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Digestive enzyme activities of Angelwing clam (*Pholas orientalis)* exhibit semi-diurnal patterns

Ruby U. Tizon, Augusto E. Serrano Jr., Rex F. M. Traifalgar

Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Philippines. Corresponding author: A. E. Serrano Jr., serrano.gus@gmail.com

Abstract. The study aimed to document the pattern of changes in the digestive enzyme activities of carbohydreases such as α-amylase, CM-cellulase, agarase, laminarinase and in protease. Results showed that the digestive enzyme activities extracted from the crystalline style of *Pholas orientalis* exhibited a semi-diurnal pattern. In a 24-h period, two peaks of digestive activities were generally observed, one at daylight and another at nighttime. Amylase, CM-cellulase, agarase and protease activities showed two peaks at 0800 and 2000 h while laminarinase activity showed peaks at 0800 and 2400 h. This pattern of enzyme activities might be related to their feeding rhythmicity and influenced by tide. It could also suggest that the Angelwing clam probably fed in a semi-diurnal pattern.
Key Words: Angelwing clam, *Pholas orientalis*, semi-diurnal pattern, digestive enzyme activities, amylase, cellulase, agarase, laminarinase, protease.

Introduction. The feeding physiology of cultured bivalves has been widely studied. Physiological indices such as clearance rate, ingestion rate, assimilation efficiency and scope for growth, have been measured in relation to their energetic budget (Aldridge et al 1995; Kuang et al 1996; MacDonald et al 1998). Very little has been done on activities of digestive enzymes as affected by various physiological factors in cultured bivalves. In our previous study, the effects of pH on the in vitro activities of three digestive enzymes namely α-amylase, cellulase and protease in the crystalline style of the angelwing clam, (*Pholas orientalis*) were studied (Tizon et al 2012). Also, the effects of microalgal diets (*Chaetoceros calcitrans, Isochrysis galbana, Thalassiosira* sp., and *Tetraselmis tetrathele*) on the activities of α-amylase, CM-cellulase, agarase, laminarinase and protease were determined (Tizon et al 2012). In order to highlight digestive capacity of the Angelwing clam under different culture circumstance, this study focused particularly on the activities of various digestive enzymes as affected by the diurnal cycle. To our knowledge, there is currently no information on this aspect of the digestive physiology of this species in the scientific literature.

Biological rhythms are of two types: endogenous, an internal physiological element acting like an “alarm clock”, and exogenous, an external synchronizer acting to correct the free-running endogenous element (DeCoursey 1983). The endogenous element is controlled by the biological clock, which in turn is affected by environmental signals, chiefly light. Circadian rhythm as defined by Campbell & Reece (2004) is a physiological cycle of about 24 h that is present in all eukaryotes and persists even in the absence of external stimulus. Cyclical variation of biochemical variables is considered part of an animal’s physiology and the rhythm in digestive enzyme activity exhibited by crustaceans is attributed to the trophic structure of the organism and its internal rhythm (Vega-Villasante et al 1999).

This study aimed to document the changes in various digestive enzyme activities within 24 h whether or not there was diurnal pattern.

Material and method

Changes in digestive enzyme activities within the 24 h period were evaluated. Fifty-four newly harvested adult *P. orientalis* (71.7 ± 7.7 g wet weight, 112.5 ± 3.7 mm standard length, SL) were acclimated to laboratory conditions for 7 d as previously described. On the 8th day, 9 animals were randomly sacrificed every 4 h, constituting 6 sampling points at 0400, 0800, 1200, 1600, 2000, and 2400 h. The crystalline styles were carefully excised and stored in ultra low freezer (-85 °C) until assay. Preparation of enzyme extract and enzyme assays were done as described previously (Tizon et al 2012).

Acclimation. All the Angelwing clams used in this study were collected from the municipal water of Brgy. Pta. Cogon, Roxas City, Philippines. The newly harvested clams were carefully packed into a styrofoam box in which sea water was added to the box enough to keep the siphon of the clams moist. Upon arrival to the university hatchery, the bivalves were cleaned of adhering mud, weighed and arranged vertically in plastic trays inside the 80 L glass aquaria. The clams were acclimated for 7 days at ambient temperature and were fed with a mixture of equal proportion of microalgae *Chaetoceros calcitrans*, *Isochrysis galbana*, *Thalassiosira* sp. and *Tetraselmis tetrathele*. They were fed continuously at approximately 2.94 x 108 cells broodstock-1 day-1 as described by Marasigan & Laureta (2001). Water was supplied via a flow-through system at a rate of approximately 110 mL min-1 and aeration was provided continuously.

Preparation of enzyme extracts. Crystalline styles were excised, weighed and stored in ultra low freezer (-85 °C) until assay. All preparation procedures were done at 4 °C unless otherwise stated. During enzyme extraction, the crystalline styles were thawed, weighed and washed with cold citrate phosphate buffer, pH 7.0. To the style was added extraction buffer at 1:30 w/v, homogenized in an Ultraturrax homogenizer, and centrifuged at 4000 rpm for 15 min. The supernatant was filtered and used as enzyme extract for the assays.

Enzyme assays. The digestive enzymes α-amylase, cellulase, agarase, laminarinase and protease activities were assayed at 25°C. All measurements were done in triplicates, with corresponding blank and control samples.

Carbohydrases. Alpha amylase, CM-cellulase, agarase and laminarinase activities were measured following the method of Areekijseree et al (2004) modified from Bernfield (1955). For amylase, the reaction mixture consisted of 0.2 mL enzyme extract, 1.8 mL phosphate and 1 mL of substrate (1.0% soluble starch dissolved in buffer) in a final volume of 2.0 mL. The reaction was stopped by adding 1.0 mL 3,5-dinitrosalicylic acid (DNS) solution after 15 min of the reaction. The solution was placed in boiling water bath for 10 min until the color of the solution turned from yellow to dark red and was allowed to cool to room temperature. The optical density (OD) of the clear solution was read at 546 nm. Mixtures with no substrate or no enzyme or both were used as blank samples for correction of innate activity in the crude extract and for the exclusion of spontaneous hydrolysis of the substrate, respectively.

Similar procedure was employed in the assays of CM-cellulase, agarase and laminarinase varying only in the substrate used. CMC, agarose, and laminarin (Sigma L-9634) were dissolved in corresponding buffers and used as substrate for CM-cellulase, agarase and laminarinase assays, respectively. The reaction mixture for the CM-cellulase assay consisted of 0.3 mL enzyme extract, 1.0 mL 0.25% CMC and 1.7 mL citrate-phosphate buffer, pH 6.0, in a final volume of 3.0 mL. That for agarase consisted of 0.1 mL enzyme extract, 1.0 mL agarose substrate (0.2%) and 1.9 mL citrate-phosphate buffer, pH 6.0, in a final volume of 3.0 mL. The reaction mixture for the laminarinase was 0.3 mL enzyme extract, 1.0 mL 0.1% laminarin, 1.7 mL of citrate-phosphate buffer, pH 6.0, in a final volume of 3.0 ml. The reactions were stopped after 15 and 30 min for CM-cellulase and agarase, and 30 min for laminarinase, respectively.

All carbohydrase activities were quantified using glucose as standard except for agarase activity in which galactose was used as standard. Protein was determined following the procedure of Bradford (1976) using bovine serum albumin as standard. Alpha amylase, CM-cellulase and laminarinase activities were expressed as µmol glucose liberated min^{-1} mg^{-1} protein while agarase activity was expressed as µmol galactose liberated min^{-1} mg^{-1} protein.

Protease. Proteolytic activity was measured following the method of Kunitz (1947) with some modifications. The reaction mixture consisted of 1.0 mL 1.0% casein dissolved in 0.01N NaOH, 1.5 mL phosphate buffer, pH 7.0 and 0.5 mL enzyme extract, in a final volume of 3.0 mL. After 60 min, the reaction was stopped by adding 1.0 mL ice-cold 5% trichloroacetic acid (TCA), allowed to stand for 15 min, centrifuged and filtered. The optical density of the clear supernatant was read at 280 nm. Mixtures with no substrate or no enzyme or both were used as blank samples. Tyrosine was used as standard for the expression of enzyme activity as µg tyrosine released hr^{-1} mg^{-1} protein.

Results and Discussion. Generally, the digestive enzyme activities extracted from the crystalline style of *P. orientalis* exhibited a semi-diurnal pattern (Figures 1 - 5). In a 24-h period, two peaks of digestive activities were generally observed, one at daylight and another at nighttime. Amylase, CM-cellulase, agarase and protease activities all showed two peaks at 0800 and 2000 h while laminarinase activity showed peaks at 0800 and 2400 h.

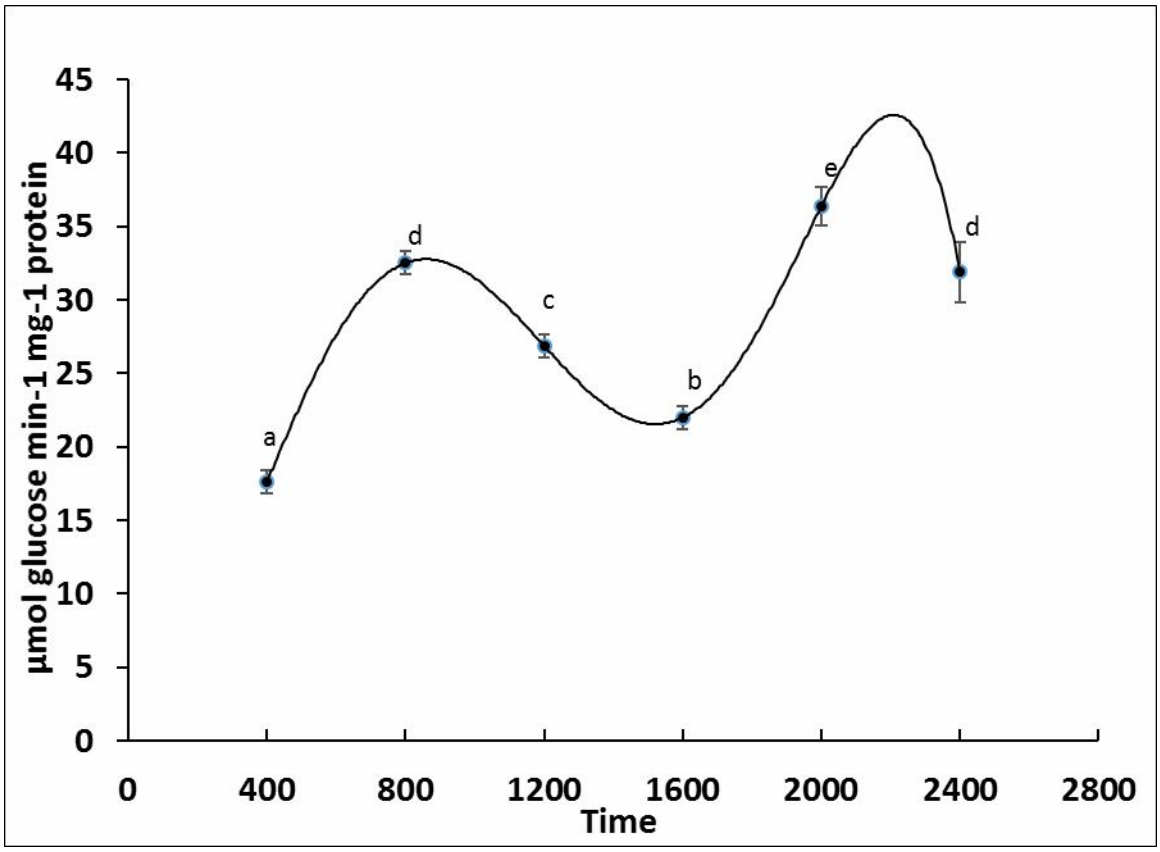

Figure 1. 24-h pattern of α-amylase activity. The assay mixture contained 0.2 mL enzyme extract, 1.8 mL buffer (pH 7.0) and 1.0 mL 1.0% soluble starch (w/v) in a final volume of 3.0 mL. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test).

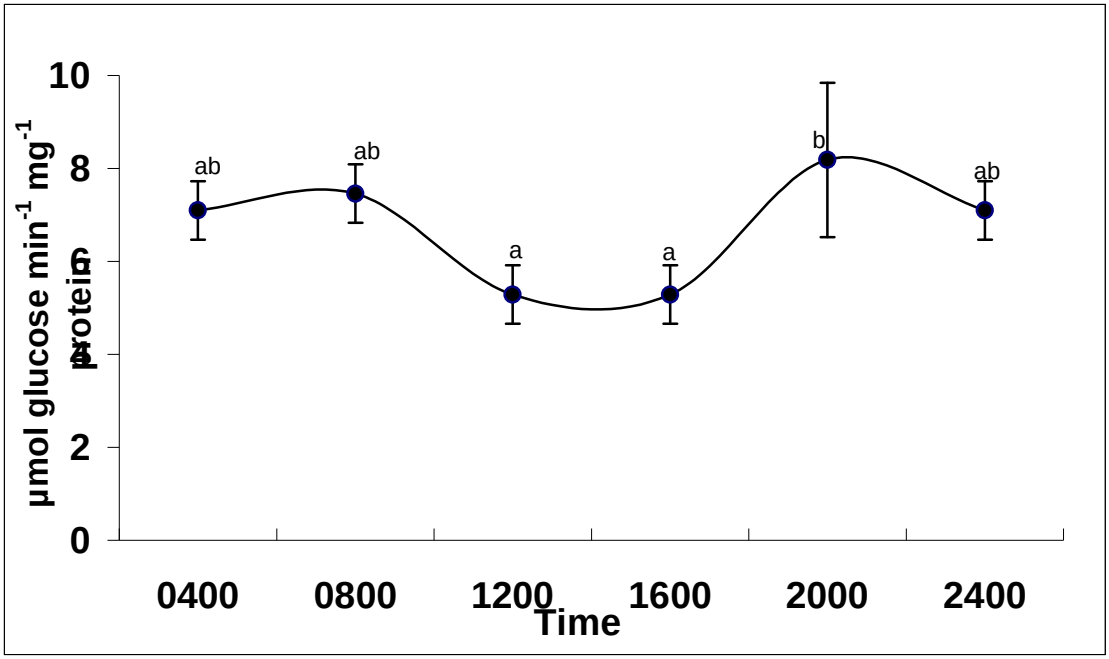

Figure 2. 24-h pattern of CM-cellulase activity. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.25% CMC (w/v) in a final volume of 3.0 mL. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test).

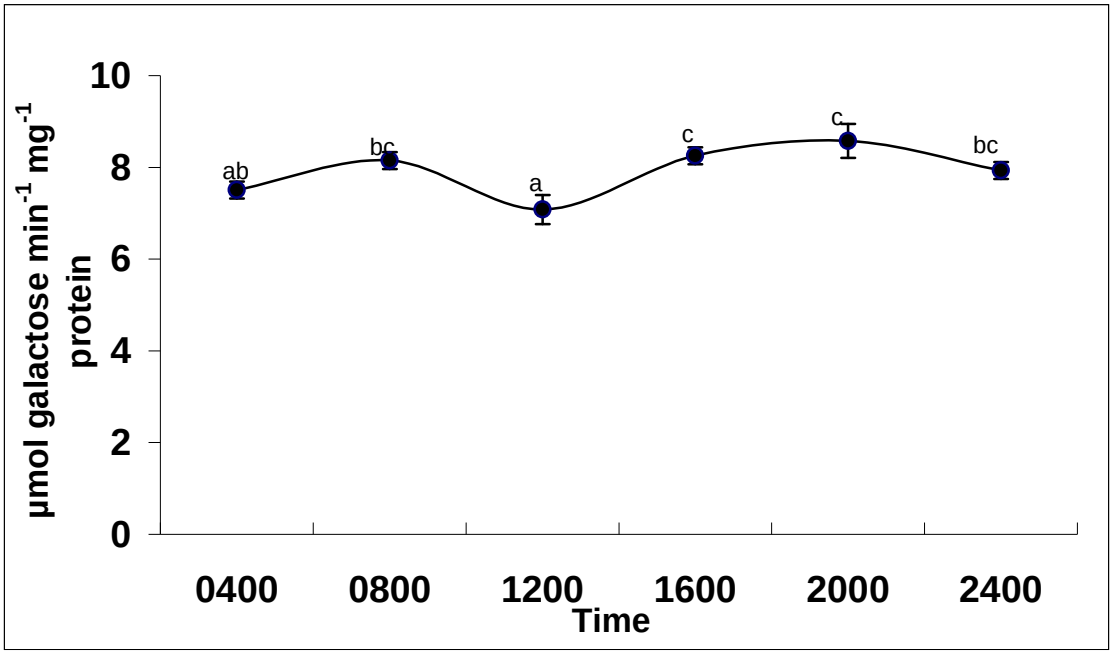

Figure 3. 24-h pattern of agarase activity. The assay mixture contained 0.1 mL enzyme extract, 1.9 mL buffer (pH 6) and 1.0 mL 0.2% agarose (w/v) in a final volume of 3.0 mL. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test).

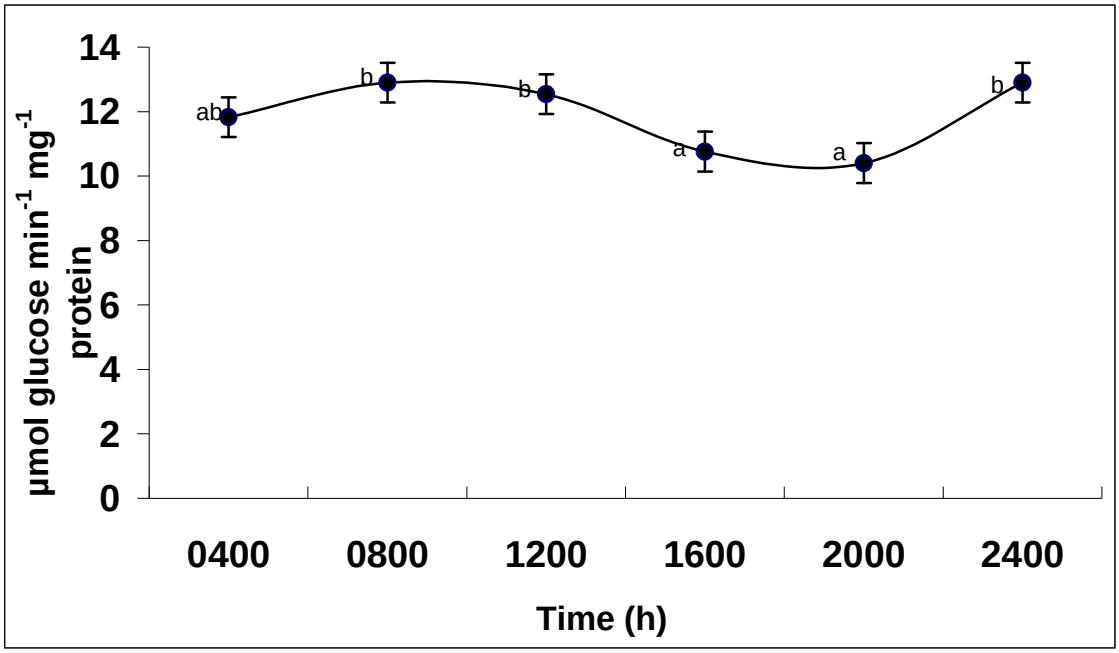

Figure 4. 24-h pattern of laminarinase activity. The assay mixture contained 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.1% laminarin (w/v) in a final volume of 3.0 mL. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test).

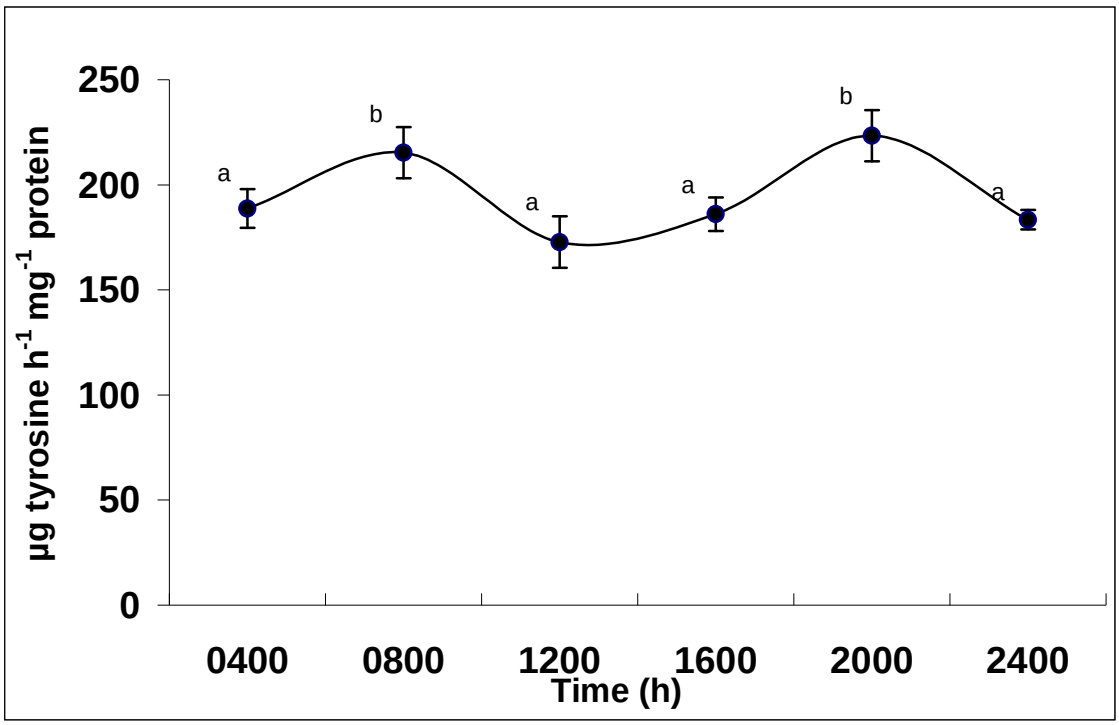

Figure 5. 24-h pattern of protease activity. The assay mixture containing 0.5 mL enzyme extract, 1.5 mL buffer (pH 7.0) and 1.0 mL 1.0% casein (w/v) in a final volume of 3.0 mL. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments ($P < 0.05$, Tukey Test).

Like the terrestrial organisms, aquatic animals including bivalves have evolved to possess the capability to perceive and transmit environmental cues like tidal changes, cycles of day and night, photoperiod, temperature, presence of food, etc., and synchronize these signals with its physiological processes. In bivalves, two major environmental cues including the tide and light-dark cycles have been identified to influence the circadian rhythm (Langton 1977). These environmental cues have been shown to affect the pattern of feeding and digestion in bivalves. The endogenous circadian oscillation is not exactly 24 h and should be reset each day to light-dark cycle to synchronize it with the natural environment and thus prevent it from drifting or free-running (Froy & Miskin 2010). The semi-diurnal patterns exhibited by the digestive enzyme activities in the present study might be related to the feeding rhythmicity of this species, suggesting in part that the Angelwing clams probably fed also in a semi-diurnal pattern.

In fish, it was shown that higher amount of melatonin is secreted during the night than at day time (Maitra et al 2006). At present, the chemical signal influencing enzyme activity in *P. orientalis* is not yet elucidated. It is previously reported that the pattern of circadian rhythmicity in invertebrates including the mollusk *Aplysia californica* (Abran et al 1994) and crayfish (*Procambarus clarkia*) (Solis-Chagoyan et al 2008) are also signaled by the hormone melatonin. Further, in *P. orientalis*, a clear rhythmic pattern was observed in its α-amylase activity. Well-defined peaks of this enzyme was observed at 0800 h and another peak at 2000 h. Of the two peaks of α-amylase activities, the activity was higher at nighttime peak than that of the daytime. Similarly, although the values were not significantly different, similar trends were observed in CM-cellulase, agarase and protease activities, wherein enzymes activities at night were higher than those recorded at daytime.

Bivalves have been found to exhibit rhythmicity in feeding, digestion, absorption, pH, oxygen consumption and protein content in relation to external factors like tide, photoperiod, and season (Hawkins et al 1983; Ibarrola et al 1998; Kim et al 1999; Langton 1977). Bivalve feeding has long been believed to be continuous, that is the animal feed most of the time (Loosanoff & Nomejko 1946) but tidal rhythmicity has been observed in *Cardium edule* (Morton 1970) and *Pecten maximus* (Mathers 1976), oysters *Crassostrea gigas* (Morton 1977) and *Crassostrea virginica* (Palmer 1980), as well as in venerid clams (*Meretrix meretrix*) and *K. opima* (Hameed & Paulpandian 1987). Similarly, fish and crustacean also exhibit rhythmicity in feeding.

There is scarcity of reports on the changes within the 24-h period of digestive enzyme activities in bivalves. In the cockle *Cerastodermia edule*, the digestive amylase, cellulase and laminarinase have been found to change with season (Ibarrola et al 1998). Tidal rhythmicity is reported in intertidal mussel (*Mytilus edulis*) having a higher digestive gland amylolytic activity when submerged in seawater during high tide, than when exposed to air at low tide (Langton 1977). In the present study, the tides were highest at 0621 h and 1930 h. At least one of the peaks (at 2000 h) of digestive activity for amylase, CM-cellulase, agarase and protease almost coincided with the time of high tide. Similarly, at least one of the two periods of lower activities (at 1200 h) of CM-cellulase, agarase and protease was recorded during the low tides which happened at 0043 h. The results of the present study indicated that the pattern of digestive activities observed in *P. orientalis* might be influenced by tide.

The pattern of digestive enzyme activities noted in the present study was similar to those of crustaceans. Cuzon et al (1982) have shown that the digestive enzyme activity of juvenile *Penaeus japonicus* reared in the laboratory follows a rhythmic cycle with two peaks happening at 0700 h and 2000-2200 h. Similarly, in intermolting crab (*Callinectes arcuatus*) peaks of higher amylase and protease activities are detected in daylight and in the evening (Vega-Villasante et al 1999). In addition, when the digestive enzyme activity of juvenile *Farfantepenaeus paulensis* shrimp is assayed on a 2-h interval, Aquilar-Quaresma & Sugai (2005) report two peaks for amylase and maltase activities both occur at 0100 h and between 1700-1900 h. The shrimps in their study have been acclimatized to laboratory conditions, (similar to the present study) and maximum digestive enzyme activities are positively correlated with the peaks of shrimp's

ingestion rate. In another experiment where juvenile *Penaeus vannamei* is exposed to continual feeding, the shrimp's total protease and trypsin activities are at 1800 h, just before the highest ingestion rate is noted, suggesting close association between feeding behavior and ingestion (Hernandez-Cortes et al 1999). The same has been observed in bivalves. Higher activities of cellulase, laminarinase and protease have been reported when *Cardium edule* cockles are given greater food quality and quantity (Ibarrola et al 2000).

The results of the present study indicated the mode of feeding of the Angelwing clam which was reflected from the semi-diurnal pattern of the enzyme activity. Enzyme secretions are known to be activated by the presence of food that act as substrate, so it is likely that feeding activity of *P. orientalis* resulted in elevated activities. The feed intake of *P. orientalis* in relation to time was not measured in the present study. Also, valuable information could have been gathered having the relationship between feed intake and diel enzyme activities, which will be further elucidated.

Conclusions. Results showed that the digestive enzyme activities extracted from the crystalline style of *P. orientalis* exhibited a semi-diurnal pattern. In a 24-h period, two peaks of digestive activities were generally observed, one at daylight and another at nighttime. Amylase, CM-cellulase, agarase and protease activities showed two peaks at 0800 and 2000 h while laminarinase activity showed peaks at 0800 and 2400 h.

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Authors:
Ruby Ursula Tizon, Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Philippines, Miagao, 5023 Iloilo, e-mail: rbytzn@yahoo.com
Augusto Erum Serrano Jr., Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Philippines, Miagao, 5023 Iloilo, e-mail: serrano.gus@gmail.com
Rex Ferdinand Mallare Traifalgar, Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Philippines, Miagao, 5023 Iloilo, e-mail: skerferd@yahoo.com

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