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Seasonal variations in the biochemical composition of the serum of Mytilus galloprovincialis Lmk. and its relationship to the reproductive cycle and parasitic load

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Abstract

The levels of carbohydrate and protein in the blood serum of mussels (Mytilus galloprovincialis Lmk.) experimentally cultured at two depths in Galicia (NW Spain) were determined over a period of 1 year. The changes in concentration of total and reducing carbohydrate and protein exhibited an annual cycle; their values varied from 0.053 to 0.700 mg/ml, from 0.050 to 0.669 mg/ml and from 0.620 to 2.830 mg/ml respectively. Also, the concentrations of total carbohydrate and protein were greater in animals from 5 m depth than those from 2 m. Total and reducing carbohydrate were the components most affected by parasitism. The greatest effect on total carbohydrate concentration was detected in mussels affected by the parasites Mytilicola intestinalis and Marteilia refringens, which presented lower values in infected mussels than in non-infected ones, mainly in the summer months,

Keywords: Mytilus galloprovincialis; Haemolymph; Diseases and their control-molluscs; Carbohydrates; Proteins and amino acids

1. Introduction

Galicia (NW of Spain) is the largest producer of cultured mussels in the world. Although no mortalities have been detected, during the last few years there has been a tendency towards a decrease in production (Figueras, 1989). One of the possible causes is the presence in certain areas of different parasites that reach prevalences of up to 95% (Figueras et al., 1991). The effects of most of these parasites remain unclear; however,

some of them have been correlated with the inhibition or retardation of bivalve gametogenesis (Figueras et al., 1991; Villalba et al., 1993).

Seasonal cycles of energy storage and utilisation are generally attributed to reproductive activity (Gabbot, 1975; Thompson, 1977; Mulvey and Feng, 1981; Fisher and Newell, 1986). On the other hand, biochemical constituents of the haemolymph have sometimes been used to describe the deleterious effects of parasites on their molluscan hosts (Fisher and Newell, 1986). In the case of mussels, these studies have been carried out using the whole animal instead of the composition of the haemolymph (Williams, 1969; Dennis et al., 1974; Ferran, 1991) and they have focused on the seasonality and/or the influence of environmental factors (Bayne, 1973; Santarem et al., 1992). Few investigations have studied the relation of the presence of the parasite to physiological aspects such as the serum carbohydrate and protein concentration (Mulvey and Feng, 1981). The majority of studies concerning the haemolymph components have been carried out on the American oyster (Crassostrea virginica) and they describe the effects of the environmental factors and parasitism, mainly by MSX (Haplosporidium nelsoni), on the host haemolymph (Feng and Canzonier, 1970; Ford, 1986; Fisher and Newell, 1986; Chu and La Peyre, 1989).

In the present study the annual cycle of concentration of serum carbohydrate (total and reducing) and protein in infected and uninfected mussels at two depths was investigated. Serum concentrations were compared with histological examinations of parasite burden and stereological analysis of the mussel gonad state through the reproductive cycle.

2. Materials and methods

The study was carried out in Meira, located in the Ría de Vigo (42º 17'N, 8º 43'W) in Spain (ambient temperature 12.3-20.3ºC and salinity 29-35 ppt; Prego and Fraga, 1992). This study site was chosen because the prevalences of several parasites detected in the mussel were intermediate between those detected in the external and internal areas of the Ría de Vigo (Robledo et al., 1994). The Ría de Vigo is the second in importance in mussel production with 23.9% of the national mussel harvest (Caceres-Martinez and Figueras, 1995).

Animals

Mussels (Mytilus galloprovincialis) with a mean total length of 41.4 mm (s.d. = 6.3), were obtained from collectors in rafts in the Ria de Vigo. The mussels were placed in oyster culture plastic baskets (42 cm diameter, 8 cm high) hung from commercial rafts at two depths (2 and 5 m) . Every month (with the exception of November) between April 1988 and December 1988, 30 mussels were taken for haemolymph samples and histological studies. Mussels were cleaned of fouling organisms and then the length, total weight, shell weight and meat weight were measured in order to obtain growth rates. The condition index was obtained following Aguirre (1979):

$$\frac{\text{Meat weight}}{\text{Total weight - Shell weight}} x100$$

Collection of haemolymph

Haemolymph samples were withdrawn from the posterior adductor muscle with a syringe attached to a hypodermic needle. The shells of the mussels were notched at the level of the posterior adductor muscle to facilitate haemolymph collection. The samples (1 ml of haemolymph per mussel) were centrifuged at 400 x g for 15 min at 4°C to remove cells and cell debris. The supernatant (cell-free haemolymph or serum) was lyophilised and then dissolved in distilled water (serum/distilled water, 1:0:5). When the analysis was not performed immediately, the serum was stored at -20°C for later analysis.

Analytical methods

The concentrated serum was used for quantitative determination of total carbohydrate, the reducing carbohydrate and total protein.

Total carbohydrate was quantified calorimetrically using the sulphuric phenol method (Dubois et al., 1956) as given by Strickland and Parsons (1968), and using sucrose in distilled water as a standard (125 μg/ml).

The Bernfeld (1951) method modified by Forohui and Gunn (1983) was used for the quantification of reducing carbohydrate. The standard was sucrose in distilled water (2 mg/ml).

Protein was quantified following the Lowry method (Lowry et al., 1951). The standard used was bovine serum albumin (fraction V) in distilled water (500 µg/ml).

Histological procedures

After haemolymph collection, mussels were removed from the shell and fixed whole in Davidson's fixative for 24 h (Shaw and Battle, 1957). An oblique transverse section, approximately 5 mm thick, was taken from each specimen so that mantle, gonad, digestive gland, kidney, gills and foot were included. The tissue samples were embedded in paraffin wax and 5-pm sections stained with iron haematoxylin, acid fuchsin and aniline blue (Gray, 1954). The slides were examined for the presence of parasites using a Nikon "Optiphot 2" microscope.

The percentages of the main gonadal components (gametes, adipogranular cells, vesicular connective tissue cells and empty spaces in the follicles) in the total volume of the gonad were assessed using a Weibel graticule (Bayne et al., 1978). Point counts were scored where the end of the test line fell on a cell or group of cells. This procedure was repeated 5 fields per mussel and the volume fraction for any cell type was given by

$$\frac{\text{Number of counts per cell type}}{210} x100$$

Statistical analysis

The values of the concentrations of total and reducing carbohydrate and proteins were $\log_{10}$-transformed prior to analysis (Sokal and Rohlf, 1979). Since no influence of sex was detected in any of the measured parameters, subsequent analyses were carried out using all individuals. The influence of the depth and the month of sampling on the levels of the different biochemical components of the haemolymph was assessed using analysis of variance (ANOVA, Systat Inc.). For each parasite or group of parasites, the influence of the presence of parasites on the biochemical levels of haemolymph

components was evaluated using ANOVA. In those cases in which the prevalence of parasites was small, the statistical analyses were carried out using the samples of surface and depth areas together.

3. Results

The values of total carbohydrate (Fig. 1A) varied seasonally from 0.053 to 0.668 mg/ml from the 2-m mussels and from 0.075 to 0.700 mg/ml from the 5-m depth samples. From the 5-m mussels there was only one peak in July and the minimum values were found in December (0.075 mg/ml). At the 2-m level the pattern was more irregular, with two higher values, the first in June (0.528 mg/ml) and the second in August (0.668 mg/ml); at the end of the experiment the values also were minimal (0.053 mg/ml). The concentration of total carbohydrate was influenced by the sampling date (ANOVA, $n = 454$, $P < 0.05$).

The concentration of the reducing carbohydrate (Fig. 1B) showed similar patterns in both groups of samples. Three peaks were observed, the first in July (0.887 and 0.669 mg/ml respectively), the second in September (1.021 and 0.835 mg/ml respectively) and the third in December (0.5 16 and 0.633 mg/ ml respectively). Reducing carbohydrate content was influenced by the depth (ANOVA, $n = 440$, $P < 0.05$) and it was found to be higher at the 2-m level than at the 5-m level. Moreover, reducing carbohydrate content was also influenced by the month (ANOVA, $n = 448$, $P < 0.05$).

Protein concentration presented a similar pattern for both depths (Fig. 1C). During the experiment the protein levels ranged from 0.620 to 1.962 mg/ml from the 2-m mussels and from 0.770 to 2.830 mg/ml from the 5-m depth samples. In mussels from 5-m depth the values were slightly higher than in mussels from 2-m and these differences were statistically significant (ANOVA, $n = 452$, $P = 0.034$). Maximum and minimum values were achieved at the same time in both samples. The first peak was in June (1.962 and 2.232 mg/ml respectively); the second appeared in August (1.610 and 1.886 mg/ml respectively) and the third was detected in October (1.232 and 2.830 mg/ml respectively). High and low values alternated in consecutive months.

Histopathological studies showed the presence of Chlamydia-like organisms in the digestive tubules, the protozoan Marteilia refringens and Ciliophora-like organisms also in the digestive tubules, ciliates in the gills, the copepod Mytilicola intestinalis in the gut, and Steinhausia mytilovum in the oocytes, as well as infiltration of haemocytes in the connective tissue of the digestive gland without any organization and the presence of both granular and agranular haemocytes accompanied by thick concentric fibrous material in the connective tissue of the digestive gland (granulocytoma) . The frequency of appearance of the parasites was not affected by depth (ANOVA, $n = 464$, $P > 0.05$),

Total carbohydrate and serum protein were affected by the presence of granulocytoma and reducing carbohydrates by Ciliophora-like organisms and Mytilicola intestinalis (Table 1) . On the other hand, statistical analysis using groups of two parasites showed that the concentration of reducing carbohydrate in the haemolymph was affected by most of the groups of two parasites where Marteilia refringens or M. intestinalis was present; however, there was no clear predominance of non-parasitized values over parasitized ones. Proteins were not affected by the combination of parasites (Table 2). Total carbohydrate was higher in non-infected mussels than in mussels with M. refringens with haemocyte infiltrations, or mussels infected by M. refringens with granulocytomas, mainly in the summer months. Uninfected mussels also showed a higher condition index than infected mussels (Fig. 2A-D).

In both places, Myths galloprovincialis exhibited a typical annual reproductive cycle for both depths. Gonadal development with production of gametes occurred throughout the year but mainly from April to June when most of the gametes were produced. There was a decrease to minimum values in October. In December, gamete production began to increase again. The mean percentage per month of adipogranular cells (AG) and vesicular connective tissue cells (VCT) was higher in summer and autumn and was at a minimum in spring (Fig. 3A and B).

The condition index presented two peaks in the year at both depths: the first in March and the second in September. Minimum values were achieved in July. No significant differences were found between the two depths (ANOVA, $n = 460$, $P > 0.05$) (Fig. 4).

4. Discussion

Carbohydrates have two major biological functions: as a long-term energy store and as structural elements. There was a clear seasonal pattern in the total carbohydrate concentration from the 5-m mussels. The values were low in April, reaching a maximum in July. This maximum coincided with the poorest condition index, perhaps because spawning had just finished and mussels would need to store energy for the next gametogenesis. In the surface sample the peak was displaced to August and there was another peak in April. Bayne (1973) found the lowest concentration of carbohydrates in Mytilus edulis serum in mid-summer and mid-winter whereas our values were highest in summer. In spring, the number of VCT cells are low because they have been utilized in gametogenesis. Ferran (1991) examined whole Mytilus galloprovincialis and found that the annual variation of sucrose and glycogen in the mantle was closely related to VCT cell numbers. In our study, the variation in total carbohydrate in the serum could also be related to VCT cell numbers. In spring, when the water temperature and salinity are favourable, the first bloom of phytoplankton occurs (Campos-Loriz and González, 1975; Iglesias and Nunes, 1982; Figueiras and Niell, 1987), and it was also in April when the mean percentage of VCT cells began to increase and the mobilisation of total carbohydrate obtained from the food to the VCT cells in formation could take place via the haemolymph.

The presence of reducing carbohydrates is indicative of carbohydrate metabolism. The concentration of reducing carbohydrate showed an irregular profile with 3 peaks in both superficial and depth samples. The levels found in the haemolymph were low during much of the year. These results suggest that until June the metabolic activity in the mussels was low, and then there was a large increase in this component in June, September and December. As no relationship was found with gonadal development, this variation could be the result of changes in enviromental conditions or mobilization of carbohydrate reserves.

Proteins have many different biological functions: they are associated with enzymatic reactions, transport, regulation of metabolism, defence, structural elements, storage. This means that a pattern can be expected, depending on the different physiological activity of individuals throughout the year. However, no annual pattern was detected in the blood protein concentration. Haemolymph protein concentration was high in the

mussels taken from the 5-m depth sample in October and these results agreed with those found by Bayne (1973) in Mytilus edulis. Another peak was found in June at both depths sampled. Santarem et al. (1992) reported different values of protein concentration depending on the sample location (estuarine or oceanic). Our results in Meira followed the pattern of the estuarine sample (April values higher than July values) and these values were higher than those previously reported (Santarem et al., 1992). The monthly alternation of high and low values in the blood protein concentration between May and October in both samples suggests a rhythmic release of such components. Fisher and Newell (1986) suggested that developing gametes could be responsible for serum protein variations. Our results cannot be explained in terms of developing gametes because there were protein concentration peaks coinciding with high and low production of gametes.

It is important to point out that in the “saw tooth” pattern found in both reducing carbohydrates and proteins, the high values of reducing carbohydrate coincided with the low values of protein and vice versa. These results suggest possible rhythmic mobilization of the reserves: when the carbohydrate reserves are depleted, mussels could use protein as a fuel and vice versa, guaranteeing energy for the mussels over the whole year.

All the haemolymph components varied according to the month sampled. Santarem et al. (1992) reported that haemolymph proteins and other haemolymph parameters were related to the humoral and cellular response and varied according to season and locality. These results suggest a high dependence of haemolymph component concentrations on temperature, salinity, and food availability, variables that are month-dependent. In most months reducing carbohydrates were higher in the 2-m depth sample and proteins in mussels from the 5-m depth sample; in both cases these differences were statistically significant. The reason for such variation remains unclear, but it is possible that environmental differences favoured an accumulation of carbohydrates at one point and of protein at another.

Histopathological studies did not show different parasites or disease conditions from those previously reported for mussels in the same area (López et al., 1990; Figueras et al., 1991; Robledo et al., 1994). The serum components were not affected by the

presence of one type of parasite. However, studying the effect of several types of parasites together, statistical analysis showed significant differences between parasitized and non-parasitized mussels in total and reducing carbohydrates. The reducing carbohydrate was the haemolymph component most affected by parasitism and Mytilicola intestinalis or Marteilia refringens was present in most cases where the difference between parasitized and non-parasitized mussels was statistically significant.

Chlamydia-like organisms, Ciliophora-like organisms and Marteilia refringens parasitize cells in the mussel digestive gland (Figueras et al., 1991) . The digestive cells are responsible for the intracellular digestion of food and are a site for the storage of metabolic reserves (Thompson et al., 1978). Digestive cells infected by any intercellular pathogen would have their capacity for food uptake considerably reduced. Villalba et al. (1993) pointed out that M. refringens significantly reduced absorption efficiency, suggesting that this parasite inhibited gonad development mainly after the spring spawning. Similarly, total carbohydrate was lower in mussels infected with M. refiingens with haemocyte infiltrations, and in mussels infected with M. refringens with granulocytomas than in non-infected ones, mainly in the summer months after spawning. Moreover, the condition index was also higher in non-infected animals than in infected ones. These results suggest that this parasite interferes with metabolism, inhibiting gonadal development and consequently reducing the condition index of infected animals. The adults of Mytilicola intestinalis have been associated with damage of the epithelium in the digestive tubule (Davey and Gee, 1988) and these infested mussels could have a reduced capacity to absorb food; however, the variation in amount of reducing carbohydrate is more difficult to explain because the values obtained in the uninfected mussels were not significantly higher than those of the infected animals.

Feng and Canzonier (1970) described changes in the haemolymph proteins of Crassostrea virginica when infected with Haplosporidium nelsoni and Bucephalus sp. Ford (1986) reported that the total serum protein concentration fell sharply and in approximate proportion to disease intensity in oysters with systemic H. nelsoni infections. When working with pairs of parasites, no differences were found in the protein concentrations of infected and non-infected mussels. In order to detect parasite effects on serum protein concentration in Mytilus galloprovincialis it is possible that a

heavy parasite burden, comparable to that examined by Ford (1986), would be necessary to detect an effect of the parasites on protein concentration.

The way in which a parasite disturbs the metabolic path of its host remains unclear. The results suggest that the synergy of different pathogens is usually more deleterious than the action of a single one. This should be investigated in molecular and physiological cellular terms under laboratory conditions using experimental infections.

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Fig. 1. Seasonal variation in total carbohydrate (A), reducing carbohydrate (B) and protein (C) (mg/ml ± SD) of mussel serum, Mytilus galloprouincialis, April to December 1988 (solid colunms, from the 2-m samples; hatched columns, from 5-m depth samples).

Fig. 2. Mean total carbohydrate and condition index values (± s.d.) for parasitized and non-parasitized mussels with Marteilia refringens and infiltration (A and B) and with M. refringens and granulocytoma (C and D) (-•- non-parasitized, -O- parasitized). Total carbohydrate was higher in non-parasitized mussels mainly in the summer months, and these mussels also showed a higher condition index.

Fig. 3. Mean monthly percentage of each cellular type of the mussel, Mytilus galloprovincialis, obtained from the 2-m and 5-m depth samples using a Weibel graticule (Bayne et al., 1978), April to December 1988. G = gametes; AG = adipogranular cells; VCT = vesicular connective tissue cells; E = empty spaces in the follicles.

Fig. 4. Mean condition index of the mussel, Mytilus galloprouincialis, obtained from the 2-m samples (-□-) and 5-m depth samples (-♦-)

Table 1

Comparison of haemolymph parameters in mussels from Ria de Vigo (NW of Spain)

Parasites or diseases	Total carbohydrate		Reducing carbohydrate		Protein	
	n	P	n	P	n	P
Chlamydia-like organisms	93	0.275	91	0.299	93	0.732
Ciliophora-like organisms	89	0.982	87	0.037*	89	0.913
Steinhausia mytilovum	38	0.748	36	0.221	38	0.580
Marteilia refringens	85	0.775	83	0.368	85	0.974
Mytilicola intestinalis	139	0.647	137	0.006*	139	0.961
Infiltration	105	0.147	103	0.465	105	0.757
Granulocytoma	80	0.030*	78	0.771	80	0.039*

One-way ANOVA was used to examine the haemolymph values with respect to the parasitized/non-parasitized status (* $P < 0.05$, statistically significant; n = number of mussels)

Table 2

Comparison of haemolymph parameters in mussels from Ria de Vigo (NW of Spain)

Parasites and/or diseases	Total carbohydrate		Reducing carbohydrate		Protein	
	n	P	n	P	n	P
M. refringens-M. intestinalis	148	0.714	146	0.004*	148	0.817
M. refringens-Chlamydia	102	0.502	100	0.152	102	0.937
M. refringens-Ciliophora	97	0.899	95	0.015*	97	0.787
M. refringens-Infiltration	124	0.033*	122	0.18	124	0.455
M. refringens-Granulocytoma	87	0.05*	85	0.844	87	0.295
M. intestinalis-Chlamydia	170	0.194	168	0.008*	170	0.971
M. intestinalis-Ciliophora	169	0.985	167	0.000*	169	0.390
M. intestinalis-Infiltration	192	0.168	190	0.004*	192	0.989
M. intestinalis-Granulocytoma	146	0.822	143	0.011*	146	0.927
Ciliophora-Granulocytoma	103	0.413	101	0.049*	103	0.853
Chlamydia-Infiltration	131	0.024*	129	0.629	131	0.864
Chlamydia-Granulocytoma	94	0.62	92	0.284	94	0.867
Ciliophora-Infiltration	124	0.703	122	0.137	124	0.683
Ciliophora-Granulocytoma	90	0.039*	90	0.546	90	0.490
Infiltration-Granulocytoma	114	0.290	112	0.282	114	0.814

One-way ANOVA was used to examine the haemolymph values with respect to the parasitized/non-parasitized status with groups of two parasites and/or diseases ($P < 0.05$).