Effects of low pH stress on shell traits of the dove snail, *Anachis misera*, inhabiting shallow vent environments off Kueishan Islet, Taiwan

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Received: 12 October 2014 – Accepted: 24 November 2014 – Published: 29 November 2014

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Published by Copernicus Publications on behalf of the European Geosciences Union

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 into 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 the GF population was greater than the 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. Overall, 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 $CaCO_3$ skeleton, e.g., mollusks, echinoderms, and corals, are likely to be among the most susceptible to ocean acidification (e.g., 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 of interest are located 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% CO_2, 0.8–8.4% H_2S, <0.03% SO_2, 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 *Tubastraea 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 the 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., 2012).

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^{o}C for later use.

2.2 Measurements of 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 with 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, and 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 were 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 13000*g* 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 30 min at 120V, and 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 utilized 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. Shell lengths of the snails ranged from 6.88 to 11.01mm. 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). 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 the GF population was significantly greater than others (Table 3 and 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 were 44.4% and 71.0%, respectively, of the *Euplica* snails from GF. Clearly, both measurements of 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). Compared 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, relative 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 characterize 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 the proteomic approach to vent mussel *Bathymodiolus azoricus* has been conducted with samples collected from three distinct hydrothermal vent fields in the Mid-Atlantic Ridge (Companya 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, with 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; whereas, in flood tide, the opposite direction dominates. Our proteomic results indicated that snails from the South were distinguished from the rest of the 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 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* and *Euplica* sp. were

from 6.88 to 11.01mm and 3.40 to 7.56mm, respectively (Tables 2 and 3). Variations in size structure among sites were also obvious. 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 that 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 data from the literature, 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 high reductions in recruitment in the acidified stations (Cigliano et al., 2010; Ricevuto et al., 2012; Milazzo et al., 2014). 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). In 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 absent in normal pH sites. The nassariid gastropods *Nassarius corniculus* and *Cyclope neritea* adapted to the Ischia vent were smaller than those found in normal pH conditions and had higher mass specific energy consumption but significantly lower whole animal metabolic energy demand (Garilli et al., 2015). 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 (3.58-31.2psu) in the Sungai Brunei estuary, Malaysia, whelk *Thais gradata* exposed to acidified sites possessed heavier shells, and the degrees of erosion were 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), revealed that 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, Ms. Yalan Chou, and Ms. 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.

Site	White vent	Yellow vent	Da-xi (DX)	Geng-fang (GF)
Latitude	24.8341°N	24.8355°N	24.9413°N	24.9046°N
Longitude	121.96196°E	121.96371°E	121.90390°E	121.87200°E
Depth (m)	17.0	9.5	3.0	3.0
Fluid flux (m^3/hr)	18.5	21.0	-	-
Temperature (°C)	55.0	115.0	27.2 ± 0.2 (27.0 - 27.4)	27.4 ± 0.5 (27.0 - 27.9)
pH	4.0	2.3	8.13 ± 0.06 (8.10-8.20)	8.13 ± 0.06 (8.10-8.20)

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.

Site	North (N)	East (E)	South (S)	Southwest (SW)	Northwest (NW)
Plume distance (m)	15.6	10.0	10.5	12.0	16.0
Depth (m)	15.0	14.5	14.2	15.7	17.4
Temperature (°C)	27	27	27	27	26
pH	7.22 ± 0.03 (7.19 - 7.25) c	7.66 ± 0.08 (7.59 - 7.75) b	7.80 ± 0.02 (7.78 - 7.82) a	7.80 ± 0.03 (7.78 - 7.83) a	7.33 ± 0.02 (7.31 - 7.35) c
No. snails (N)	0	7	65	33	36
SL (mm)	-	9.23 ± 0.63 (8.23 - 9.97)	9.01 ± 0.89 (6.88 - 11.01)	9.14 ± 1.11 (6.93 - 10.84)	9.13 ± 0.56 (7.81 - 10.40)
SW (mm)	-	4.54 ± 0.32 (4.16 - 5.05)	4.42 ± 0.29 (3.65 - 4.96)	4.41 ± 0.30 (3.71 - 5.16)	4.30 ± 0.72 (3.86 - 4.93)
TW (mg)	-	125 ± 18 (104 - 152)	121 ± 22 (67 - 188)	137 ± 23 (91 - 213)	113 ± 20 (75 - 153)
T1 (um)	-	199 ± 56 (136 - 285)	225 ± 69 (109 - 481)	200 ± 56 (118 - 290)	168 ± 49 (79 - 276)
T2 (um)	-	188 ± 44 (109 - 248)	200 ± 51 (112 - 328)	205 ± 55 (117 - 354)	180 ± 55 (79 - 325)

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.

Site	V-South (S)	V-Rest (R)	Da-xi (DX)	Geng-fang (GF)
Snail sp.	*Anachis misera*	*Anachis misera*	*Euplica* sp.	*Euplica* sp.
No. snails (N)	65	76	16	30
SL (mm)	9.01 ± 0.89 (6.88 - 11.01)	9.14 ± 0.84 (6.93 - 10.84)	7.33 ± 1.34 (5.92 - 10.58)	9.61 ± 1.75 (6.74 - 13.19)
SW (mm)	4.42 ± 0.29 (3.65 - 4.96)	4.37 ± 0.29 (3.71 - 5.16)	4.14 ± 0.87 (3.40 - 6.34)	5.50 ± 1.01 (3.62 - 7.56)
LSMean of SW (mm)	4.42 ± 0.32 c	4.33 ± 0.35 c	4.77 ± 0.40 b	5.27 ± 0.38 a
TW (mg)	121 ± 22 (67 - 188)	124 ± 24 (75 - 213)	84 ± 70 (39.3 - 294.3)	195 ± 105 (42 - 436)
LSMean of TW (mg)	118.07 ± 8.30 b	117.59 ± 8.98 b	105.94 ± 4.28 b	149.66 ± 5.70 a
T1 (um)	225 ± 69 (109 - 481)	232 ± 31 (141 - 299)	385 ± 113 (243 - 653)	536 ± 171 (201 - 852)
LSMean of T1 (um)	255 ± 73 c	228 ± 70 d	446 ± 76 b	514 ± 71 a
T2 (um)	200 ± 51 (112 - 328)	234 ± 36 (148 - 304)	241 ± 104 (147 - 588)	343 ± 124 (157 - 702)
LSMean of T2 (um)	256 ± 56 b	230 ± 52 c	295 ± 60 a	324 ± 55 a

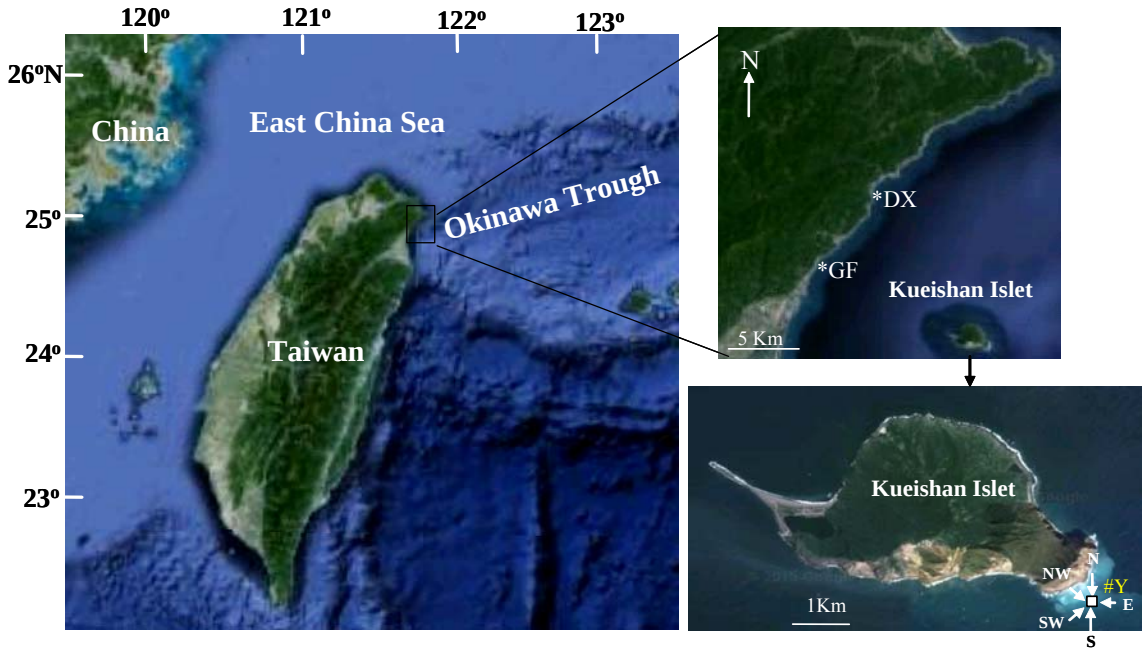

Figure 1. Map showing the sampling sites. *DX: *Euplica* sp. from Da-xi (24.9413°N, 121.90390°E); *GF: *Euplica* sp. from Geng-fang (24.9046°N, 121.87200°E); □: *Anachis misera* from the white vent (24.8341°N, 121.96196°E); #Y: yellow vent (24.8355°N, 121.96371°E); N: north; E: east; S: south; SW: southwest; NW: northwest (Source: Google Maps).

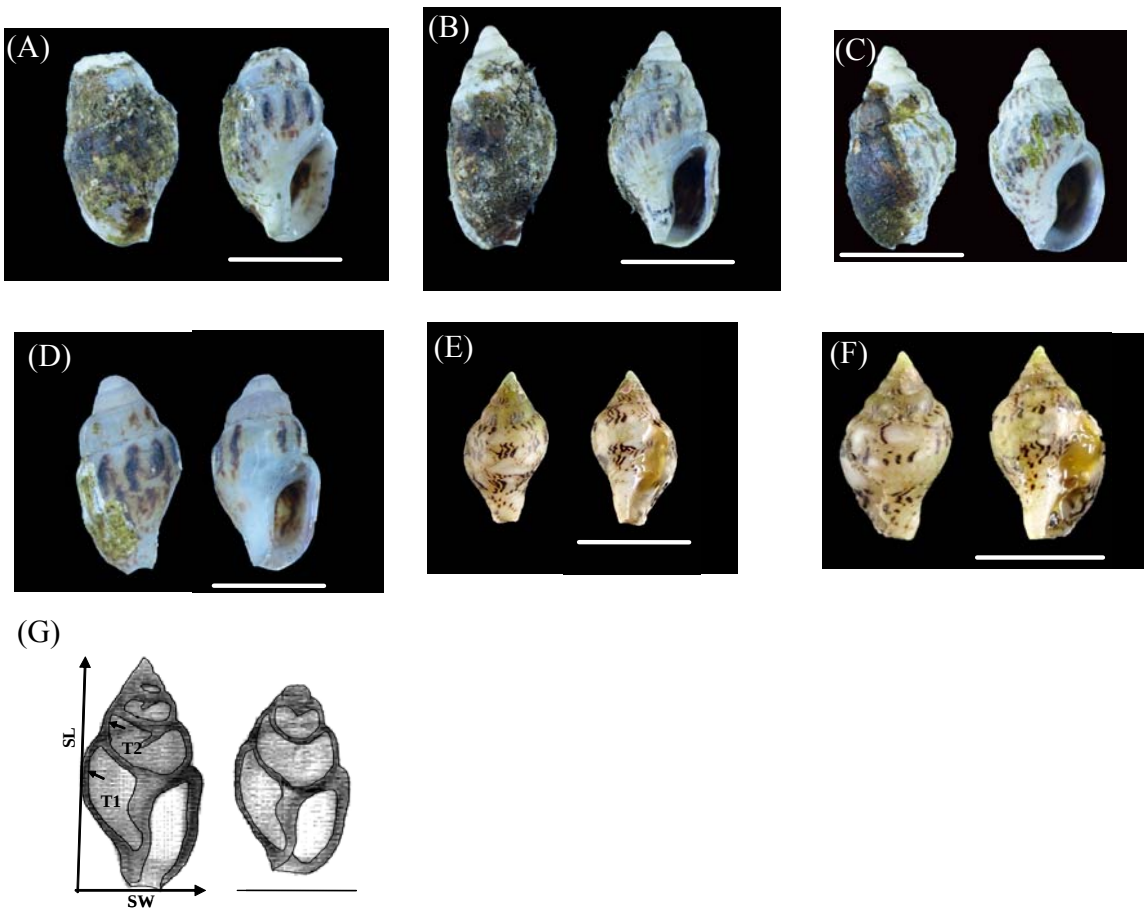

Figure 2. Shell morphology and X-ray photos of *Anachis misera* around the vent off Kueishan Islet and *Euplica* sp. from non-vent control sites of Da-xi and Geng-fang. (A) *A. misera* from the East; (B) *A. misera* from the South; (C) *A. misera* from the Southwest; (D) *A. misera* from the Northwest; (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.

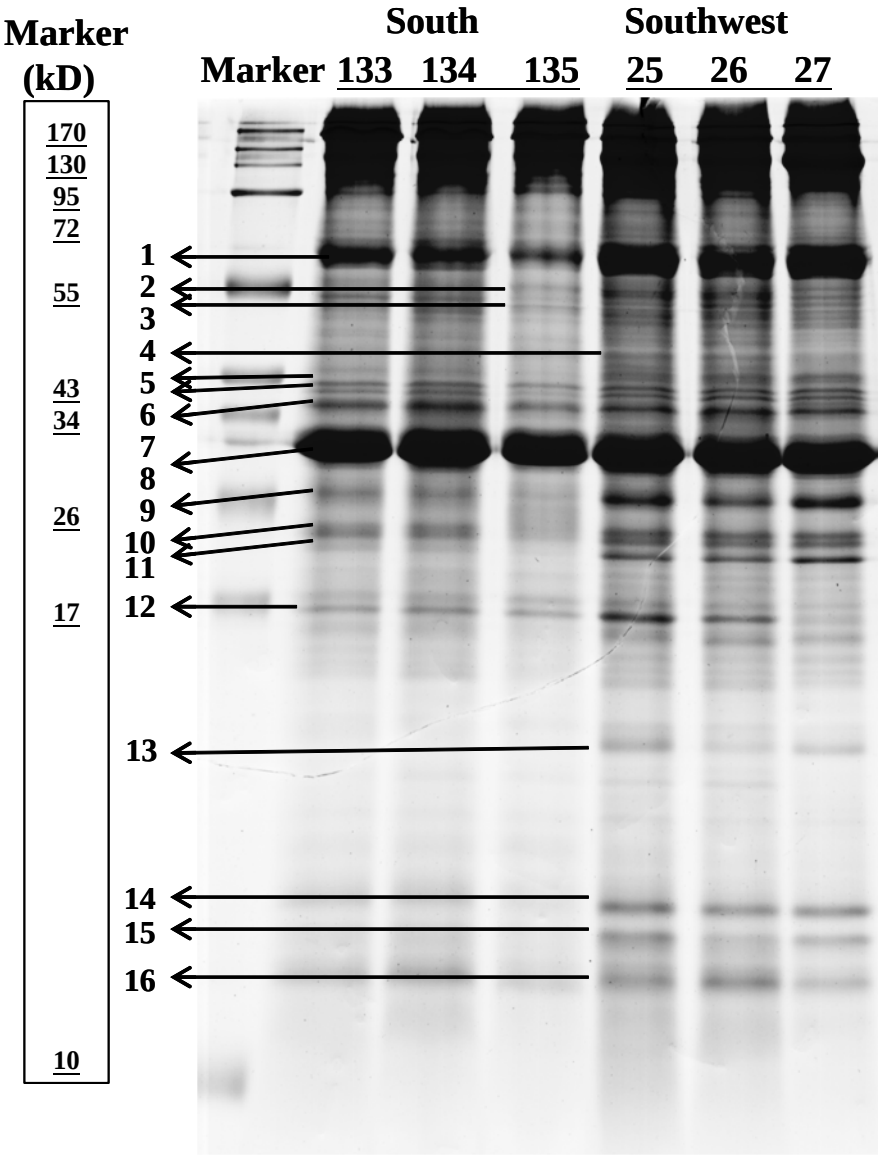

Figure 3. Gel electropherogram with molecular markers of *Anachis misera*. Number: protein band serial number.

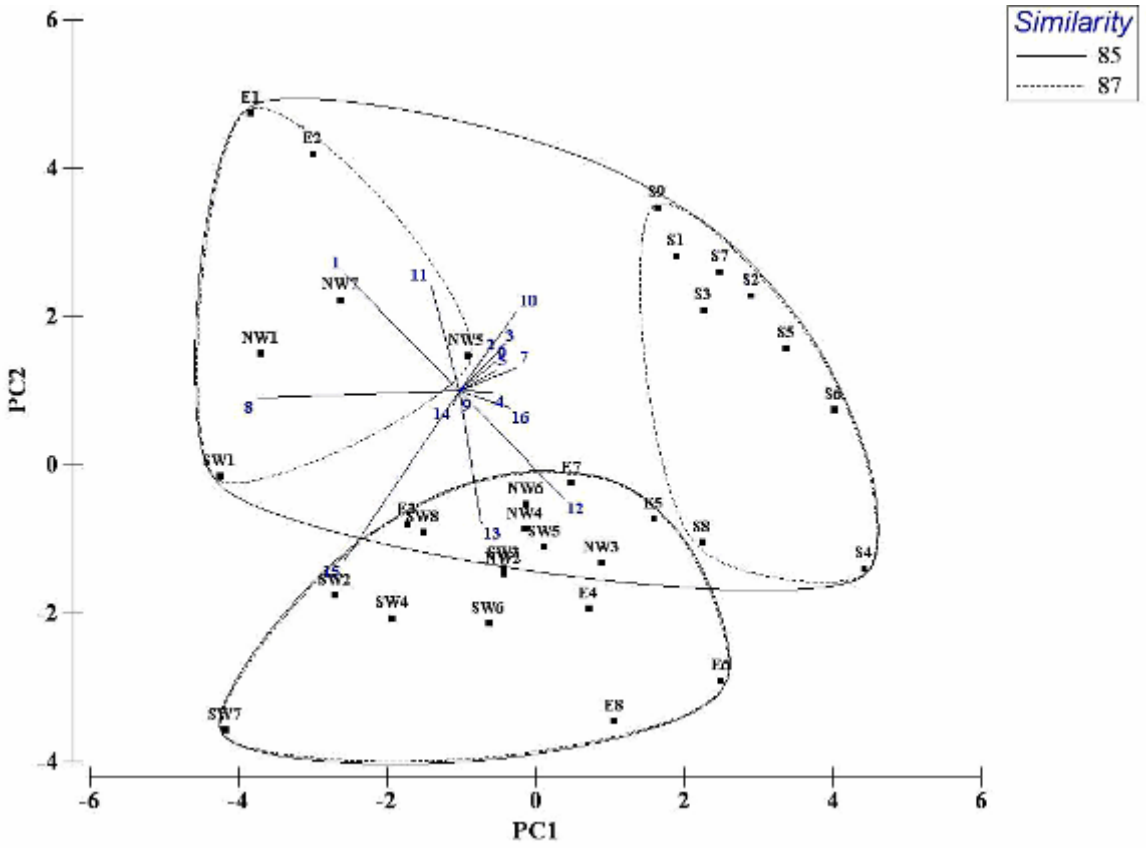

Figure 4. Results from the combined principal component analysis (PCA) and cluster analysis. The cluster analysis of Bray–Curtis similarity (BCs) indices using standardized overall protein expressions of *Anachis* snails from different sampling sites. E: east; S: south; SW: southwest; NW: northwest; 1-16: protein spot variable.

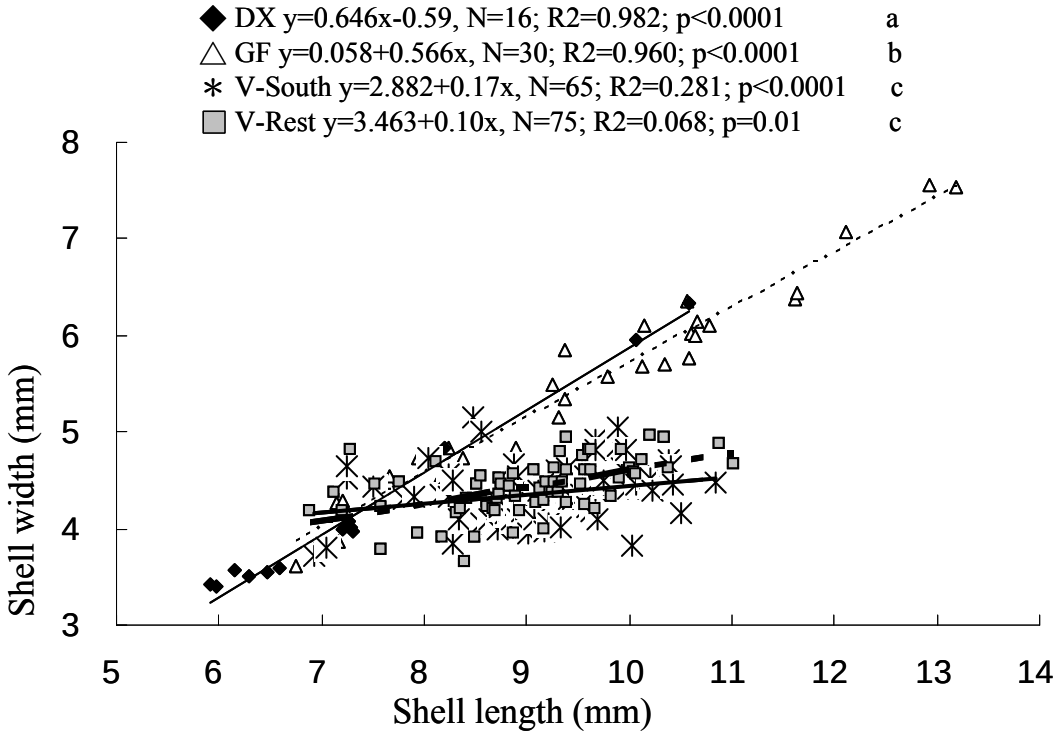

Figure 5. Relationship between shell length and shell width of *Anachis* and *Euplica* snails from different sites. Different letters indicate that the regression lines differ significantly ($p<0.05$).

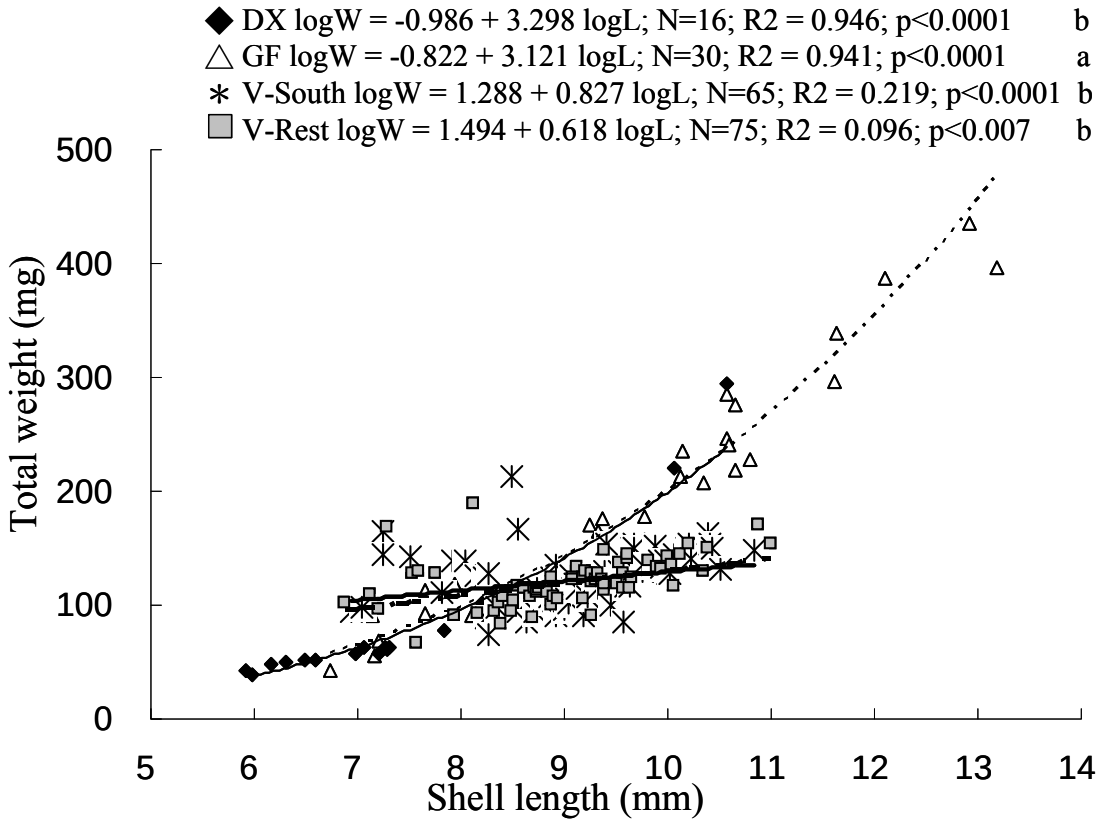

Figure 6. Relationship between shell length and total weight of *Anachis* and *Euplica* snails from different sites. Different letters indicate that the logarithmic transformed regression lines differ significantly ($p<0.01$).

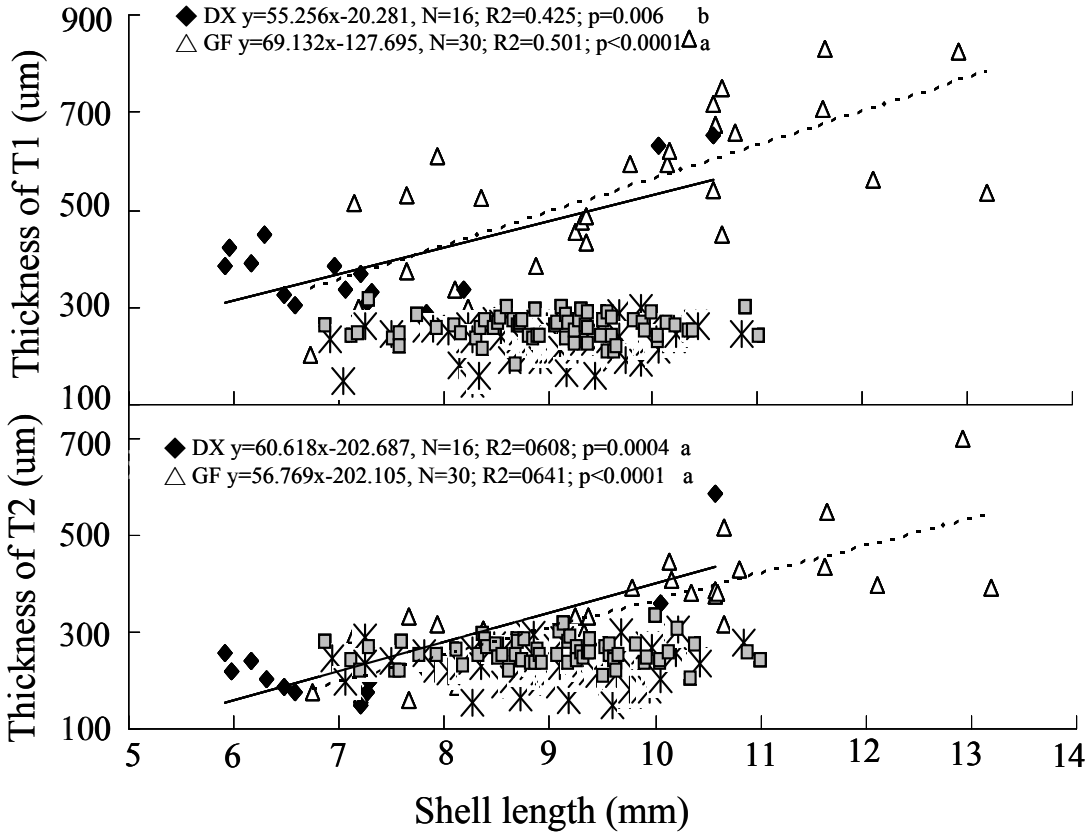

Figure 7. Shell thickness of *Anachis* and *Euplica* snails. (A) Thickness of body whorl (T1); (B) Thickness of penultimate whorl (T2). *: V-South; □: V-Rest; Different letters indicate that the regression lines differ significantly ($p<0.05$).