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## Influence of photoperiod on digestive enzyme activities of the Angelwing clam (*Pholas orientalis*)

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**Abstract**. Thirty adult Angelwing clams, *Pholas orientalis*, (89.78  $\pm$  6.60 g wet weight; 118.13  $\pm$  2.92 mm standard length) were acclimated in the laboratory for 7 d, randomly segregated into 3 groups which were exposed to different photoperiod regimes (24 h light, 12 h light - 12 h dark, 24 h dark). After 7 d, the clams were sacrificed and their crystalline styles excised and the digestive enzymes a-amylase, CM-cellulase, agarase, laminarinase and protease were assayed. All the digestive enzymes, amylase, cellulase, agarase, laminarinase and protease exhibited significantly the highest activities when the clams were exposed to 24 h darkness for 7 d and those exposed to continuous artificial light or to equal light and dark hours exhibited lower activities but were not significantly different from each other. **Key Words**: Photoperiod, *Pholas orientalis*, digestive enzyme activities, alpha amylase, cellulase, agarase, laminarinase.

**Introduction**. Differences in day lengths or photoperiods have been used by many marine invertebrates as an external factor to start biological and physiological processes (DeCoursey 1983). Photoperiod can regulate reproduction and growth of marine species. Manipulation of light-dark cycles has been shown to increase the growth of fish (Stefansson et al 1989) and shrimp (Withyachumnarnkul et al 1990).

In fish, photoperiod is a factor triggering melatonin production that regulates secretions of releasing hormones in the hypothalamus, which in turn control the production of hormones in the pituitary, eventually leading to gonadal maturation (Devauchelle & Mingant 1991; Maitra et al 2006). Melatonin, the endocrine signal of photoperiod, is produced by the pineal gland and retina of the eye at significantly higher amounts during the night than at daytime (Maitra et al 2006). Its presence has been detected in marine invertebrates like freshwater prawn Macrobrachium rosenbergii (Withyachumnarnkul et al 1992), giant tiger shrimp Penaeus monodon (Withyachumnarnkul et al 1995), sea hare Aplysia californica (Abran et al 1994), fiddler crab Uca pugilator (Tilden et al 2001), snail Helix aspersa (Blanc et al 2003), and rotifer Philodina sp. (Hardeland & Poeggeler 2003). Melatonin is reported to translate environmental signals like photoperiod into rhythmic messages in fish (Maitra et al 2006). Although the chemical signal regulating enzyme activity has not been elucidated yet in bivalves, melatonin has been shown to modulate the circadian rhythm in invertebrates like crayfish Procambarus clarkii (Solis-Chagoyan et al 2008).

There is scarcity of reports elucidating the influence of photoperiod on the digestive capacity of bivalve species. Expression of photoperiod phenomena is possible in animals possessing some sort of a photoreceptor (Cronin 1986). Hecht (1927) has reported that the photoreceptors in *Pholas* are located in the siphon and exposed parts of the mantle. Bivalves respond to light stimulation. The shells of *Mytilus edulis* grown in darkness are thin and brittle (Stromgren 1976b), a characteristic of *Pholas orientalis*. The same author has demonstrated that when *M. edulis* are grown in the dark, the clam has lighter pigmentation, again similar to Angelwing clams. Furthermore, Hecht (1927) has

demonstrated that the siphon of *Pholas dactylus* is very sensitive to light. Similarly, Corda et al (1998) have shown that direct light exposure causes shortening of P. orientalis siphon, resulting to lower filtration rate. The decrease in the enzyme activity observed in the present study among bivalves exposed to continuous 24 h light was in agreement with these findings. The decrease may be related to the depressed ability of the animal to feed and since enzyme activity is considered to be related to availability of food substrate in the gut. Continued darkness results in a higher shell length growth in mussel *M. edulis* which is correlated with higher defecation rate than in natural daylight and continuous light (Nielsen & Stromgren 1985). Moreover, the growth rate of *M. edulis* (Seed 1969; Stromgren 1976a, 1976c), Modiolus modiolus (Stromgren 1976b) and snail Heliosoma duryi (Kunigelis & Saleuddin 1978) are significantly improved when the animals are kept in dark environment. Nielsen & Stromgren (1985) have suggested that light affects the bivalve's filtering capacity, leading to reduced shell formation since calcification is dependent upon filtration rate and food intake. Moreover, Nielsen & Stromgren (1985) propose that the bivalves showing positive correlation with darkness (M. edulis and M. modiolus) are pigmented and epifaunal while light-insensitive clams like Cerastoderma edule are unpigmented and sand burrower. In contrast, several authors have reported beneficial effects of lighted environment. Increasing light exposure to 15 h d<sup>-1</sup> resulted in a higher percentage of spawning in *Pecten maximus* scallop (Devauchelle & Mingant 1991). Sick et al (1973) report that ingestion rate of pelleted food by juvenile shrimp *Penaeus setiferus* is directly proportional with light intensity.

This study aimed to measure the influence of photoperiod on the Angelwing clams in laboratory condition on the activities of digestive enzymes namely a-amylase, CM-cellulase, agarase, laminarinase and protease.

## Material and method

**Experimental set up**. Thirty adult Angelwing clams, *P. orientalis*, (89.78  $\pm$  6.60 g wet weight; 118.13  $\pm$  2.92 mm standard length) were acclimated in the laboratory for 7 d, randomly segregated into 3 groups and each group exposed to different photoperiod regimes (24h light, 12h light - 12h dark, 24h dark). Angelwing clams were placed in aquaria and fed equal proportions of the four algal diets (*Chaetoceros calcitrans, Isochrysis galbana, Thalassiosira* sp., and *Tetraselmis tetrathele*). Those exposed to 24 h-light treatment were provided with 40 W fluorescent lamp (5.7 Klux), while aquaria of those in 24 h dark treatment was totally covered with black cloth. Those in the 12 h light - 12h dark were exposed to 12 h with 40 W fluorescent lamp and 12 h totally covered with black cloth. After 7 d treatment, the clams were excised, the crystalline styles removed and stored in ultra low freezer (-85°C) until assay.

**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 a-amylase, cellulase, agarase, laminarinase and protease activities were assayed at 25 °C. All measurements were done in triplicates, with corresponding blank and control samples.

<u>Carbohydrases.</u> 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 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 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  $\mu$ mol glucose liberated min<sup>-1</sup> mg<sup>-1</sup> protein while agarase activity was expressed as  $\mu$ mol galactose liberated min<sup>-1</sup> mg<sup>-1</sup> protein.

<u>Protease</u>. 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 0.01 N 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, 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  $\mu$ g tyrosine released hr<sup>-1</sup> mg<sup>-1</sup> protein.

**Results and Discussion**. All the digestive enzymes, amylase, cellulase, agarase, laminarinase and protease exhibited significantly the highest activities when the clams were exposed to 24 h darkness for 7 d and those exposed to continuous artificial light or to equal light and dark hours exhibited lower activities but were not significantly different from each other (Figures 1-5).

Although the Angelwing clam is a mud burrower and has minimal pigmentation, digestive capacity was enhanced in dark environment probably because it resembled the clam's natural setting being found in subtidal areas of about 8 m from the water surface (Laureta & Marasigan 2000). In addition, it burrows itself into muddy substrate at a depth of 0.3-0.6 m with only its siphon sticking out of the substrate to feed on suspended particles. According to Loosanoff & Nomejko (1946), natural oyster beds are situated at a considerable depth (10-30 ft, a depth comparable to that of P. orientalis bed) with suspended matter blocking the sun rays. Thus, oyster exists in near-darkness even during very strong daylight, a condition not so dissimilar to the dark regime in the present study, and the natural setting of *P. orientalis* beds. Another possible reason for increased enzyme activity at dark environment is the nightly vertical migration of phototactic algae in the natural environment. Phytoplankton are concentrated on the water surface at daylight, but migrate to deeper areas at night time (Staker & Bruno 1980), making the algae available for the filter feeding bivalves. Thus, the bivalves might have been accustomed to feeding at dark times, triggering the significant increase of digestive enzyme activities in the dark photoperiod regime.

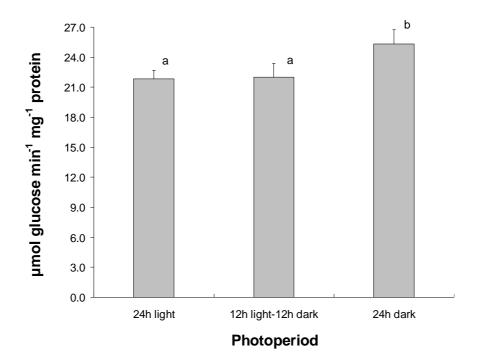


Figure 1. Alpha amylase activities of clams exposed to different photoperiods. The assay mixture containing 0.2 mL enzyme extract, 1.8 mL buffer (pH 7.0) and 1.0 mL 1.0 % soluble starch (w/v) was incubated for 15 min. Bars represent mean  $\pm$  standard deviation; means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

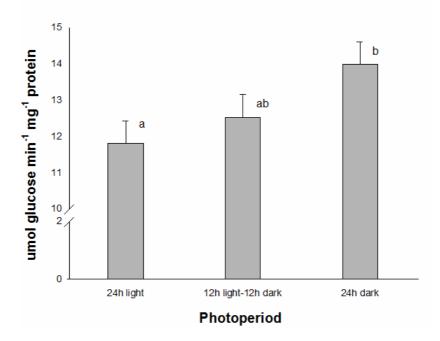


Figure 2. CM-cellulase activities of clams exposed to different photoperiods. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.25 % CMC (w/v) was incubated for 15 min. Bars represent mean  $\pm$  standard deviation; means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

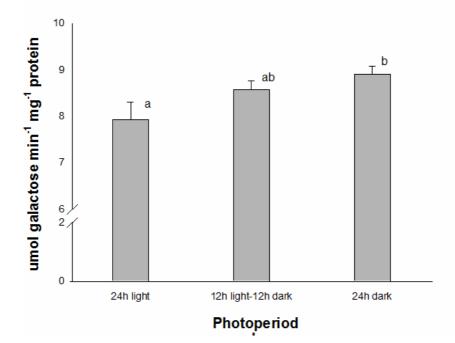


Figure 3. Agarase activities of clams exposed to different photoperiods. The assay mixture containing 0.1 mL enzyme extract, 1.9 mL buffer (pH 6) and 1.0 mL 0.2 % agarose (w/v) was incubated for 15 min. Bars represent mean  $\pm$  standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

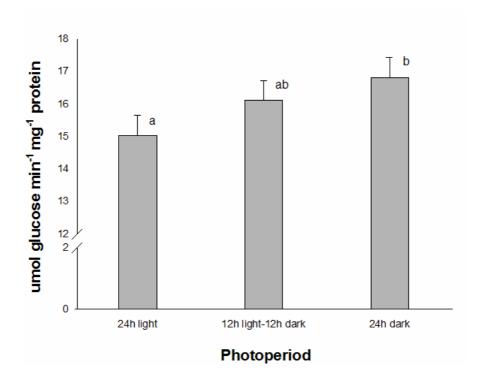


Figure 4. Laminarinase activities of clams exposed to different photoperiods. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.1 % laminarin (w/v) was incubated for 30 min. Bars represent mean  $\pm$  standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

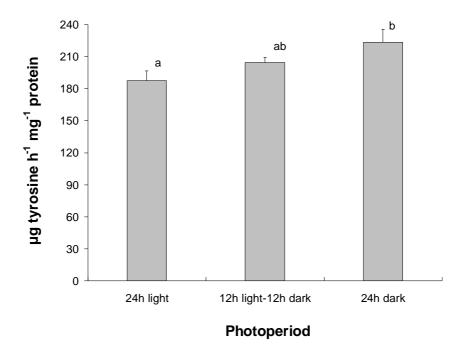


Figure 5. Protease activities of clams exposed to different photoperiods. The assay mixture containing 0.5 mL enzyme extract, 1.5 mL buffer (pH 7.0) and 1.0 mL 1.0 % casein (w/v) was incubated for 1 h. Bars represent mean  $\pm$  standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

The natural feeding activity of *P. orientalis* in the wild has not been elucidated to date. The present results showed higher enzymatic activity at low light setting, indicating that the clam exhibited a heightened feeding activity at low-light conditions. The present findings agreed with previous works in other bivalve species, *M. edulis* (Stromgren 1976a, 1976c) and *M. modiolus* (Stromgren 1976c) elucidating that exposure to low light levels enhances growth and metabolic activities. Although these earlier reports have shown the positive effects of low light exposure to growth of bivalves, the physiological mechanism involved in such effects remains unclear. Nielsen & Stromgren (1985) have demonstrated that the improvement of *M. edulis* growth due to continuous low light exposure is attributed to higher feed intake and to higher defecation rate, indicating a heightened feeding activity. Similarly, Hecht (1927) has shown that the siphon of Pholas dactylus is extremely sensitive to light. In addition, Corda et al (1998) have demonstrated that the filtration rate of *P. orientalis* is influenced by light exposure. Angelwing clams exposed to continual light exhibited a decreased filtration rate. The present study agreed with these earlier reports elucidating the enhanced digestive activities of clams maintained in dark environment. The present work was the first evidence indicating that digestive enzyme activity in a bivalve species can be influenced by light exposure. Moreover, it could provide additional evidence on previous works, showing faster growth rates in bivalves maintained in low lighted environment.

**Conclusions**. All the digestive enzymes, namely, a-amylase, CM-cellulase, agarase, laminarinase and protease exhibited significantly the highest activities when the clams were exposed to 24 h darkness for 7 d and those exposed to continuous artificial light or to equal light and dark hours exhibited lower activities and were not significantly different from each other.

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