Biota Vol. 14 (3): 156-165, Oktober 2009
ISSN 0853-8670

Seasonal Activity of Metabolic Enzymes of *Littorina littorea* (Gastropoda: Mollusca)

Aktivitas Musiman Enzim Metabolisme dari *Littorina littorea* (Gastropoda: Moluska)

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Abstrak

Aktivitas musiman enzim pyruvate kinase (PK), dan citrate synthase (CS) pada otot kaki siput, *Littorina littorea* (Gastropoda, Littorinidae) telah diteliti. Enzim-enzim ini berperan sebagai enzim kunci pada rangkaian metabolisme (*metabolic pathways*). Perbedaan yang signifikan terlihat pada aktivitas adalah CS dan PK sepanjang musim. Aktivitas spesifik dari CS bervariasi antara 0,04 dan 0,4 µg berat basah dan PK antara 0,1 dan 1,7 µg berat basah. Aktivitas enzim tersebut lebih tinggi pada musim dingin dibandingkan dengan musim panas. Hubungan negatif antara suhu dan aktivitas enzim terdeteksi dalam penelitian ini. Terdapat perbedaan yang nyata antara suhu air dan aktivitas enzim CS, dan suhu air dan udara pada enzim PK ($P<0.05$). Teramati tendensi yang sama untuk prosentasi perubahan pada aktivitas enzim PK dan CS pada bulan pertama pengamatan dan bulan berikutnya. Hasil penelitian setuju dengan hipotesis yang mengatakan bahwa terdapat kemungkinan perubahan aktivitas enzim CS dan PK selama siklus musiman. Fenomena ini dibahas dalam pembahasan tulisan ini.

Kata kunci: enzim (CS, PK), metabolisme, aktivitas musiman, suhu, *Littorina littorea*

Diterima: 12 Mei 2009, disetujui: 31 Juli 2009

Introduction

Enzyme is used to asses metabolic rates, or responses to environmental changes (e.g. Childress and Somero, 1990; Simpfendörfer *et al*., 1995; Salomon and Buchholz, 2000). Metabolic temperature adaptation can be achieved by ways, namely, the organism can either reduce metabolism to save energy in the cold or metabolic activity is increased at low temperatures, compensating for rate limiting effects and maintaining metabolism at almost constant level (Vetter and Buchholz, 1998). Irrespectiveness of the adaptive mechanism, enzymes is always involved in the biochemical adaptation (Vetter and Buchholz, 1998; Mangunwardoyo *et al*., 2007).

Snails need to acclimate or adapt to changes in the environment. One way of this adaptation is to change the activity and/or properties of metabolic enzymes. Activities of metabolic enzymes in turn may be considered as indicators of metabolic adaptability. The use of enzyme to asses metabolic rates, or responses to environmental changes by different animals has been previously studied (e.g. Childress and Somero, 1990; Simpfendörfer *et al*., 1995; Dahlhoff and Menge, 1996; Greenway and Storey, 1999; Salomon and Buchholz, 2000; Saborowski and Buchholz, 2000; Sokolova and Pörtner, 2001). Generally, enzymes such as pyruvate kinase (PK) and citrate synthase (CS) are widely studied, since these enzymes are key enzymes in metabolic pathways. PK is a regulatory enzyme of the glycolytic pathway and involved in ATP production (Buchholz and Saborowski, 2000), while CS is a key regulatory enzyme in

the citric acid cycle and can give a quantitative estimate of aerobic capacity of an organism (Somero and Childress, 1980). Buchholz and Saborowski (2000) noted that CS is an enzyme instrumental in energy metabolisms that has a control function in the citric acid cycle. Accordingly, the present study was conducted since there was no information about the activities of these enzymes in the periwinkle, *L. littorea* in relation with environmental factor.

In the present study, the seasonal activity of metabolic enzymes (CS dan PK) of foot muscles of *L. littorea* was studied. The study was aimed to test the hypothesis that there were possible changes in these enzyme activities of the foot muscle of the intertidal snail, *L. littorea* during seasonal cycles.

Materials and Methods

Enzyme Activity Determination

Sample

The snails were collected alive from the pier of Helgoland Island, Germany. The collection was done once a month during springtide started from May 2000 to April 2001. Samples were then brought to the laboratory for dissection. The animals were placed in the aquaria without water. During dissection procedure, the animals which were not yet dissected were acclimatized to the respective field temperature at the time of collection to minimize variation. This was done by opening the windows or door in the culture hall. The dissection was done on ice. The foot muscle was cut, and the operculum was discarded. The muscle was then weighted, and put in Eppendorf tube. The tube was then thawed on ice. The rest of the body was placed on the plastic plate to examine for sex and parasite infestation. Sometimes snail infested could be recognized from the colour of hepatopancreas. This snail was immediately discarded. After dissecting 3 animals, the foot muscles were shortly stored at –80°C until analysis. The process from sampling to storage was done within two hours for at least 20 uninfested snails. This duration of time was taken into consideration in order to avoid denaturation of the tissue.

Tissue Preparation

Six to ten specimens representing monthly samples with a weight range of 50-100 mg were taken from –80°C cool storage and thawed on ice. Each specimen was homogenized with an Ultra-Turrax (Janke and Kunkel, Staufen, Germany) in 1 ml ice-cold extraction Tris/HCl-buffer (50 mmol l^{-1}, pH 7.5). During this process, the sample was maintained cooled in an ice to avoid denaturation due to temperature increase. The resulting homogenate was centrifuged at 13,000 g (4°C) for 15 min. The supernatant was used for determination of citrate synthase and also pyruvate kinase.

Activity Determination

Citrate Synthase (CS, E.C. 4.1.3.7)

CS [citrate oxaloacetic-lyase (pro-3S-CH2COO → acetyl-CoA)] as condensing enzyme catalyses the reaction by which acetyl-CoA condenses with oxaloacetate and enters the Kreb cycle, and is located in the matrix of the mitochondria. Standard assays for CS determination were run as follows: 520 µl of buffer (50 mmol l^{-1}, pH 7.5, supplemented with 100 mmol l^{-1} KCl and 1 mmol l^{-1} EDTA, adjusted to pH 7.5), 20 µl DTNBA (5.025 mmol l^{-1} buffer), 20 µl Acetyl-CoA (2.5 mmol l^{-1} A.dest.) and 20 µl sample (supernatant). After 5 min of pre-incubation the reaction was initiated by addition of 20 µl oxaloacetate (5.0 mmol l^{-1} A.dest.). The changes of absorbance were recorded continuously at 410 nm in a Lambda 2 photometer, which was equipped with a thermostated cuvette holder controlled by a peltier temperature programmer (both Perkin Elmer, Überlingen, Germany). The specific activity (related to wet mass) was determined at 25°C.

Pyruvate Kinase (PK, E.C. 2.7.1.40)

PK [ATP: pyruvate 2-O-phosphotransferase] catalysis the conversion of phosphoenolpyruvate (PEP) to pyruvate with regeneration of ATP.

$$\text{(a) Phosphoenolpyruvat + ADP} \xrightarrow[Mg^{2+},\ K^{+}]{\text{Pyruvat-Kinase}} \text{Pyruvat + ATP}$$

$$\text{(b) Pyruvate + NADH + H}^{+} \overset{\text{LDH}}{\rightleftharpoons} \text{L-Lactate + NAD}^{+}$$

The equilibrium (a) lies on the side of pyruvate and ATP with an equilibrium constant $K=6x10^3$. Pyruvate is rapidly removed in the indicator reaction (b). The amount of PEP converted to pyruvate per unit time, as determined by the decrease of absorbance due to oxidation of NADH, is a measure of the PK activity.

In this study, PK was measured via the coupled reaction with LDH. Standard assays contained 500 µl buffer (50 mmol l^{-1} Tris/HCl, supplemented with 60 mmol l^{-1} KCl and 4 mmol l^{-1} $MgSO_4$, adjusted to pH 7.5), 20 µl NADH (7 mmol l^{-1} A.dest.), 20 µl PEP (16 mmol l^{-1}), 10 units LDH and 20 µl of sample. After 5 min of pre-incubation the reaction was started with addition of 20 µl ADP (17 mmol l^{-1} A.dest.) and monitored at 340 nm at constant temperature.

Data Analysis

Enzyme activities were normalized to the fresh weight (FW) of the tissue sample and expressed as μg^{-1} FW. Data were presented as means ± standard deviation. The relation between specific activity and weight was expressed by linear regression.

The level of significance is labeled by asterisk and the probability levels are as follows:

* $0.05 \geq p \geq 0.02$

** $0.02 \geq p \geq 0.002$

By $p > 0.05$ means that the relation is not significant (ns)

Results and Discussion

Allometric Relation

In order to study to what extent the enzyme activities depend on the weight of the animals' tissue, the specific activities of the enzymes were plotted against the weight of snail foot muscle, and this relationship was tested by linear regression analysis. To determine the allometric relation was important in order to be able to decide which range of weight of the animals could be used for determination of enzyme activity. In the present study the range of the foot muscle weight of *L. littorea* used for seasonal study was 50 – 100 mg wet weight (Fig. 1).

Citrate Synthase

The relationship between CS activity and weight of animal's tissue is shown in Fig. 1. The statistical analysis showed that this relationship was significant ($p<0.0001$), with a correlation coefficient of 35%. CS-activities ranged between 0.07 U g^{-1} fresh wt and 2.47 U g^{-1} fresh wt while sample size (foot muscle) was between 35 mg and 208 mg.

Pyruvate Kinase

The relationship between PK activity and weight of snail foot muscle is shown in Fig. 2. The statistical analysis showed that the relationship was not significant ($p=0.27$). The activities ranged between 0.08 U g^{-1} fresh wt and 2.7 U g^{-1} fresh wt.

Analysis of the activities of CS and PK of the foot muscle of male and female *L. littorea* is shown in Fig. 3. A significant increase of CS activity with increasing weight of foot muscle was found ($p<0.05$). The correlation coefficient (r^2) were 0.23 CS-male and 0.45 CS-female. Accordingly, the weight dependence explained 23 percent (male) and 45 percent (female) of variation in CS activity.

The relationship between PK activity and tissue weight showed no significance ($p>0.05$) with a correlation coefficient (r^2) for PK-male of 0.006 and PK-female of 0.04.

Seasonal Activities

The seasonal activities of enzymes of the foot muscle of *L. littorea* are shown in Fig. 4. Statistical analysis showed that there were

significant changes of enzyme (CS, PK) activities among seasons (ANOVA:$p<0.05$; Table 1). The specific activities of CS ranged between 0.04 and 0.4 U g^{-1} fresh wt. The specific activities of PK ranged between 0.1 and 1,7 U g^{-1} fresh wt. The enzyme activities of winter and spring animals were higher compared to summer and autumn ones. The *in situ* temperatures during time of collecting samples were indicated. Negative relationships between temperature and the activities of CS and PK were detected in the study.

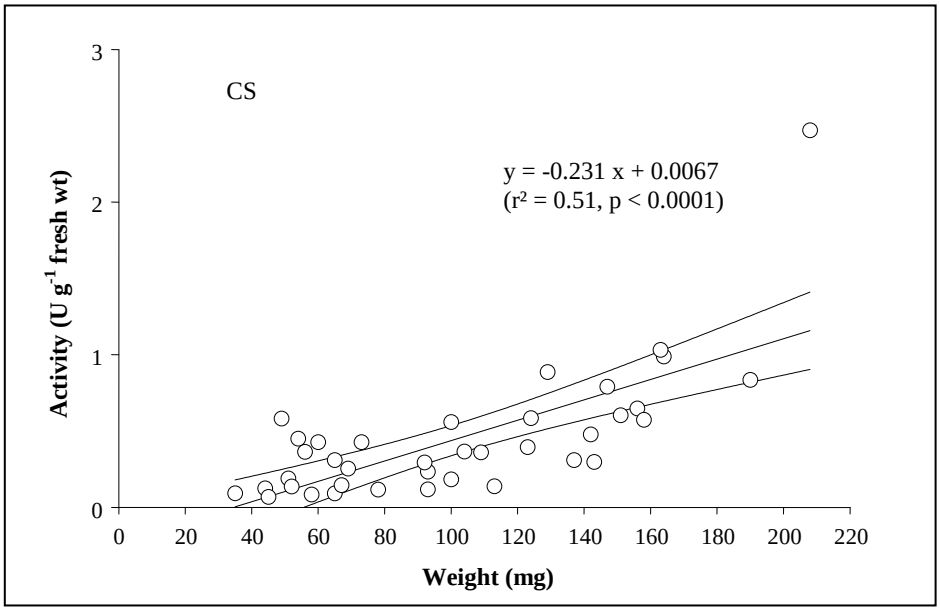

Figure 1. Specific activity of citrate synthase in relation to the weight of foot muscle of the snails. The range of the foot muscle weight used (50 –100 mg FW) for seasonal activity determination is indicated.

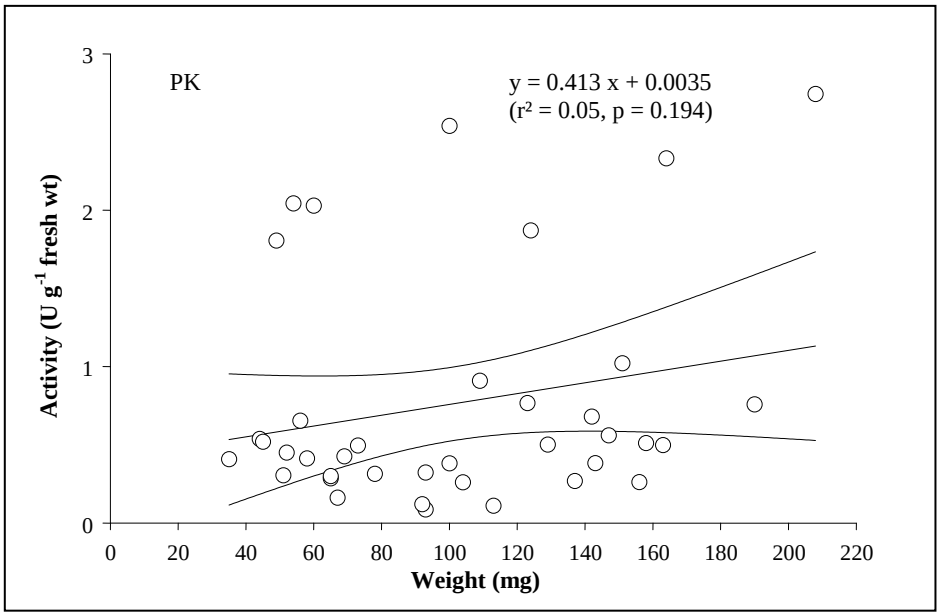

Figure 2. Specific activity of pyruvate kinase in relation to the weight of foot muscle of the snails. The range of the foot muscle weight used (50 –100 mg FW) for seasonal activity determination is indicated.

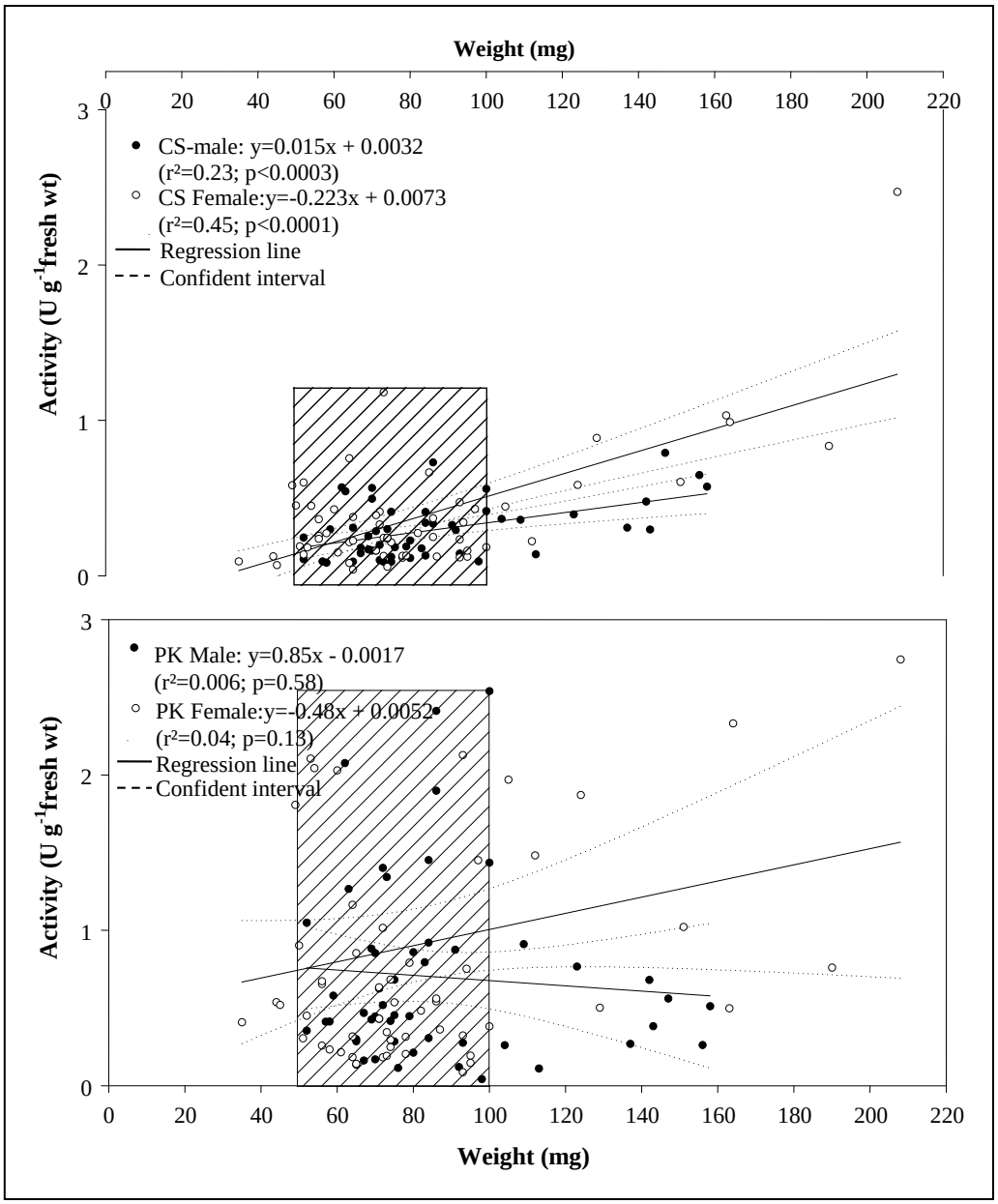

Figure 3. Specific activity of CS and PK in relation to the weight of foot muscle of the snails (different sex). The range of the foot muscle weight used (50 –100 mg FW) for seasonal activity determination is indicated.

Table 1. Results of statistical analysis of enzyme activities (CS and PK: May'00 – April'01) for the whole seasons, using one way analysis of variance (ANOVA).

Enzymes	Number of samples (monthly)	Degrees of Freedom	P
CS	6-10	11	0.009**
PK	6-10	11	<0.001**

Pairwise Multiple Comparison Procedures (Dunn's Method) were used to test which comparisons were statistically significant (p<0.005). The results were as follows:
CS: Apr. vs Nov.; Apr. vs Jul.; Mar. vs Nov.
PK: Dec. vs Nov.; Dec. vs Oct.; Dec. vs Aug.; Apr. vs Nov.

It was also evaluated the percentage of changes in seasonal enzyme activities, and the relation between migratory activity (total number of snails occurring on the pier monthly) and the enzyme activities. The activity of the first month of observation was considered as 100 percent activity. In this respect, CS and PK are plotted together in order to show the tendency of both enzyme activities. The results showed that there was a change in enzyme activity during the season, with relative similar tendency for both enzymes, CS and PK. The enzyme activities were high in spring, and then decreased to relatively constant levels in summer and autumn, and then started to increase again in winter and spring, reaching maximal values in early spring. The total number of snails showed a parallel tendency.

Snails need to acclimate or adapt to changes in the environment. One way of this adaptation is to change the activity and/or properties of metabolic enzymes. Activities of metabolic enzymes in turn may be considered as indicators of metabolic adaptability (Mangunwardoyo *et al*., 2007). PK and CS were chosen to be studied, since these enzymes are key enzymes in metabolic pathways. PK is an indicator of glycolytic capacity and involved in ATP production (Buchholz and Saborowski, 2000) and CS is a key regulatory enzyme in the citric acid cycle and can give a quantitative estimate of aerobic capacity of an organism (Somero and Childress, 1980; Buchholz and Saborowski, 2000). This particular study was conducted to evaluate possible activity changes of these enzymes in the foot muscle of the intertidal snail, *L. littorea.* Furthermore it was intended to link changes in activities to the number of snails prevailing in the study area (on the pier) during seasonal cycles. The results of the present study showed that the enzyme activities (CS and PK) differed during the whole seasons (see Fig. 4, Tab.1), supporting the hypothesis that there were changes in enzyme activities of the foot muscle of intertidal snail, *L. littorea* during seasonal cycles. The result, especially in CS, agrees with the one found by Dahlhoff and Menge (1996) who reported that metabolic activity of *Mytilus californianus*, as indexed by enzyme activities of gill and adductor muscle, differed with site and exposure by season. However, their observations showed that mussels collected in summer had higher enzyme activities than mussels collected in autumn and winter. This was reported to be due to the chlorophyle a concentrations that were higher in summer (high phytoplankton productivity) than in autumn and winter (low phythoplankton productivity) (Dahlhoff and Menge, 1996). Accordingly, they suggested that seasonal variation in the quantity of available food, as well as seasonal and spatial variation in the composition of phytoplankton and the availability of other food sources, is likely to have strong effects on mussel physiology. In contrast, the present study showed that snails collected in winter and spring had higher enzyme activities than snails collected in summer and autumn. However, the factor availability of food which influences the mussel's physiology as indicated by Dahlhoff and Menge (1996) was not considered in the present study because technically, this was difficult to study in a benthic grazer like *L. littorea*. Nevertheless, food was found to be an important factor to affect enzyme activities not only in molluscs, but also in crustaceans as reported by Buchholz and Saborowski (2000) who found that Mediterranean krill, *Meganyctiphanes norvegica*, appear to be more influenced by the seasonal trophic regime than krill at northern sites, where temperature effects were more important. However, in the case of the snail, a synergistic effect of activity enhancement may have taken place (see below).

On the other hand, Sokolova and Pörtner (2001) found no seasonal effect in the activity of CS in *L. saxatilis* (at least no differences between summer and winter). The missing difference in CS activity reported by Sokolova and Pörtner (2001) may have been due to the frequency of taking samples. Their samples were taken in October 1999 and April 2000. In this way, the authors may have missed a possible mid-winter peak as it was the case here, in *L. littorea*. However, it can not be excluded that different species may have a different enzymatic response in seasonal acclimatisation (at least for CS).

The analysis of enzyme activity as percentages suggested that the activity of CS and PK of foot muscle of *L. littorea* was relatively stable in summer and autumn, whereas in winter and spring, the activity was significantly higher. This supports the above results that there is a change in enzyme activity (CS and also PK) during the complete seasons. A similar tendency was also shown by the total number of snails occurring on the pier at the time of collecting the samples for enzyme determination. Accordingly, the parallelity of metabolic enzyme activities and migratory activity may have been functionally related. However, the increase of activities in late winter and spring may probably also be related to the onset of reproduction of the snails during these seasons. Accordingly, the concomitant increase in enzyme activities (CS and PK) may have been associated with the beginning of active gametogenesis and spawning in winter/ spring. The results of the population study showed that the size spectrum of 50% of the snails in winter were at 17-22 mm shell length which corresponded to year classes 1.5-2.5. Enzymes were determined in 50-100 mg foot muscle corresponding to this size-range in turn. Consequently, the snails sampled could be considered sexually mature and at an age of the first considerable spawn (Moore, 1937). Linke (1933) reported that at Helgoland the spawning season of *L. littorea* is from January to June, and Reid (1996) suggested that there is a defined breeding season of the snail, with peak spawning in late winter and spring and most of adults being spent by late summer and autumn. Accordingly, regards the given size range, the spring increase in enzyme activity may have been well relatable to the seasonal onset of reproductive activity. In this case, the enhancement of metabolic enzymes would have underpinned physiological performance for the sake of reproduction of the snails. Concomitantly, migratory activity may have been enhanced and again supported by increasing metabolic enzyme activity. At the same time, environmental temperatures were lowest. Accordingly, the activity increase of enzymes may have been also related to physiological or metabolic compensation of the rate-reducing effects of the low temperatures. Presumably, both, the increase in migratory and reproductive activity coupled with possible temperature compensation may have depended on a synergistic effect of increasing metabolic enzyme activities during spring.

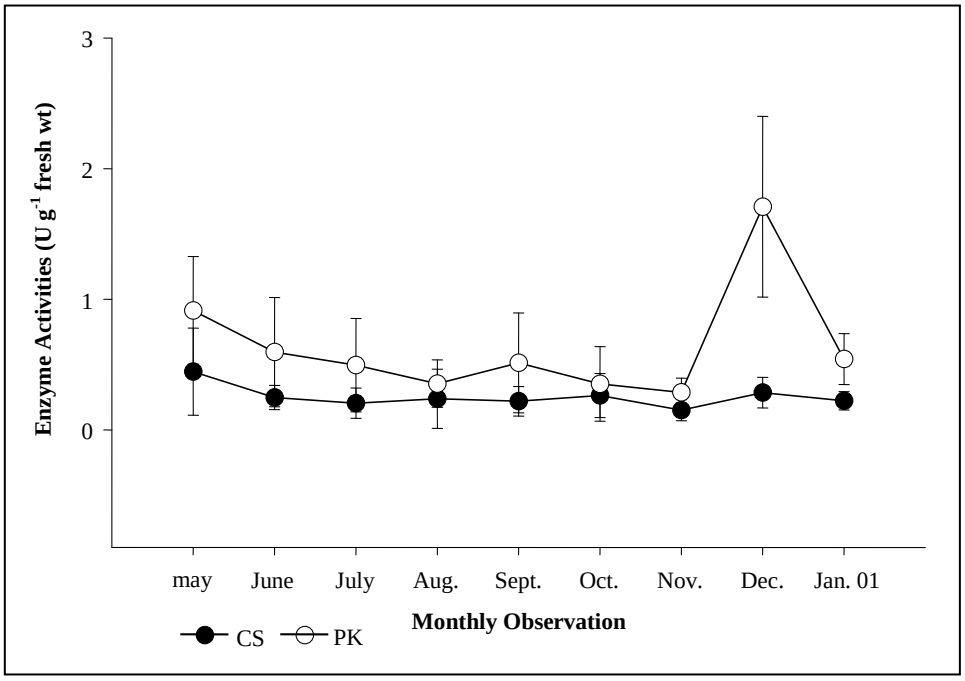

Figure 4. The seasonal activity of CS and PK of the foot muscle of the *L. littorea* (n=6-10).

Comparing the values (CS and PK) found by Sokolova and Pörtner (2001) for *L. saxatilis* and *L. obtusata* sampled in the North-Sea, and by Dahlhoff and Menge (1996) for *M. californianus* (CS), the CS and also PK activities of the present study were lower. Comparably, however, CS values found in the present study were in the range of the ones found by Baldwin *et al*., (1981). The different values of enzyme activities found in the present study compared to other literature might probably be due to different temperatures of incubation and substrate concentrations. Sokolova and Pörtner (2001) suggested the observed differences in enzyme activities were due to the adaptation of snails to different thermal environments, and in the case of their experimental animals where specimens were acclimated for longer times (4-6 weeks) it was the most plausible explanation. The experimental animals of the present study however, were taken from the pier and dissected directly to separate the foot muscles without acclimating them in the lab. In this way the ambient temperature should have caused and reflected possible thermal effects as related to season.

Statistical analysis was done to analyze the relationship between enzyme activities and environmental temperature recorded at the time of collecting the samples for enzyme determination. The results showed that there was a tendency of negative relationship (however, with low correlation coefficients) between temperature and the activities of CS, and PK. This supported the hypothesis that there was a temperature compensation of metabolic enzyme activity in snails, *L. littorea*. These results agreed with the fact that in molluscs, aestivation, anoxia, temperature stress and air exposure lead to a decrease in PK activity, most likely due to phosphorylation of the active non-phosphorylated form of the enzyme (Field and Ellington, 1992; Simpfendörfer *et al*., 1997; Sokolova and Pörtner, 2001). Changes in enzyme kinetics or synthesis are directly related to acclimation and adaptation to ambient temperature, since enzyme activity is involved at all levels of metabolism (Clarke, 1983). Regarding CS and PK, the adjustments of the basic characteristics temperature maximum, energy activation, and thermal stability are unimportant in temperature acclimatization in Northern krill, *Meganyctiphanes norvegica* (Vetter and Buchholz, 1997). However, maintenance experiments showed that the species adjusts specific activity of CS to different temperatures during acclimation over 11 days. Such a capacity to compare thermal influences by maintaining a similar level of activity irrespective of the ambient temperature would be expected to play a role in longer term adjustments to seasonal temperature changes (Buchholz and Saborowski, 2000). Pierce and Crawford (1997) found significant negative correlation between enzyme activities (PK) of *Fundulus* and mean annual temperature. This indicates that activity of PK increase with decreasing ambient temperature and should compensate for the reduced activity of each enzyme at colder temperatures (Sokolova and Pörtner, 2001). Furthermore, according to Buchholz and Vetter (1993) and Vetter and Buchholz (1997) adaptive properties of metabolic enzyme at low temperatures lead to increasing substrate affinity as a compensating effect. The animals studied here were from the same population and hence the results of the present study fit with this pattern. Accordingly, the present study agreed with the hypothesis that there were possible adaptive changes in enzyme activities (PK) of the foot muscle of *L. littorea* during seasonal cycles.

Besides the above observations, other factors that could have influenced enzymatic activities of *L. littorea* in this particular study area were food availability, desiccation, mobility, and parasites. These factors are necessary to take into account in future studies in more detail. It would also be helpful to compare other *Littorina* populations (including related tropical species) from different shore levels and latitudes to obtain more information on the overall ecophysiological capability of the species or genus.

Conclusion

The enzyme activities were found to differ during the year. Activity of CS and PK in the foot muscle was relatively stable in summer and autumn, but higher in winter and spring. A

similar trend was shown in the total number of snails occuring on the pier at the time of collecting the samples for enzyme determination. This correspondence between metabolic and migratory activity may have been functionally related. The increase of activities in late winter and spring may also be related to the onset of active gametogenesis and spawning in these seasons. The enhancement of metabolic enzyme activity would underpin physiological performance for the sake of both reproduction and migratory activity of the snails.

Acknowledgement

My special thanks go to Prof. Dr. Friedrich Buchholz for his continuous support, discussion, constructive criticism and also patience and advise to supervise me during my study. I thank Prof.Dr.Harmut Kausch (IHF-Uni Hamburg) for being my second supervisor. I am grateful to Dr. David Reid, Department of Zoology (Mollusca Research Group) of the Natural History Museum, London, England, to read, correct, and approve the English Language. Biologische Anstalt Helgoland (BAH), Foundation Alfred-Wegener Institute (AWI) has allowed me to use its facilities, I really appreciate this support. The financial support from DAAD during my stay in Germany is gratefully acknowledged.

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