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

Y. J. Chen, J. Y. Wu, C. T. A. Chen, and L. L. Liu

Department of Oceanography, National Sun Yat-sen University, Kaohsiung 804, Taiwan

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Correspondence to: L. L. Liu (lilian@mail.nsysu.edu.tw)

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Abstract

The effects of naturally acidified seawater on a snail species, *Anachis misera* (Family: Columbellidae) were quantified in five shallow vent-based environments off Kueishan Islet, Taiwan. An absence of *Anachis* snails was observed in the most acidic North site (pH 7.22), and the size structure differed among the remaining East, South, Southwest and Northwest sites. 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 T1 (thickness of body whorl : shell length) and T2 (thickness of penultimate whorl : shell length) from the Northwest site showed a decrease of 6.3 and 9.4 %, respectively, compared to the South ones. 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. As a whole, the shallow vent-based findings provide new information from subtropics on the effects of ocean acidification 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 (Fabry et al., 2008), 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

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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 < 50 ppm 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 *Anachis* snails distributed around vents exposed to varying degrees of plumes would exhibit different ecophysiological performance.

2 Materials and methods

2.1 Field study

The sampling area was a shallow-water vent with white plumes in Kueishan Islet, Taiwan (Fig. 1). *Anachis* snails were collected around the vent (121°96′19.1″ E, 24°83′41.4″ N), including the directions of North (N), East (E), South (S), Southwest (SW), and Northwest (NW) during the period of 28 June to 1 July 2011. The distance

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of the collection sites to the vent center was 10–16 m, and the water depth was in the range of 14.5–17.5 m. 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 and penultimate whorl, as well as the total weight of the intact individual, were determined (Fig. 2). Shell thickness was measured through enlarged X-ray radiographs which were produced by exposing snail shells to X-ray with the settings of 80 kVp and 1 mA for 116.7 ms. The distance between the X-ray source and the objects was 50 cm. The relationship between shell length and shell width or total weight was evaluated by the linear regression analysis. The data of pH and shell thickness were analyzed using a one-way ANOVA and Tukey's multiple-comparisons test.

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 13 000 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 25 µg protein in each sample lane, electrophoresis was run for 30 min at 120 V then 4 h at 180 V. 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 spots were assigned a spot number, and their

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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 spot was further estimated by principal component analysis (PCA).

3 Results

3.1 Morphological traits of snails from different sites

Temperature ranges of the sampling sites were from 26 to 27 °C (Table 1). Spatial variability in pH among sites was clearly observed, and the lowest one was 7.22 ± 0.03 at the North site ($p < 0.0001$) which is close to the very acidic yellow plumes north of the sampling white vent.

Anachis snails were found around the vent, except for the most acidic North site. Snails with eroded apex were more commonly seen at the Northwest site (pH 7.33) than at the South (pH 7.80) (Fig. 3). Shell lengths of the snails were from 6.88 to 11.01 mm (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).

3.2 Comparison of shell thickness among sites

Shell shape (shell width : shell length), standardized shell thickness T1 (thickness of body whorl : shell length), and T2 (thickness of penultimate whorl : shell length) varied among sites. Snails at the Northwest site (pH 7.33) exhibited significantly greater shape

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ratio (more globular) than those of the South site (pH 7.80) (Fig. 7). Within each sampling site, shell thickness between T1 and T2 was insignificantly different (paired t test, $p > 0.05$). In contrast, among sites, both measures of standardized shell thickness decreased under acidic environments. By comparison, T1 and T2 of the Northwest snails were 93.7 and 90.6 %, respectively, of the South ones.

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.

4 Discussion

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.

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4.1 Comparison with other ocean acidification studies

To date, most ocean acidification studies have been conducted in the laboratory or controlled environments for a short period of time. As a result, it has been firmly concluded 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. 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).

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). 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 *L. littorea* were observed (Melatunan et al., 2013). 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*

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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.

In this study, compared to the South site (pH 7.80), snails from the East, Southwest, and Northwest (pH 7.66, 7.80, and 7.33, respectively) changed shell shape in the ranges of 0.5–4.3 % to become more rounded (Fig. 6). In addition, snails in the acidic Northwest site exhibited a 6.4 and 9.6 % decrease in shell thickness of body whorl (T1) and penultimate whorl (T2), respectively, compared to snails from the South. 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.

4.2 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., $Y = -0.025 + 0.003\,\mathrm{SL}$ ($R^2 = 0.88$; $N = 26$) and $Y = -2.39 + 1.04\,\ln\mathrm{SL}$ ($R^2 = 0.92$), 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

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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.

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.

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Table 1. Environmental parameters and shell traits of *Anachis misera* around the vent off Kueishan Islet. Data are shown as Mean ± SD and ranges. T1: thickness of body whorl; T2: thickness of penultimate whorl; –: no data; Means that differ significantly from each other are indicated by different letters.

Site	North	East	South	Southwest	Northwest
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 c	7.66 ± 0.08 b	7.80 ± 0.02 a	7.80 ± 0.03 a	7.33 ± 0.02 c
	(7.19–7.25)	(7.59–7.75)	(7.78–7.82)	(7.78–7.83)	(7.31–7.35)
No. snails (N)	0	7	65	33	36
Shell length (mm)	–	9.23 ± 0.63	9.01 ± 0.89	9.14 ± 1.11	9.13 ± 0.56
	–	(8.23–9.97)	(6.88–11.01)	(6.93–10.84)	(7.81–10.40)
Shell width (mm)	–	4.54 ± 0.32	4.42 ± 0.29	4.41 ± 0.30	4.30 ± 0.72
	–	(4.16–5.05)	(3.65–4.96)	(3.71–5.16)	(3.86–4.93)
Total weight (mg)	–	125 ± 18	121 ± 22	137 ± 23	113 ± 20
	–	(104–152)	(67–188)	(91–213)	(75–153)
T1 (um)	–	199 ± 56	225 ± 69	200 ± 56	168 ± 49
		(136–285)	(109–481)	(118–290)	(79–276)
T2 (um)	–	188 ± 44	200 ± 51	205 ± 55	180 ± 55
		(109–248)	(112–328)	(117–354)	(79–325)

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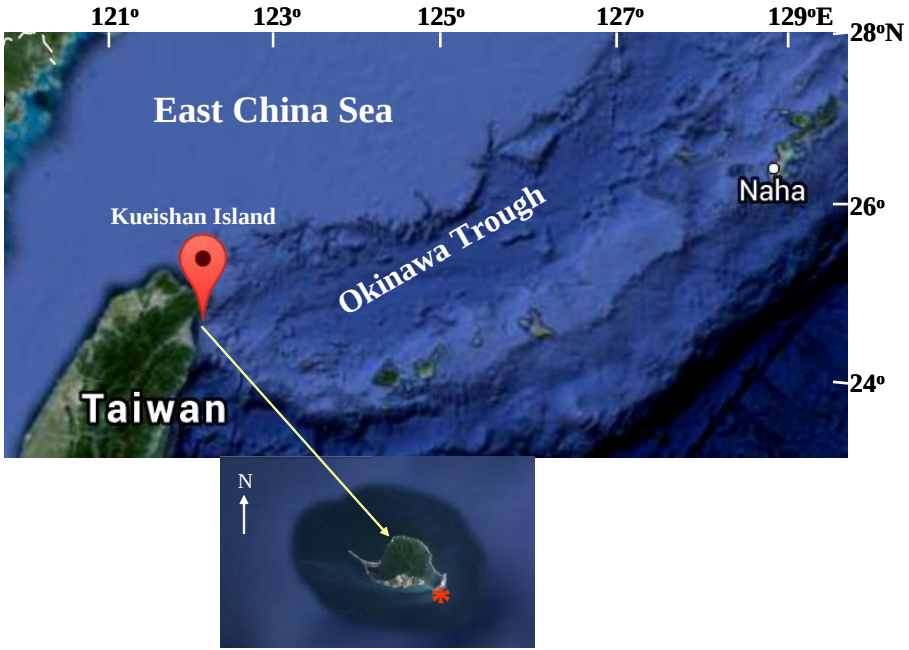

Figure 1. Map showing the collection site of *Anachis misera* (Source: Google map).

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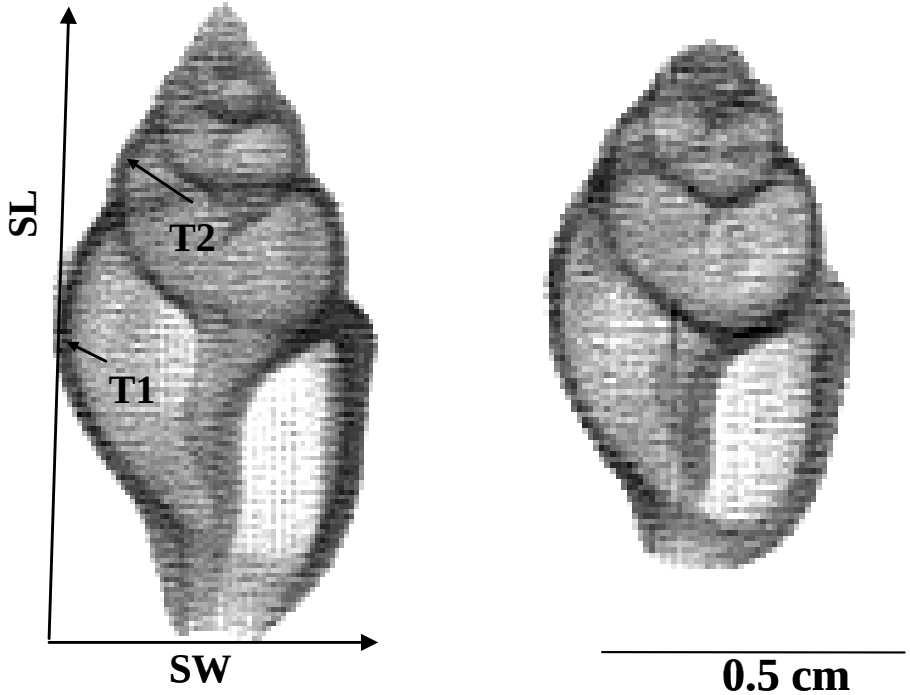

Figure 2. Biometric measurements of the shell of *Anachis misera*. SL: shell length; SW: shell width; T1: thickness of body whorl; T2: thickness of penultimate whorl; Left: Snail from the South; Right: Snail from the Northwest.

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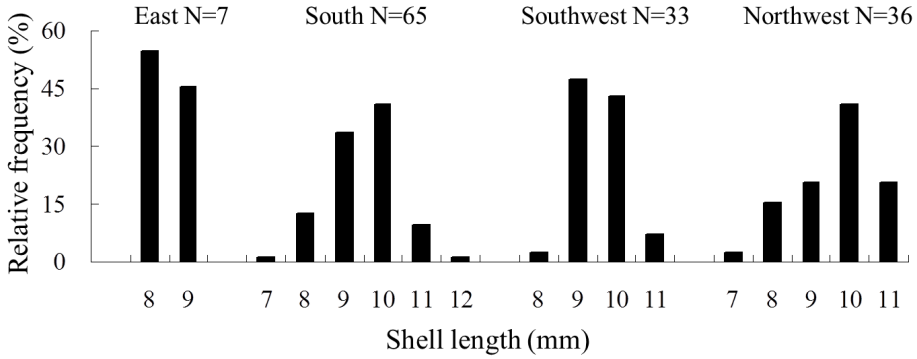

Figure 3. Length frequency distribution of *Anachis misera* around the vent off Kueishan Islet. N= sample size.

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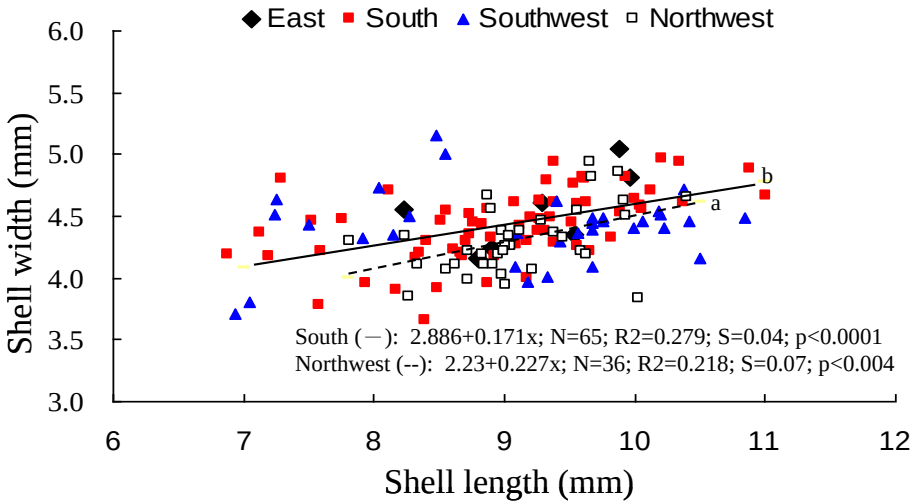

Figure 4. Relationship between shell length and shell width of *Anachis misera* from different sites. S: standard error of the regression; different letters indicate that the regression lines differ significantly ($p < 0.05$).

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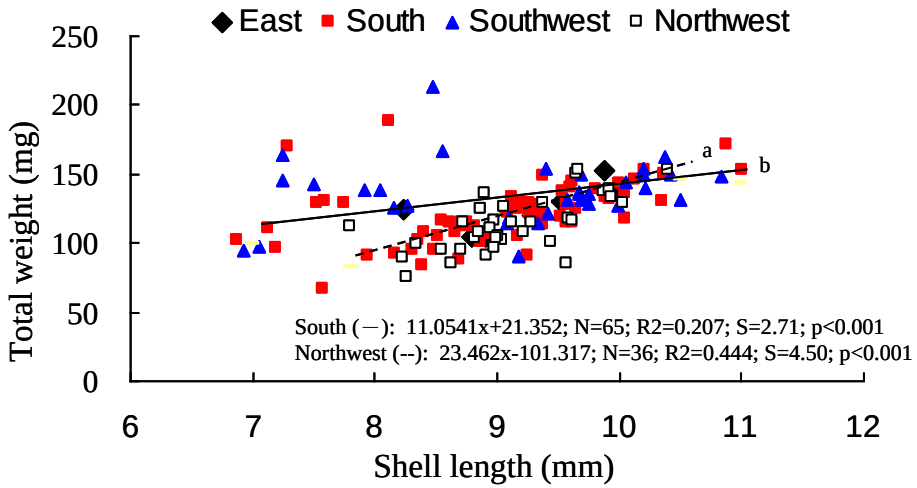

Figure 5. Relationship between shell length and total weight of *Anachis misera* from different sites. S: standard error of the regression; different letters indicate that the regression lines differ significantly ($p < 0.01$).

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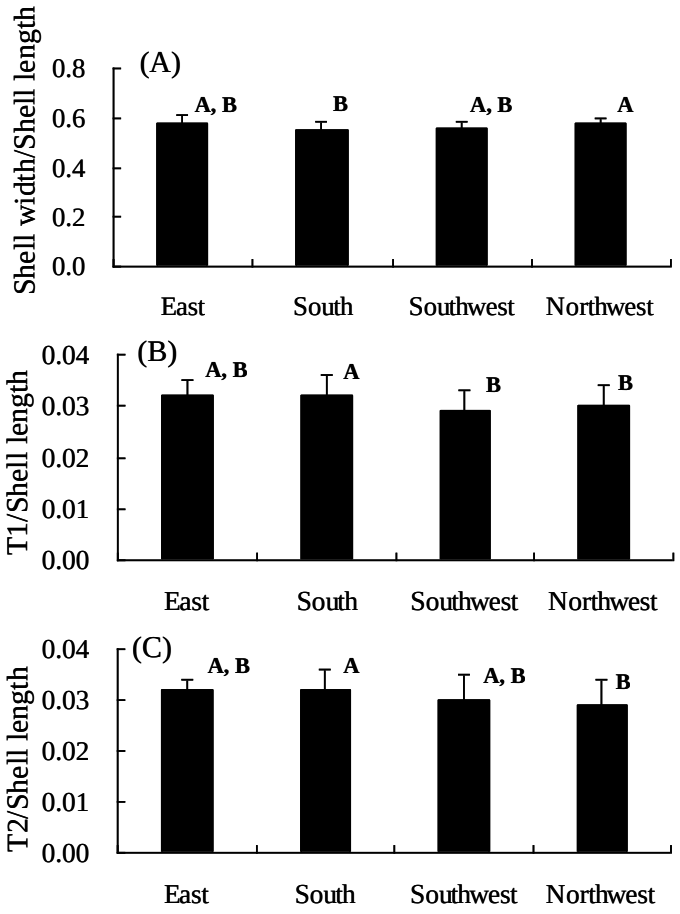

Figure 6. Standardized shell traits of *Anachis misera*. **(a)** Shell shape; **(b)** Thickness of body whorl (T1); **(c)** Thickness of penultimate whorl (T2). Data are shown as Mean ± SD and ranges. Means that differ significantly from each other are indicated by different letters ($p < 0.05$).

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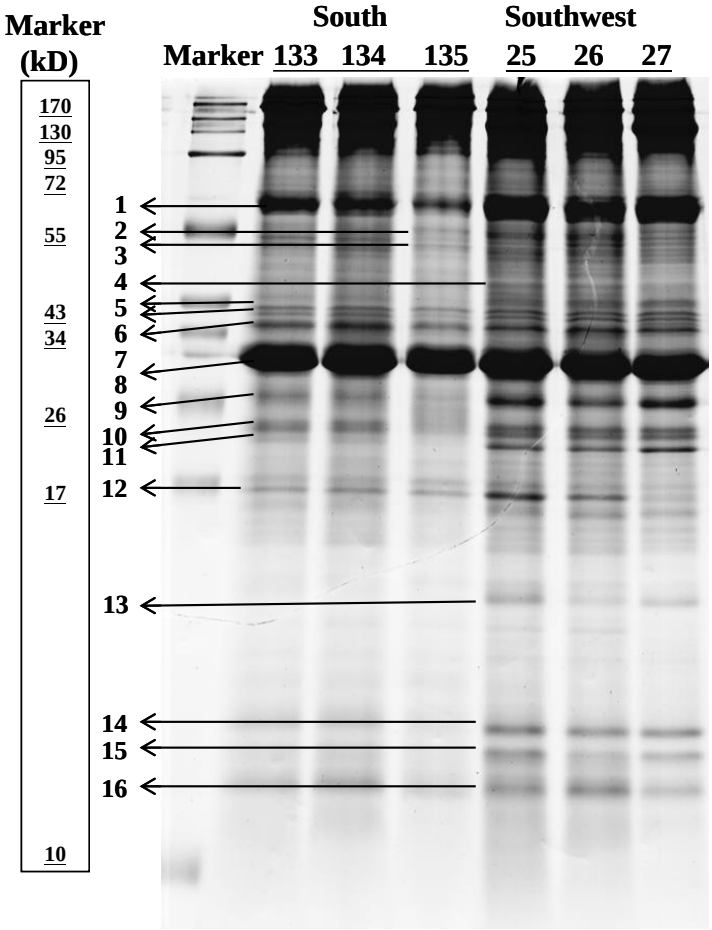

Figure 7. Gel electropherogram with molecular markers of *Anachis misera*. Number: protein band serial number.

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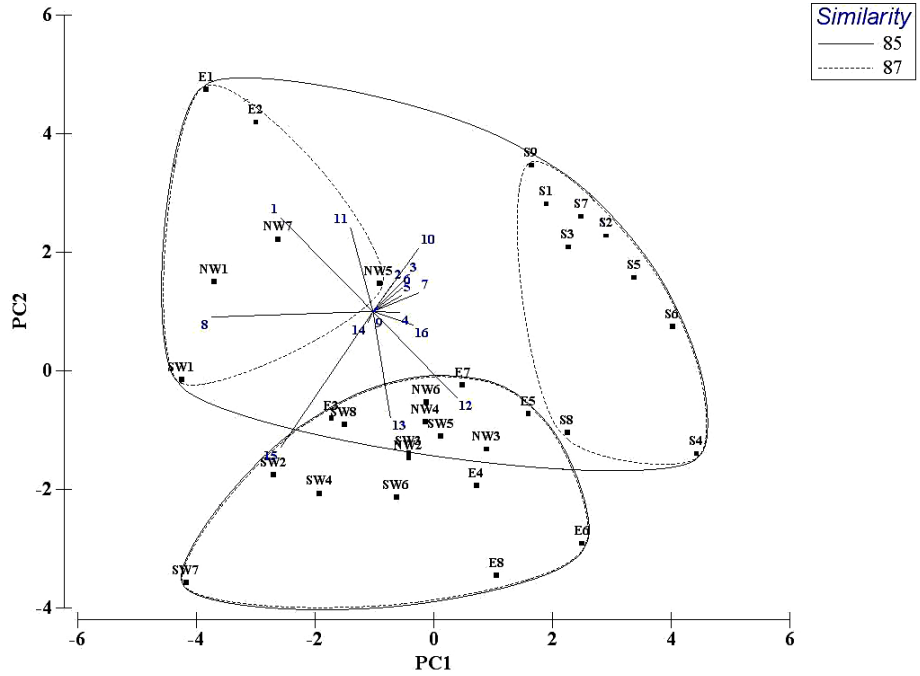

Figure 8. Results from the combined principal component analysis (PCA) and cluster analysis. The cluster analysis of Bray–Curtis Similarity (BCs) Indices using standardized overall protein expressions of *Anachis* snails from different sampling sites. E: East, S: South; SW: Southwest; NW: Northwest; 1–16: protein spot variable.

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