snails from different sites. Different letters indicate that the logarithmic transformed regression lines differ significantly (p<0.01).

- **DX**: log\(W = -0.986 + 3.298 \log L\); N=16; \(R^2 = 0.946; p<0.0001\)  
- **GF**: log\(W = -0.822 + 3.121 \log L\); N=30; \(R^2 = 0.941; p<0.0001\)  
- **V-South**: log\(W = 1.288 + 0.827 \log L\); N=65; \(R^2 = 0.219; p<0.0001\)  
- **V-Rest**: log\(W = 1.494 + 0.618 \log L\); N=75; \(R^2 = 0.096; p<0.007\)
Figure 7. Shell thickness of *Anachis* and *Euplica* snails. (A) Thickness of body whorl (T1); (B) Thickness of penultimate whorl (T2). *: V-South; #: V-Rest; Different letters indicate that the regression lines differ significantly (p < 0.05).

\[
\begin{align*}
\text{DX} & : y = 55.256x - 20.281, N=16; R^2=0.425; p=0.006 \\
\text{GF} & : y = 69.132x - 127.695, N=30; R^2=0.501; p<0.0001
\end{align*}
\]

Figure 8. Results from the combined principal component analysis (PCA) and cluster analysis. The cluster analysis of Bray–Curtis similarity (BCs)
If a positive correlation between shell length and shell width or total weight existed, the coefficient of determination ($R^2$) of the equations was low, i.e., 0.207-0.444. Snails from the Northwest site (pH 7.33) exhibited a more globular shape than those of the South ones (pH 7.80). Standardized shell thickness (thickness of body whorl:shell length)

(thickness of penultimate whorl:shell length)

In a similar vein, based on the 16 examined protein spots, protein expression profiles of snails in the South were distinct. With further characterization by principle component analysis, the separation was mainly contributed by the first (i.e., spots 8, 1, 15, and 12) and second (i.e., spots 15, 13, 12, 1, and 11) principal-components.

Shells lengths of the snails were from 6.88 to 11.01mm (Table 1). Based on the length-frequency histograms, it is found that the relative proportions of size classes differed among sites (Fig. 4). Positive correlation between shell length and shell width was only observed in the South and Northwest samples, and their slopes were significantly different (Fig. 5). The same pattern was also observed in the relationship between shell length and total weight (Fig. 6). It is noticed that the coefficient of determination ($R^2$) of the equations was low, i.e., 0.207-0.444 (Figs. 5 and 6).

### 3.3 Protein expression profiles

In the protein expression profiles of *Anachis* snails, 16 protein spots were selected for cluster analysis (Fig. 8). Based on Bray-Curtis Similarity (BCs) Indices, classification of the snails falls into three clusters. Snails from the high pH South were all within one cluster. In contrast, snails from the remaining sites were indistinguishable in other clusters. With further determination of the contribution of each protein variable, the data were characterized by principle component analysis (PCA). The first to the fifth principal components accounted for 35.4, 28.5, 13.2, 8.8, and 4.2% of the total variance, respectively. The separation was mainly contributed
by the first (i.e., spots 8, 1, 15, and 12) and second (i.e., spots 15, 13, 12, 1, and 11) principal-components.

Our results showed that *Anachis* snails survived and differed in their ecophysiological performance under varying degrees of low pH stress.

More specifically, snails in the acidic Northwest site (pH 7.33) possessed thinner shells and were more globular in shape than those of the South (pH 7.80) (Fig. 7). At the biochemical level, the protein expression profiles of snails from the South were distinguished from the others (Fig. 8). Overall, the effects of vent environments on snails at physiological and biochemical levels were comparable.

Marine snails possessing shells with a more elongated shape are found to be more vulnerable to crab predation, possibly due to higher handling efficiency compared with a more globular shell (Cotton et al., 2004).

This reduction in shell thickness may increase the organism’s susceptibility to crushing predators (Boulding and Van Alstyne, 1993; Trussell and Etter, 2001). As shell thickness is reduced under low pH and elevated temperature, acquiring a more globular shape could enable snails to compensate better (Melatunan et al., 2013).

Compared with deep-sea vent studies, in the northwest Eifuku volcano, Mariana arc, the vent mussel, *Bathymodiolus brevior* inhabiting low pH environments (pH 5.36-7.29), exhibited shell thickness and daily growth increments in shells of only about half of the ones with pH>7.8 (Tunnicliffe et al., 2009). Along the Mid-Atlantic Ridge, the expression profiles of 35 proteins from the gill of *Bathymodiolus azoricus* revealed clear separation among sites, which indicates that specific adaptations of *B. azoricus* depend on local conditions (Companya et al., 2011). Moreover, it has been reported that snails of *Melaraphe neritoides*, *Patella caerulea*, and *Patella rustica* distribute in shallow vents off Ischia (pH 6.53) (Hall-Spencer et al., 2008). However, shell traits of these snails were not evaluated.
Northwest (pH 7.66, 7.80, and 7.33, respectively) changed in the acidic Northwest site

4.2 Comparison with other Anachis studies
Among the Anachis species (Family: Columbellidae), Anachis avara is a common one living on the coast of the eastern United States (Scheltema, 1968; Hatfield, 1980). At Bear Cut, FL, the population of A. avara showed seasonal fluctuation in its size structure (Hatfield, 1980). It reached a mean terminal size of 10.50 mm (8.00-13.29 mm) and matured quickly at the age of six to seven months. The estimated life span was less than two years. It is suggested that the fluctuation in size structure was primarily the result of seasonal recruitment, and the abundance was probably determined by predation.

In Anachis fluctuate, the regression equation of shell length (mm) and dry tissue weight or shell weight (g) had been reported, i.e., $Y = -0.025 + 0.003SL (R^2 = 0.88; N=26)$ and $Y = -2.39 + 1.04\ln SL (R^2 = 0.92)$, respectively (Bertness and Cunningham, 1981). By comparison, in this study, shell lengths of A. misera were from 6.88 to 11.01 mm (Table 1). Variations in size structure among sites were also obvious. In addition, if a positive correlation between shell length and shell width or total weight in A. misera was present, the $R^2$ of the equation was low, i.e., 0.207-0.444 (Figs. 5 and 6). The standard error of the regression varied from 0.04 to 4.50, which indicated that the precision of the regression models was low, particularly in the relationship of shell length and body weight. Although differential recruitment and acidic stress are potential factors to account for such discrepancy, further study is needed to address this question.
Figure 8. Results from the combined principal component analysis (PCA) and cluster analysis. The cluster analysis of Bray–Curtis similarity (BCs) indices using standardized overall protein expressions of *Anachis* snails from different sampling sites. E: east, S: south; SW: southwest; NW: northwest; 1-16: protein spot variable.