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Effects of animal size and nutritional status on the RNA/DNA ratio in different tissues of the green-lipped mussel *Perna viridis*

JAMIUS W.Y. YEUNG AND KENNETH M.Y. LEUNG

The Swire Institute of Marine Science and School of Biological Sciences, the University of Hong Kong, Pokfulam, Hong Kong, People's Republic of China

This study aimed to examine the responses of RNA/DNA ratio in Perna viridis *under different nutritional status via both field and laboratory studies, and hence evaluate the usefulness of this ratio as a rapid growth biomarker in the mussels. First, the effects of size (small: 30–40 mm; medium: 40–50 mm; large: >50 mm) and tissue type (adductor muscle, foot, gill and hepatopancreas) on the RNA/DNA ratio were investigated in* P. viridis *collected from three different sites with different degrees of eutrophication in Hong Kong waters. Across all sizes, the mussels collected from a fairly 'eutrophic' mariculture zone had significantly higher RNA/DNA ratios in their gills than those from the other two relatively clean sites. The RNA/DNA ratio in small mussels was generally higher than in medium and large individuals, though such a size effect significantly interacted with tissue type and site. Second, we conducted a 10-day comparative laboratory study to elucidate the influence of starvation and feeding on the RNA/DNA ratio in the mussels. We observed that both hepatopancreas and foot muscle generally exhibited significant and rapid response to such a short-term starvation or food addition. The present results confirmed that the RNA/DNA ratio in* P. viridis *is a sensitive biomarker to gauge their growth and general health condition in accordance with food availability and/or eutrophication condition.*

Keywords: RNA/DNA ratio, *Perna viridis*, biomonitoring, biomarker, eutrophication, mussel

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INTRODUCTION

Mussels inhabiting the natural environment are subjected to the changes in food availability (Wong & Cheung, 2001) which eventually effect their growth and health condition (Menge *et al.*, 2007). In South-east Asia, the green-lipped mussel, *Perna viridis* (Linnaeus, 1758), plays an important role in molluscan culture by serving as an abundant and cheap protein source in this region (Menzel, 1988; Chou & Lee, 1997; Rajagopal *et al.*, 1998; Wong & Cheung, 2001). Regular growth and health assessment would be necessary for quality assurance in the mussel farming industry. Although physiological responses, such as change in biomass and condition index, have been used for assessing growth and health condition in bivalves (Widdows, 1978), the responses usually required a longer exposure duration to reveal measurable effects. Recently, the nucleic acid ratio between RNA content and DNA content in tissues (i.e. RNA/DNA ratio) has been proposed as an efficient index for evaluation of the general health condition of marine organisms when exposed to rapidly changing natural and anthropogenic stressors (Dahlhoff & Menge, 1996; Wo *et al.*, 1999; Yang *et al.*, 2002; Dahlhoff, 2004). The RNA/DNA ratio has demonstrated its versatile utility in assessing growth rate and condition of fish (Buckley, 1979) and subsequently other marine organisms such as bivalves (Menge *et al.*, 2007), copepods (Wagner *et al.*, 1998) and crustaceans (Moss, 1994).

Previous studies have reported that the RNA/DNA ratio is a sensitive biomarker to food availability in different species (Parslow-Williams *et al.*, 2001; Okumura *et al.*, 2002; Speekmann *et al.*, 2006). However, the intrinsic biological variations, such as size and tissue types, could mask the response in the RNA/DNA due to food availability (Mayrand *et al.*, 1994; Norkko & Thrush, 2006). Therefore, the present study involved both field and laboratory aspects to examine the effects of endogenous factors (animal size and tissue types) and food availability on the RNA/DNA ratio of *P. viridis*. The results would provide a sound basis for more accurate interpretation of the RNA/DNA ratio when it is adopted as a rapid growth biomarker in *P. viridis* cultured in the mussel farming industry or used in a biomonitoring programme for marine pollution.

Corresponding author:
K.M.Y. Leung
Email: kmyleung@hku.hk

MATERIALS AND METHODS

Field study

Various sizes of the green-lipped mussel *Perna viridis* were collected from three sites in Hong Kong, namely Hoi Ha Wan (HHW), Bluff Island (BI) and Lamma Island (LI), between September and October 2008 (Table 1; Figure 1); LI is located within a mariculture zone. Mussels at HHW and

Table 1. General hydrographical and geographical information of the three study sites.

Site	Geographical coordinate	Water zone	Nutrient gradient	Human activities	References
Hoi Ha Wan	22°28′N 114°19′E	Tolo Harbour and Channel	Oligotrophic/mesotrophic	Marine park with coral protection area nearby	Chau, 2007; Miao *et al.*, 2006
Bluff Island	22°19′N 114°11′E	Mirs Bay water control zone	Oligotrophic	Coral protection area	Wu *et al.*, 2003
Lamma Island	22°11′N 114°7′E	Southern water control zone	Eutrophic	Aquaculture	Liu *et al.*, 2000; Lee *et al.*, 2005

BI were found attaching to the submerged chain of floating buoys near a coral protection area, while those collected from LI were attached to the raft of an open-sea-cage fish farm where nutritional fish feed in the form of pellet or trash fish were commonly applied. To verify if RNA/DNA ratios of mussels collected from these three different sites (i.e. HHW, BI and LI) were influenced by the degree of nutrient enrichment, secondary water quality monitoring data on the nutrient enrichment related parameters including turbidity, volatile suspended solids, total inorganic nitrogen, chlorophyll-*a* and total suspended solids were extracted from the Marine Water Quality Database (2004–2007) of the Environmental Protection Department (EPD) of the Hong Kong Special Administrative Region Government (HKEPD, 2008). As there were three, two and two marine water sampling stations for LI, BI and HHW, respectively, all available data for each of the three sites were pooled for statistical comparison.

No significant difference in salinity, temperature and dissolved oxygen levels was found among the study sites during the sampling period. All collected mussels were transported to the laboratory in a cool box with ambient seawater. Upon arrival at the laboratory, any epibiotic organisms on the mussel shells were removed. To investigate the effect of animal size on the RNA/DNA ratio, mussels were divided into three size-classes, 30–40 mm (small), 40–50 mm (medium) and >50 mm (large), according to the shell length measured with calipers (accuracy: ±0.5mm). For each size-class, 15 mussels were randomly selected from each sampling location. Adductor muscle (AD), foot, gill and hepatopancreas (HP) were excised and weighed from each mussel and snap frozen in liquid nitrogen in an autoclaved microcentrifuge tube. Frozen samples were stored at −80°C until nucleic acid analysis.

Laboratory study

To investigate the effect of nutritional status (i.e. starving versus feeding) on the RNA/DNA ratio, mussels with initial shell length of 30–40 mm were collected from HHW and used for the laboratory experiment. As a previous study has demonstrated that the RNA/DNA ratio in marine mussels can be influenced by size and vary significantly between different sites (Norkko *et al.*, 2006), this study was restricted to *Perna viridis* with a similar size (i.e. shell length) collected from HHW only. This size-range was chosen based on the results of the above field study and previous literature showing that small immature mussels generally exhibited the highest RNA/DNA ratio in their tissues when compared with the larger mature mussels and provided a more sensitive

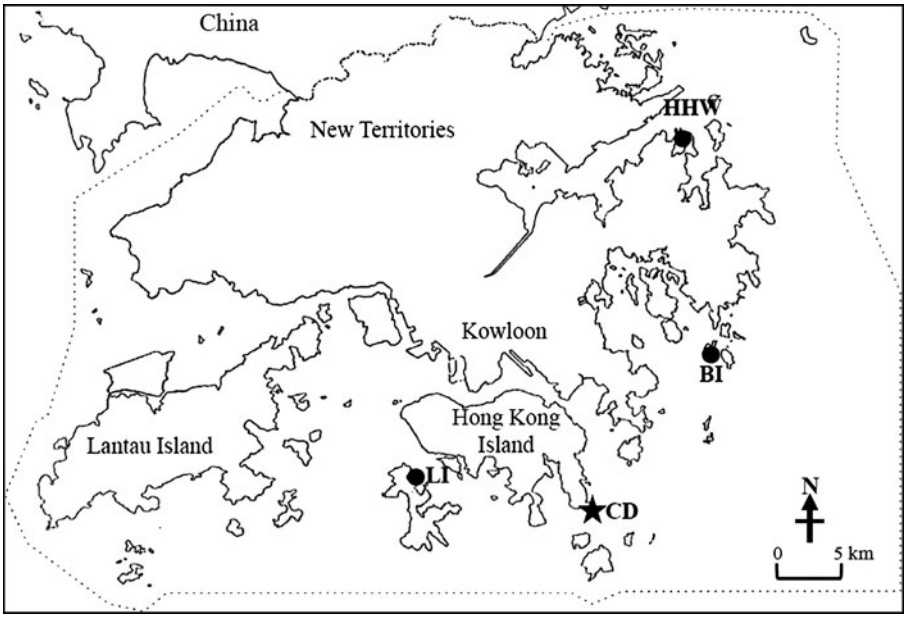

Fig. 1. Map of Hong Kong showing the sampling sites where the mussels *Perna viridis* were collected at Hoi Ha Wan (HHW), Bluff Island (BI) and Lamma Island (LI), respectively. A filled star indicates the location of the Cape D'Aguilar Marine Reserve (CD) where natural seawater was taken, filtered and used for the laboratory study.

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response in terms of the magnitude of change to the ambient environment (Chícharo & Chícharo, 2008). Any epibiotic organisms on the mussel shells were removed. The mussels were then acclimated to laboratory conditions (salinity at 33‰ (±2; ±SD), temperature at 25°C (±1)) for 2 weeks before experimentation. During the acclimation period, the level of dissolved oxygen was maintained at >90% saturation with running natural seawater from the Cape D'Aguilar Marine Reserve (CD: 22°11′N 114°13′E; Figure 1); the mussels were initially starved for the first week and then fed with the marine microalgae *Tetraselmis* spp. in the second week. After the acclimation period, 25 mussels were randomly selected and allocated to each of the two experimental treatments (i.e. starving versus feeding). In the starving treatment, the mussels were placed in an 8 l glass tank filled with membrane filtered seawater (Millipore 0.22 μm membrane, Billerica, MA, USA) collected from Cape D'Aguilar Marine Reserve and were not fed with the marine microalgae for 10 days. In the feeding treatment, mussels were placed in the same condition as the starved mussels except that they were provided with *Tetraselmis* spp. at a density of 5×10^6 cells/ml on a daily basis. The ration concentration was comparable with the cell density observed during algal bloom in the eastern waters of Hong Kong (Yung *et al.*, 1997; Wong & Wong, 2009). The experimental conditions were maintained at 12h:12h light:dark cycle, salinity at 33‰ (±2; ±SD), pH at 8.7 (±1), temperature at 25°C (±1) and dissolved oxygen level >90% saturation through aeration. Water was renewed once every two days. The exposure duration of 10 days was chosen as the endpoint, based on the results of a pilot study where mortality was observed in starved mussels after 10 days. Five mussels were randomly collected from each treatment tank by the end of Day 10 and AD, foot, gill and HP of each selected mussel were dissected and stored at −80°C until the nucleic acid analysis.

Nucleic acid analysis

The fluorometric method was used to measure the concentration of nucleic acids while extraction and quantifying procedures followed those described by Caldarone *et al.* (2001) with some modifications. In brief, a sample of snap frozen tissues was homogenized by Ultra Turrax homogenizer (IKE Werke, Germany) in 200μl of 1% STEB (1 g N-sarcosil in 100 ml 1 × TE buffer) in 1.5 ml microcentrifuge tube. The homogenate was then shaken at 4°C for 1 hour. After shaking, 1 ml 1 × TE buffer was added to the homogenate and mixed thoroughly by vortex or mechanical inversion for 40 times. This made up the final concentration of the mixture with 0.2% STEB. The mixture was then centrifuged at 10,000 rpm for 30 minutes. To avoid the re-suspension of tissue particulates, 1 ml of supernatant was transferred to an autoclaved microcentrifuge tube for storage at −80°C before analysis.

Upon analysis, 75 μl of the supernatant was loaded to a well of a 96-well plate and 75 μl of ethidium bromide fluorescence was added to the supernatant. After 30 minutes, fluorescence of total nucleic acid was measured using the spectrofluorometer with a microplate reader (M2e, Molecular Devices Corporation, USA) and the SOFTmax Pro. 5.1. software (Molecular Devices Corporation, USA) at 525 nm excitation wavelength and 590 nm emission wavelength. After the first fluorescence measurements, 7.5 μl RNase of 200 μg/ml was added and 45-minute incubation time was followed to ensure that all the RNA was hydrolysed by the enzyme. The fluorescence was subsequently measured as above for the second reading. In essence, RNA fluorescence values were the difference between the first and second fluorescence readings; and DNA fluorescence values were the second fluorescence reading. RNA and DNA concentrations in the samples were determined from the standard calibration curves which were constructed by running the same analysis with standards of known DNA and RNA contents (Sigma calf thymus DNA, 1.5–15 μg/ml; Sigma calf liver RNA, Type IV, 5–40 μg/ml).

Statistical analyses

Levene's test was used to test for homogeneity of variance of the RNA/DNA ratio among treatment groups and the results indicated that all treatment groups shared an equal variance for the field study but not for the laboratory study. To correct the heterogeneity of variance, datasets from the laboratory study were log-transformed. For the field study, a 3-way analysis of variance (ANOVA) was used to test the variation in the RNA/DNA ratio in field collected mussels among sites (3 levels: BI, HHW and LI), animal sizes (3 levels: small, medium and large) and tissue types (4 levels: AD, foot, gill and HP), while all three factors were treated as fixed factors. For the laboratory study, a 3-way ANOVA was used to compare the log-transformed RNA/DNA ratios among the four tissue types (AD, foot, gill and HP), two time points (Day 0 versus Day 10) and the two feeding treatments (starving versus feeding). For ANOVA, a *post-hoc* Student–Newman–Keuls (SNK) multiple comparison test was used to identify any significantly different means. A multivariate analysis of variance (MANOVA) was used to verify if there was site difference in the nutrient enrichment related parameters measured by Hong Kong Environmental Protection Department (HKEPD, 2008) with a *post-hoc* SNK test to identify any significantly different means. Spearman's correlation analyses were performed on the mean values of environmental parameters and RNA/DNA ratios of different tissues from field collected mussels. All data analyses were performed using SPSS (Version 17.0, Chicago, IL, USA).

RESULTS

Field study

The RNA/DNA ratio of *Perna viridis* varied significantly between the three sites, four tissue types and three size-classes, and these factors also significantly interacted with each other (Table 2; Figure 2), though the effects of tissue type and site were more apparent than the influence of size as indicated by the F value of each fixed factor (Table 2). In AD, RNA/DNA ratios were similar among all size-groups for each of the three sites while both medium and large mussels collected from BI had a significantly lower RNA/DNA ratio than those from LI and HHW (Figure 2A). The RNA/DNA ratio in foot muscles of the small BI mussels was significantly higher than that in the medium animals (Figure 2B), whereas RNA/DNA ratios in foot muscles of mussels collected from HHW and LI were fairly similar across all size-classes (Figure 2B).

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Table 2. Results of 3-way analysis of variance of RNA/DNA ratio of four tissues (adductor muscle, foot, gill and hepatopancreas) from three size-classes (small, medium and large) of *Perna viridis* collected from three sampling sites (Hoi Ha Wan, Bluff Island and Lamma Island). Bold *P* values indicate factors with significant effect at $P < 0.05$.

Factor	SS	df	MS	F	*P*	Power
Site	7.797	2	3.898	38.063	**<0.0001**	1.000
Tissues	13.144	3	4.381	42.776	**<0.0001**	1.000
Size	1.219	2	0.610	5.952	**0.0028**	0.879
Site × tissues	7.025	6	1.171	11.431	**<0.0001**	1.000
Site × size	2.488	4	0.622	6.073	**<0.0001**	0.987
Tissues × size	0.340	6	0.057	0.553	0.7679	0.223
Site × tissues × size	2.420	12	0.202	1.969	**0.0252**	0.919
Error	50.187	490	0.102			
Total	466.752	526				

Irrespective of size and site effects, RNA/DNA ratios of the gills of *P. viridis* were significantly and consistently lower than those measured in other tissues (Figure 2). There was a significant and consistent site effect on the RNA/DNA ratio in the gills across all size-classes with LI mussels having the highest ratio, BI mussels being the intermediate and followed by those from HHW (Figure 2C). Nonetheless, effect of size on RNA/DNA ratios in the gills was insignificant (Figure 2C). Variation of RNA/DNA ratios of HP was site specific; size effect was statistically significant in HHW mussels only in which the RNA/DNA ratio decreased with increasing size (Figure 2D; Table 2). In general, mussels of all size-classes from BI consistently showed the lowest RNA/DNA ratio in all tissue types except the gills when compared with that of the other two sites (Figure 2D).

Spatial variation in nutrient-related parameters

Based on the HKEPD data (Table 3), waters nearby LI consistently showed significantly higher values for all of the nutrient enrichment related parameters when compared with those measured nearby BI and HHW (Table 4). These results suggested that there was considerably higher nutrient levels and greater food availability (as reflected by chlorophyll-*a*) to filter feeding *Perna viridis* in LI than in the other two sites.

Spearman's correlation analyses, however, revealed significant positive correlations between the mean RNA/DNA ratios of AD, gill and HP tissues, and the averages of nutrient-related environmental parameters (Table 5). Specifically, the mean RNA/DNA ratios in both AD and HP were positively correlated with turbidity and chlorophyll-*a* concentration,

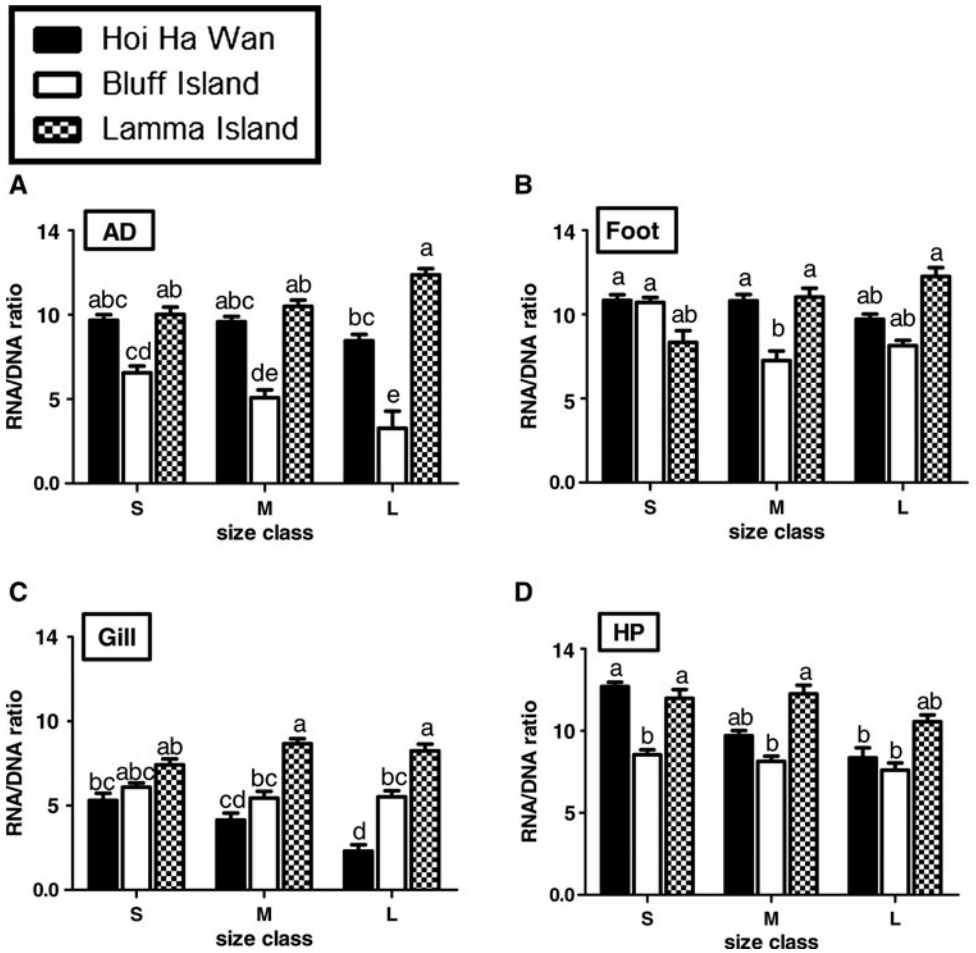

Fig. 2. Mean RNA/DNA ratios (+ SEM, N = 13–15) in the four tissue types (A) adductor muscle (AD), (B) foot, (C) gill and (D) hepatopancreas (HP) in three different size-classes (i.e. S, small, 30–40 mm; M, medium, 40–50 mm; and L, large, >50 mm) of *Perna viridis* collected from three different sampling sites including Hoi Ha Wan, Bluff Island and Lamma Island. Bars labelled with different letters indicate significantly different means at $P < 0.05$ (Student–Newman–Keuls test).

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Table 3. Environmental data from sampling sites in this study (mean ± SEM). Data were collected from the Marine Water Quality Database (2004–2007) of the Environmental Protection Department (EPD) of Hong Kong Special Administrative Region Government (HKEPD, 2008).

Site	Sampling station by EPD	Depth	Turbidity (nephelometric turbidity units)	Volatile suspended solids (mg/l)	Total inorganic nitrogen (mg/l)	Chlorophyll-*a* (μg/l)	Suspended solids (mg/l)
Lamma Island	SM3, SM4, SM5	Middle water*	10.30 ± 4.39	1.60 ± 0.78	0.15 ± 0.10	3.27 ± 4.05	3.99 ± 2.86
Bluff Island	PM11, MM14	Middle water*	8.48 ± 3.20	0.54 ± 0.58	0.06 ± 0.04	1.83 ± 2.30	1.90 ± 2.11
Hoi Ha Wan	MM17, MM6	Middle water*	8.49 ± 3.86	0.45 ± 0.53	0.05 ± 0.04	2.41 ± 2.38	1.59 ± 1.71

*Notes: according to the marine water quality report from Hong Kong Environmental Protection Department (HKEPD, 2008), 'middle water' is defined as the water column between 1 m below sea surface and 1 m above seabed.

Table 4. Results of multivariate analyses of variance on nutrient-related environmental variables of three sampling sites (Lamma Island (LI); Bluff Island (BI); Hoi Ha Wan (HHW)) from HKEPD data between 2004 and 2007 (HKEPD, 2008). Factors with significant effect at $P < 0.05$ are indicated by bold P values.

Factor	Variables	df	MS	F	*P*	Student–Newman–Keuls test
Site	Turbidity (NTU)	2	135.112	8.756	**<0.0001**	LI > BI = HH
	Volatile suspended solids (mg/l)	2	13.463	30.650	**<0.0001**	
	Total inorganic nitrogen (mg/l)	2	0.375	69.494	**<0.0001**	
	Chlorophyll-*a* (μg/l)	2	62.480	6.149	**0.002**	
	Suspended solids (mg/l)	2	209.830	37.347	**<0.0001**	
Error	Turbidity (NTU)	333	15.432			
	Volatile suspended solids (mg/l)	333	0.439			
	Total inorganic nitrogen (mg/l)	333	0.005			
	Chlorophyll-*a* (μg/l)	333	10.162			
	Suspended solids (mg/l)	333	5.618			

NTU, nephelometric turbidity units.

respectively; whereas the mean RNA/DNA ratios of gill were positively correlated with volatile suspended solid, total inorganic nitrogen and suspended solids, respectively. Surprisingly, none of the environmental parameters showed a significant correlation with the mean RNA/DNA ratios in gill tissues (Table 5).

Laboratory feeding experiment

No mortality of *P. viridis* was observed throughout the experiment. This experiment primarily tested the hypothesis that differences in food availability (i.e. starved versus fed mussels) could influence the RNA/DNA ratio in their tissues. In general, RNA/DNA ratios of starved mussels were significantly lower than those in fed mussels at the end of experimentation after ten days (Figure 3 & Table 6; ANOVA: $F_{1,47} = 4.849$, $P = 0.033$), but the magnitude of change in the ratio greatly varied among different tissue types (ANOVA: $F_{3,47} = 6.321$, $P = 0.001$).

RNA/DNA ratios of AD between starved and Day 0 mussels were quite consistent, while the difference between starved and fed mussels on Day 10 was significant (Figure 3; Table 6; SNK test: $P < 0.05$). The coefficients of variation (CV) in the ratios of AD were found to be 26% and < 40% for starved and fed mussels, respectively. Foot muscle exhibited the most significant difference between starved and fed mussels in which the mean ratios of fed mussels were significantly higher than those in starved mussels (Figure 3; Table 6; SNK test; $P < 0.05$). However, a high variability in RNA/DNA ratios of foot muscle was noted as reflected by the CV (>67% for fed mussels and >80% for starved ones). In gills, RNA/DNA ratios of fed and starved mussels were fairly stable (CV < 35% for both starved and fed mussels) during the experimental period (Figure 3; Table 6; SNK test: $P > 0.05$).

Irrespective of food availability, RNA/DNA ratios of the gills were generally lower among all tissue types (Figure 3). This observation was consistent with those observed in the field experiment. Similar to the foot muscle, RNA/DNA ratios of HP in fed mussels (CV = 45%) were significantly higher than those in starved mussels (CV = 51%) and slightly higher than the ratio initially measured on Day 0 (Figure 3; Table 6; SNK test: $P < 0.05$).

DISCUSSION

It has been widely suggested that the RNA/DNA ratio in sessile marine organisms may be a useful biomarker in coastal habitats that are constantly fluctuating in food dynamic, and changes in magnitude of the RNA/DNA ratio are often representative of the ambient condition (Chícharo *et al.*, 2001; Dahlhoff *et al.*, 2002; Dahlhoff, 2004; Norkko *et al.*, 2005). Before making proper use of this biomarker, the background of natural variability in their responses needs to be better understood. The current results demonstrated that the RNA/DNA ratio varied significantly among sites with different levels of nutrient enrichment, among tissue types and to a lesser extent among different size-classes. Our observations have provided useful background information on the use of the RNA/DNA ratio as a growth (or stress) biomarker in the mussel farming industry or a biomonitoring programme for marine pollution.

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Table 5. Results of Spearman's correlation analyses on the relationships between RNA/DNA ratios of different tissues (i.e. AD, adductor muscle; foot; gill; HP, hepatopancreas) in field collected *Perna viridis* and nutrient-related environmental parameters (N = 9).

Tissue	Turbidity (nephelometric turbidity units)	Volatile suspended solids (mg/l)	Total inorganic nitrogen (mg/l)	Chlorophyll-*a* (μg/l)	Suspended solids (mg/l)
AD					
$^{*}r_s$	0.949**	0.474	0.474	0.949**	0.474
P	<0.001	0.197	0.197	<0.001	0.197
Foot					
r_s	0.211	−0.053	−0.053	0.211	−0.053
P	0.586	0.893	0.893	0.586	0.893
Gill					
r_s	0.474	0.949**	0.949**	0.474	0.949**
P	0.197	<0.001	<0.001	0.197	<0.001
HP					
r_s	0.738*	0.211	0.211	0.738*	0.211
P	0.023	0.586	0.586	0.023	0.586

$^{*}r_s$, Spearman's correlation coefficient. $^{*}P < 0.05$; $^{**}P < 0.001$.

Food availability and RNA/DNA ratio

It is a well-known fact that there is a strong link between coastal food availability and mussel physiology (Dahlhoff & Menge, 1996). The results from the present field and laboratory studies supported this postulation and confirmed that the level of the RNA/DNA ratio in the mussel is tissue-specific and can reflect nutritional status (well-fed versus starvation), which is directly related to food availability. Mussels collected from BI always had significantly lower RNA/DNA ratios in HP, adductor and foot muscles than mussels collected from LI and HHW, while there was a consistent spatial variation of the ratio in the gills (i.e. LI > BI > HHW). Such observed spatial variations in the RNA/DNA ratio could be partially attributed to the difference in nutrient-related hydrographical condition among sites. For instance, BI is located in the southeastern waters of Hong Kong subjected to inputs from the oligotrophic South China Sea (Wu *et al.*, 2003) with relatively limited freshwater and nutrient input (e.g. nitrogen and silicon) from terrestrial runoff (Miao *et al.*, 2006; HKEPD, 2008). Under such a low nutrient condition, mussels at BI would have comparatively lower food availability, slower growth rate and hence a lower RNA/DNA ratio in contrast to those living in the eutrophic LI site.

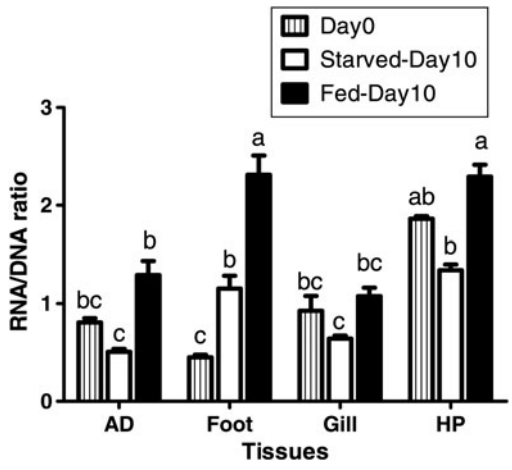

Fig. 3. Mean RNA/DNA ratios (mean + SEM, N = 5) in different tissues, adductor muscle (AD), foot, gill and hepatopancreas (HP), between fed and starved *Perna viridis* after the 10-day experimental period. Black bars represent fed mussels, whereas white bars represent starved mussels. Bars with vertical line represent reference mussels on Day 0. Bars labelled with different letters indicate significantly different means at $P < 0.05$ (Student–Newman–Keuls test).

On the contrary, high RNA/DNA ratios were frequently registered in LI mussels, especially in the gills, where there appears to be the most 'eutrophic' environment among the three study sites because there were significantly higher concentrations of nutrient-related parameters in the water column (HKEPD, 2008). Mussels from LI were, in fact, situated at an open-sea-cage fish farm where fish meals (in the form of pellets) and minced trash fish with high protein content are commonly applied as fish feed (Rumsey 1993). Any unconsumed fish food and fish faecal egestion can accumulate as organic matter on the seabed which can be re-suspended in the water column to serve as additional nutrient supply for filter feeding mussels to promote their growth (Hylland *et al.*, 1996; Leung *et al.*, 1999) and result in elevated RNA/DNA ratios in the mussels (Norkko *et al.*, 2006). Conceivably, the RNA/DNA ratio in mussels could be used as a sensitive biomarker to differentiate sites with different levels of nutrient enrichment.

The present laboratory experiment was designed to test the hypothesis that the RNA/DNA ratio decreases with starvation in the mussel. This is an important consideration for inferring mussel's metabolic activity in the field while the results would be useful to evaluate the sensitivity of different tissues towards poor nutritional status in terms of the RNA/DNA ratio. After starving for ten days, *Perna viridis* showed a significant decrease in RNA/DNA ratios in AD, HP and gill tissues when compared with the fed individuals. On the contrary, the RNA/DNA ratio of foot muscles was still elevated in the mussels even under starvation. Such an increase in the amount of RNA abundance may be associated with the continuous synthesis of proteinaceous byssus threads required for settlement on substrate, irrespective of food availability or energy supply (Shin *et al.*, 2002; Wang *et al.*, 2010). By comparing between starved and well-fed mussels, RNA/DNA ratios in foot muscle, HP and AD were very sensitive to a short-term change in food availability (i.e. 10 days) and such results reinforce the findings obtained in the field study (as discussed above). Like *P. viridis*, previous studies also reported that starvation can result in reduced RNA/DNA

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Table 6. Results of 3-way analysis of variance on RNA/DNA ratios in four different tissues of fed and starved *Perna viridis* (i.e. 2 feeding treatments) over the 10-day experiment experimental period (i.e. 2 time points: Day 0 versus Day 10). Factors with significant effects at $P < 0.05$ are indicated by bold P values.

Factor	SS	df	MS	F	*P*	Power
Feeding treatment	2.629	1	2.629	4.849	**0.033**	0.578
Time	1.513	1	1.513	2.791	0.101	0.373
Tissues	10.280	3	3.427	6.321	**0.001**	0.953
Feeding treatment × time	2.629	1	2.629	4.849	**0.033**	0.578
Feeding treatment × tissue	0.248	3	0.083	0.152	0.928	0.076
Time × tissues	4.688	3	1.563	2.882	**0.046**	0.652
Feeding treatment × time × tissues	0.248	3	0.083	0.152	0.928	0.076
Error	25.479	47	0.542			
Total	136.338	63				

ratios in bivalves (Wright & Hetzel, 1985; Dahlhoff *et al.*, 2002), gastropods (Okumura *et al.*, 2002) and fish (Ferron & Legett, 1994). The decrease in the RNA/DNA ratio is related to the reduction or inhibition of protein synthesis and thus the ratio can be used as an indication of hindered growth and metabolism (Dahlhoff, 2004; Chícharo & Chícharo, 2008). Thereby, the lower RNA/DNA ratio may signify the slow growth rate (or poor health, i.e. stress condition) in the mussels that are perhaps under deprived food availability coupled with other physical–chemical stresses.

Size effect on RNA/DNA ratio

It is a general physiological rule that juvenile animals usually have a higher metabolic rate and faster growth (Chícharo & Chícharo, 2008). As such, one would predict that small mussels should present a higher RNA/DNA ratio reflecting a high rate of protein synthesis for somatic growth (Hawkins, 1991). This study, however, did not show and support the hypothesized size-dependent trend that the RNA/DNA ratio increases with decreasing size in *Perna viridis*. Instead, the current results showed that the size effect on the RNA/DNA ratio in *P. viridis* is highly tissue-specific and site-specific. Thus, it is not possible to make tangible generalizations on the size effect. The size effect was only significant in AD and foot muscle of BI mussels and in gill and HP of HHW mussels, in which small mussels exhibited a higher RNA/DNA ratio than in medium and large mussels (Figure 2). Referring to the HKEPD data, BI and HHW are relatively 'oligotrophic'. The decline of RNA/DNA ratios with increasing size observed in BI and HHW mussels could be the combined consequence due to the limited exogenous food available and the allocation of endogenous energy reserves from somatic growth to reproduction (Roddick *et al.*, 1999; Mouneyrac *et al.*, 2008). Also, it should be noted that a decrease in the RNA/DNA ratio with increasing age most likely reflects a decrease in growth rate but may not necessarily indicate a decrease in condition (Chícharo & Chícharo, 2008).

The discrepancy of the size effect between sites is probably associated with the better nutritional status in LI mussels contrasting with BI and HHW mussels. The elevated food availability associated with fish farming activities might have promoted fast growth and thus overridden the size effect on RNA/DNA ratios in LI mussels. Norkko *et al.* (2006) also reported a similar observation that the effect of enhanced food availability could override the negative effect of hypoxia on the RNA concentration in marine benthic bivalves.

Moreover, it has been reported that there were significant differences in nucleic acid concentration between males and females of marine organisms during the spawning season (Chícharo & Chícharo, 2008). The current study did not take gender effect into account, as it was not possible to identify the sex of *Perna viridis* solely based on the colour of the gonad without in-depth histological studies (Lee, 1988). Based on the field study conducted by Cheung (1991), *P. viridis* in Hong Kong exhibited year-round spawning and thus gender effect might influence the interpretation of the RNA/DNA ratio to a certain extent. However, several studies also elucidated that food availability is the primary controlling factor for growth and gonad development in mussels (Sreenivasan *et al.*, 1989; Rajagopal *et al.*, 2006). Hence, gender and size could be comparatively minor factors influencing the result and interpretation of the RNA/DNA ratio in the mussels under eutrophic condition. A further study on the gender effect on the ratio in *P. viridis* is still needed to verify this postulation.

Nonetheless, the current results did show that small mussels in two sites (BI and HHW) exhibited the highest RNA/DNA ratio in most cases. Therefore, selecting small fast-growing individuals for measurement of the RNA/DNA ratio is still preferable as it may provide a more sensitive response (in terms of the magnitude) to the change of ambient levels of nutrients and toxicants.

Sensitivity of RNA/DNA ratio in different tissues due to food availability

In the present field study, the correlation analyses demonstrated that the level of the RNA/DNA ratio, particularly in HP, AD and gill tissues of the mussels, is strongly associated with nutrient levels in the marine environment. In our laboratory study, RNA/DNA ratios of HP, AD and gill tissues of the mussels considerably reduced after starvation for ten days, while feeding could promote the elevation of the ratio in all tissues, in particular more pronounced increases were observed in foot muscle and HP tissues. RNA/DNA ratios of HP and foot are comparatively more sensitive to nutritional status because they are characterized by dynamic protein-based activities, but in different aspects.

Hepatopancreas serves as an energy storage organ to allocate energy utilization among tissues (Mayrand *et al.*, 1994; Segnini de Bravo, 2003). The increase in the RNA/DNA ratio of HP is more directly related to energy acquisition and facilitated growth. HP consists of digestive glands which produce digestive enzymes and also act as a buffer organ to store and distribute metabolites to other parts of the organism (Pease, 1976;

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Mayrand *et al.*, 1994). Since HP is actively involved in the metabolic and enzymatic activities, a higher rate of protein turnover is expected (Bayne *et al.*, 1976) and this is reflected in our results that HP tissues have a significantly higher RNA/DNA ratio over other tissue types in most of the cases.

As mentioned above, a high RNA/DNA ratio in foot muscles of *P. viridis* is probably related to the secretion of proteinaceous byssal threads (Coan *et al.*, 2000). Under a desirable environmental condition, mussels are more likely to attach on the substratum by pressing foot muscles firmly on the surface and secreting adhesive protein for byssus production (Nishida *et al.*, 2003) and lead to elevated RNA/DNA ratio. Given that there was high variation (shown by the coefficient of variation) in the RNA/DNA ratios in foot muscle and no significant correlation between the ratio in this tissue and all of the nutrient-related environmental parameters, the RNA/DNA ratio of foot muscle may be less directly related to the energy acquisition or nutritional associated factors. Wang *et al.* (2010) proposed that byssus thread production has to compete with other physiological processes for energy which may reduce the tolerance of the mussels to stress, and consequently inhibit the chance of their survival. However, the present laboratory study was a short-term experiment in which survival was not threatened by energy depletion. It would be worthwhile and interesting to further investigate the potential energetic trade-off between byssus production and survival under prolonged starvation when growth becomes negative.

CONCLUSION

To summarize, the RNA/DNA ratio in the mussel *Perna viridis*, particularly in HP tissues, is sensitive to nutritional-related stresses. Spatial or temporal comparison of the ratio between different groups of *P. viridis* should be carried out by selecting the appropriate types of tissues and to a lesser extent a confined size-class for analysis. Although RNA/DNA ratios of AD and gill tissues were relatively less sensitive to short-term changes in food availability in the laboratory experiment, their ratios in field collected mussels displayed a clear spatial variation pattern corroborated by the difference in nutrient-related hydrographical condition in a more long-term time scale. Given that considerable differences in the RNA/DNA ratio were found between tissue types, conversion factors could also be estimated and adopted to facilitate the comparison between studies using different tissues of the same species for nucleic acid analysis (Olivar *et al.*, 2009). By choosing suitable tissues at a confined size of the same population of *P. viridis* (e.g. 30–40 mm in shell length) with optimum sample size, the RNA/DNA ratio could be a useful biomarker for indicating the cellular growth of the mussels and indirectly reflecting the propensity of primary productivity in the marine ecosystem.

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Correspondence should be addressed to:
K.M.Y. Leung
School of Biological Sciences
University of Hong Kong
Pokfulam, Hong Kong, People's Republic of China
email: kmyleung@hku.hk

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