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SEASONAL VARIATIONS IN BIOCHEMICAL COMPOSITION OF MYTILUS EDULIS WITH REFERENCE TO ENERGY METABOLISM AND GAMETOGENESIS

by

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CONTENTS

I. INTRODUCTION

Early investigations on seasonal variations in biochemical composition of *Mytilus edulis* and *Mytilus galloprovincialis* have been reviewed by GIESE (1969). More recent studies on *Mytilus edulis* are those of LUBET & LE FERON DE LONGCAMP (1969), WILLIAMS (1969), DE ZWAAN (1971), DE ZWAAN & ZANDEE (1972), DARE (1973), GABBOTT & BAYNE (1973) and DARE & EDWARDS (1975). These papers are mainly concerned with a seasonal variation in glycogen content which reflects

the interaction between food availability, temperature, growth and reproductive cycle.

In *Mytilus edulis* glycogen is an important source of energy (DE ZWAAN & ZANDEE, 1972). Studies by GABBOTT & BAYNE (1973) showed that protein and lipid reserves are also built up, but mainly in the non-mantle tissues. The seasonal changes in lipid level are inversely related to the changes in glycogen level (LUBET & LE FERON DE LONGCAMP, 1969; WILLIAMS, 1969). This relation was also found by PIETERS *et al.* (1979) and WALDOCK (1979).

Seasonal changes in biochemical composition may be of great importance in relation to (anaerobic) energy metabolism. Studies on anaerobic energy metabolism in bivalve molluscs have been recently reviewed by DE ZWAAN (1977). He discussed the formation of multiple end products and the dependence of the proportional accumulation on the duration of anaerobiosis. We also found indications for differences in the relative amounts in which end products accumulate in different seasons.

In order to obtain more detailed information about the effects of the variations in environmental factors like temperature, salinity and food availability on the biochemical composition and (anaerobic) energy metabolism, we started in February 1977 an extensive seasonal cycle experiment lasting nearly one and a half year. The present paper deals with the results of this investigation.

Acknowledgements.—The authors gratefully acknowledge the technical assistance of A. M. T. de Bont, A. Kok, the late F. J. de Koning, E. C. J. Kruitwagen, H. J. L. Ravestein and P. R. Veenhof. We are also indebted to Dr A. C. Drinkwaard of the Netherlands Institute for Fisheries Investigations, IJmuiden for his interest and cooperation in making this research possible. The crews of the M.S. Eider and the former M.S. Griend of the Netherlands Institute for Sea Research, Texel are acknowledged for the collection of the mussels.

II. MATERIALS AND METHODS

1. FIELD MEASUREMENTS

During the experimental period, lasting from February 1977 until July 1978, samples of mussels (*Mytilus edulis* L.) were collected from a culture bed in the Dutch Wadden Sea at intervals of about 3 weeks. The mussel bed, marked TX 41A, 1500 m off shore, east of the island Texel, was about 3.5 m below high tide level. Samples of mussels and water (from near the bottom) were taken 2 to 3 h before high tide.

Temperature, salinity and nutrient levels of the water samples were determined. The nutrient levels were estimated by measuring total chlorophyll pigments photometrically at a wave length of 440 nm after extraction with 70% ethanol in water and expressed in arbitrary units. With this method only relative differences in chlorophyll content could be observed. Determinations of ammonia, nitrate and nitrite were carried out according to STRICKLAND & PARSONS (1968), including some modifications.

2. SELECTION OF EXPERIMENTAL ANIMALS

After arrival at the laboratory the animals were cleaned of encrusting organisms and grouped according to shell length. The mussels were kept for 6 days in a tank with running sea water at a temperature and with a food supply almost identical to that of the sampling place. Fig. 1 shows the shell length distribution at 4 moments during the investigation. The class width amounted to 0.5 cm.

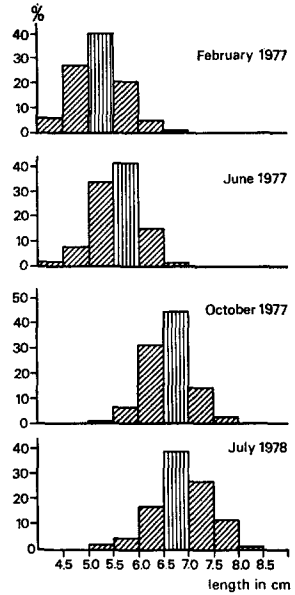

Fig. 1. Shell length distributions of the mussels (in %) (after PIETERS *et al.*, 1979).

At the starting point of our experiment in February the smallest animals measured 4.0 cm and the largest ones 7.0 cm. Animals from the class with the highest frequency were used for the experiments. The mean shell length increased from 5.2 cm in February 1977 to 7.0 cm in July 1978.

3. TREATMENT OF THE EXPERIMENTAL ANIMALS

A first group of 50 animals, coming directly from the running sea water (aerobic sample), was removed from their shells. After determination of the amounts of enclosed sea water and wet weight of the tissues the fractions were combined and homogenized with 1 volume of ice-cold bicarbonate buffer (0.05 M, pH 9.5) in a Sorvall Omnimixer during 3 min at maximum speed. From a second group of 100 animals several organs were excised, *viz.* gills, mantle, posterior adductor muscle, while the remaining parts were combined in a rest fraction. The obtained fractions were also homogenized in bicarbonate buffer. A third group of 50 to 100 mussels was used to collect haemolymph by a syringe from the sinus in the posterior adductor muscle. The syringe was inserted through a notch in the shell adjacent to the muscle. Per mussel about 1 ml haemolymph was obtained. The haemolymph was centrifuged to remove the blood cells and stored at −20° C until analysis. Finally, a fourth group of 50 animals was exposed to air, just above the water level of the tank, for 48 h (anaerobic sample) at ambient temperature, which was 1° to 2° C above the sea water temperature of that moment. At the end of the exposure period, animals were removed from their shells and treated identically to those of the first group.

4. LABORATORY MEASUREMENTS

Dry weight, water content and ash weight.—From the homogenates aliquots of about 10 ml (in case of total animals) or 5 ml (in case of organ homogenates) were taken, weighed and dried at 90° C for 24 h and subsequently kept over P_2O_5 in a desiccator until the weight was constant. Dry weight and tissue water content were calculated after corrections for enclosed sea water and buffer.

Ash weight was determined by subjecting aliquots of dried homogenate in an electric furnace to 700° C during 12 h. The weight of salts from the enclosed sea water and homogenization buffer was subtracted from the measured values.

Glycogen.—Immediately after homogenization, aliquots (about 2 ml) of homogenates were weighed, 2 ml of 40% KOH added and the mixture saponified by heating to 60° C for 1 hour. Unless KOH was added as quickly as possible, rapid hydrolysis of glycogen occurred, even when the homogenates were stored at −20° C. After 1 hour the partially saponified mixture was neutralized with HCl and remaining proteins were removed by adding 1 ml of 40% (w/v) trichloroacetic

acid and centrifuging for 10 min at 10 000 *g*. Glycogen was determined in appropriate dilutions of the supernatant according to VAN HANDEL (1965).

Proteins.—Proteins were determined by the method of LOWRY *et al.* (1951).

Total lipids and volatile fatty acids.—Total lipids and volatile fatty acids (VFA) were extracted from weighed aliquots of about 50 ml (total tissues) or 25 ml (organs) of homogenate, after addition of 100 µl of 0.1 M valeric acid per 25 g homogenate as an internal standard, with chloroform-methanol (2 : 1, v/v) according to FOLCH, LEES & SLOANE-STANLEY (1957). After equilibrium was reached the chloroform and methanol-water layers were separated.

Total lipids were subsequently isolated from the chloroform fraction by evaporation under reduced pressure. The residue was dried over P_2O_5 during one night and the total lipid weight determined. The neutral lipids were separated from the polar lipids by silicic acid chromatography as described by SWEELEY (1969). The obtained neutral lipids as well as the phospholipids were saponified with 2 N KOH in methanol (80%) at 80° C. The unsaponifiable material was extracted with petroleum ether (b.p. 40° to 60° C), and the remaining fraction was acidified to pH 1 in order to isolate the fatty acids with b.p. petroleum ether. All samples were dried and weighed.

Fatty acids.—The isolated fatty acid mixtures were methylated after the method of SCHLENK & GELLERMAN (1960) and purified by passing through a silicic acid column and finally analyzed in a gas-liquid chromatograph (Packard-Becker, Model 420) with a flame ionization detector. The columns (150 cm, internal diameter 4 mm) were packed with 20% poly-ethylene glycol adipate (PEGA), with 2% phosphoric acid, on Chromosorb W (100–200 mesh) and 10% Apiezon L on Gaschrom Q (80–100 mesh). Identification of the fatty acids in the chromatograms was done by comparison with standards, using the graphic procedure of the log relative retention times versus chain length (JAMES, 1960). To estimate the chain length of the unsaturated fatty acids, the samples were hydrogenated by the method of FARQUHAR *et al.* (1959) and rechromatographed.

Volatile fatty acids.—For the isolation procedure and the quantitative determination of the volatile fatty acids is referred to KLUYTMANS, VEENHOF & DE ZWAAN (1975).

Free amino acids.—For the determination of free amino acids (FAA) 10 ml aliquots of homogenate were acidified with 1 ml 70% perchloric acid. The precipitated proteins were removed by centrifugation (10 min at 10 000 *g*). The pH was subsequently adjusted to pH 2 to 3 with 5 M K_2CO_3 and the insoluble $KClO_4$ crystals removed by centrifugation for 10 min at 48 000 *g*.

The supernatant was analyzed for FAA by injecting 25 µl samples into a LKB-3201 amino acid analyzer.

Qualitative and quantitative determination of amino acids (including taurine) was carried out by comparison with standard amino acid mixtures. In addition L-alanine was also determined spectrophotometrically according to WILLIAMSON (1974a).

Succinate.—Succinate was determined according to WILLIAMSON (1974b). The homogenate was deproteinized with perchloric acid as described for the free amino acids. After centrifugation the supernatant was adjusted to pH 7.5 with 5 M K_2CO_3, stored overnight at −20° C, thawed and used in the assay after centrifugation for 10 min at 20 000 *g*.

III. RESULTS

1. ENVIRONMENTAL CHARACTERISTICS

The seasonal variations in temperature and salinity at the sampling place are presented in Fig. 2. The highest temperatures were measured during the months of July and August and the lowest during February.

The salinity curve shows about the same fluctuations as the temperature curve. The salinity changes were the result of the sluicing

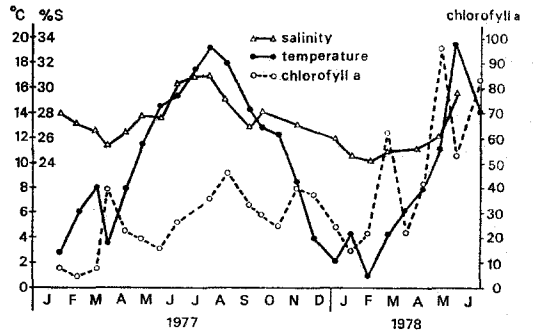

Fig. 2. Seasonal variation in temperature (°C), salinity (‰ S) and chlorophyll a (arbitrary units) in the Wadden Sea at the sampling place (redrawn from PIETERS *et al.*, 1979 and ZURBURG *et al.*, 1979).

regime of the IJsselmeer. During winter the amount of fresh water sluiced to the Wadden Sea is higher than in summer.

The food availability was estimated by measuring the chlorophyll content (Fig. 2) in the water samples from near the bottom. The phytoplankton bloom in April 1978 corresponded with an increase of the chlorophyll a content of the water. The main factor for these changes was the enormous bloom of the colony alga *Phaeocystis pouchetii* (PIETERS, 1978; PIETERS *et al.*, 1980).

2. GROWTH AND CONDITION OF THE MUSSELS

From early spring through early autumn an almost linear growth was observed for both tissue and shell (Fig. 3). In spite of a decrease in the sea water temperature from August, growth continued until October. Afterwards the shell growth slowed down until February 1978, and then resumed at a less pronounced rate. The average growth of the shells amounted to about 2 cm during the growth season of 1977.

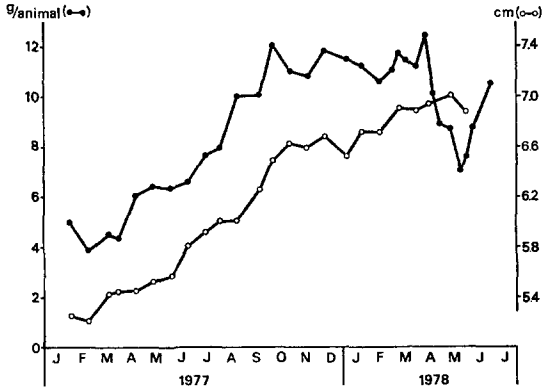

Fig. 3. Growth of the mussels expressed by wet weight determinations (g per animal) and by shell length measurements (cm) (after PIETERS *et al.*, 1979).

No increase of wet weight occurred during autumn and winter. Dry weight decreased gradually (Fig. 4a). Since the wet weight did not change in this period the decrease of dry weight must be compensated by an increase of water content (Fig. 4b). The water content of the gills was the highest of all organs, about 90%, and showed only minor annual fluctuations. On the contrary, in mantle, posterior adductor muscle, rest and total tissues the water content fluctuated between 70 and 82% and was at minimum in October and November when the dry weights of the examined tissues had reached their maxima. Of the organs investigated the posterior adductor muscle and the gills contrib-

uted much less to the body weight than the mantle and the rest: 3–4% and 4–7% versus 13–25% and 69–75%, respectively (see also ZURBURG *et al.*, 1979). A marked growing period was observed in all organs from early summer to November. Afterwards the weight decreased during winter and early spring 1978 to about twice the value in February 1977.

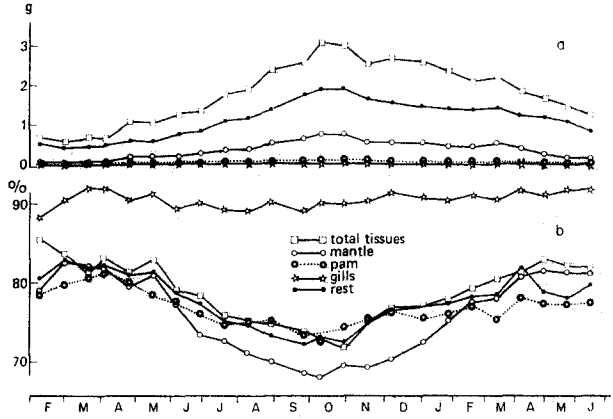

Fig. 4. a. Mean dry weight (grams) of total tissues and of isolated organ fractions. b. Mean water content (%) of same tissues (averages of total and different tissues of 50 and 100 animals, respectively).

The lowest water content was found simultaneously with the highest glycogen content (Fig. 7). It could be that glycogen partly replaces the water in the tissue cells.

The condition of the experimental animals was expressed according to the method of KORRINGA (1955) by using the formula:

$$\text{condition index} = \frac{\text{dry weight in g}}{\text{shell cavity volume in cm}^3} \times 1000$$

The condition index and the data necessary for its determination are collected in Table I. The results show that the highest values were found from July through November. From November until next February a gradual decrease was observed which was followed by a dramatical decline during the spawning period from March through June.

3. BIOCHEMICAL COMPOSITION OF TOTAL TISSUES AND DIFFERENT ORGANS

The ash content in mg per animal (Fig. 5), exhibited a pattern resembling that of the wet weight (Fig. 3). Expressed as percentage of

TABLE I

Growth and condition index of *Mytilus edulis* from a location in the Dutch Wadden Sea; data are means of 50 animals (PIETERS, 1978).

Sampling date	*Mean shell length (cm)*	*Shell cavity volume (cm^3 $mussel^{-1}$)*	*Dry weight (g $mussel^{-1}$)*	*Condition index*
1977 Feb 04	5.25	6.79	0.69	102
24	5.21	7.84	0.63	80
Mar 17	5.42	8.38	0.71	85
31	5.44	8.16	0.70	86
Apr 21	5.45	9.34	1.11	119
May 12	5.52	10.37	1.09	105
Jun 02	5.57	11.22	1.28	114
23	5.80	11.01	1.41	128
Jul 14	5.91	11.29	1.80	159
Aug 04	5.99	11.39	1.90	167
25	5.99	14.73	2.47	168
Sep 22	6.25	15.23	2.60	171
Oct 06	6.49	18.04	2.94	163
27	6.62	18.00	3.01	167
Nov 17	6.59	18.32	2.52	138
Dec 08	6.68	18.30	2.67	146
1978 Jan 05	6.52	19.10	2.58	135
26	6.71	18.06	2.38	132
Feb 16	6.71	18.09	2.13	118
Mar 09	6.90	18.08	2.21	122
30	6.88	20.31	1.90	94
Apr 20	6.93	19.03	1.68	88
May 11	7.00	18.96	1.56	82
Jun 01	6.87	18.29	1.35	74
29		16.79	2.30	137

dry weight a more or less inverse correlation with the dry weight curve of the total tissues (Fig. 4a) was observed. Minima of about 4 to 5% of dry weight were observed from early summer 1977 through December 1977. Maxima were found in early spring 1977 (7%) and 1978 (6.5 to 8%). DARE & EDWARDS (1975) measured ash contents between 9 and 15% in *Mytilus edulis* from North Wales.

The changes in biochemical constituents of the total tissues are shown in Fig. 6. In the growing mussel from spring to autumn a gradual increase of protein, glycogen and lipid contents was observed. During that period enough food was available. From November through next April protein and glycogen contents declined. Low food levels combined with gametogenesis will be reasons for the decrease in protein and glycogen contents. However, the lipid content remained rather constant until the beginning of the spawning period in which protein and glycogen contents reached minimum values.

The relative amounts of glycogen in total tissues and different organs are shown in Fig. 7. In the different organs the highest glycogen level was found from June through October. The glycogen level in gills and posterior adductor muscle (PAM) reached maximum values of 10 and

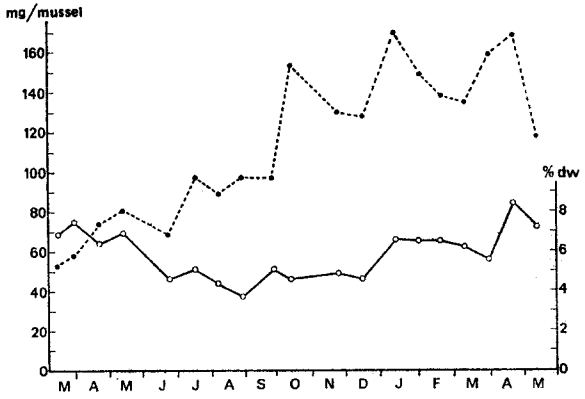

Fig. 5. Ash content of total tissues expressed in mg per mussel and as percentage of dry weight.

18%, respectively. These organs retained their glycogen level from summer to next February while in the mantle and rest tissues the levels were decreasing earlier, namely from October to April from 40 to 8% and 35 to 5%, respectively. Mantle tissue showed the highest glycogen

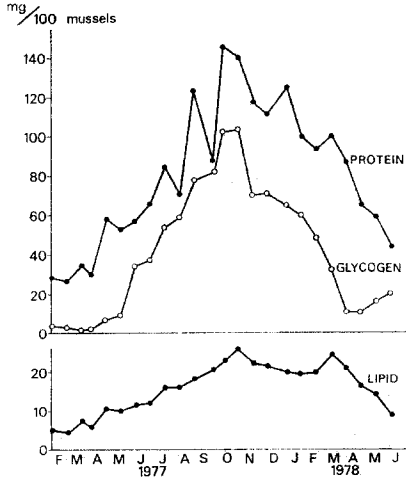

Fig. 6. Seasonal variation in total protein, glycogen and lipid content (mg per 100 mussels).

percentage followed by the total tissues and rest tissues which exhibited almost identical curves. The rapid decline of glycogen level (three upper curves, Fig. 7) in November could be partly caused by the known rapid production of byssus threads in this season (PIETERS *et al.*, 1979).

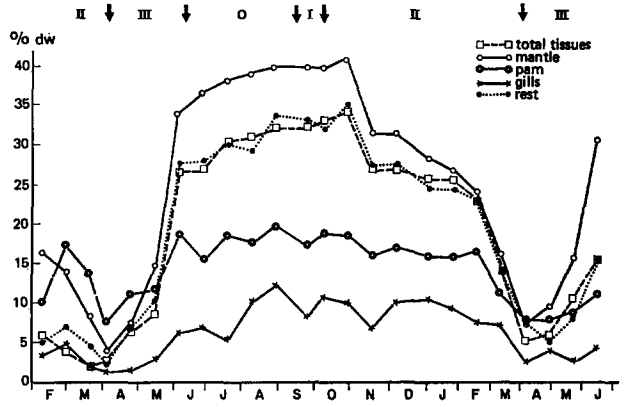

Fig. 7. Seasonal variation in relative amounts of glycogen (% of dry weight) in total tissues and different organs (the stages of gonad development O to III indicated).

The seasonal variation in protein levels, expressed as percentage of dry weight, in different organs and total tissues, is presented in Fig. 8. All the different tissue fractions, except the posterior adductor muscle, appeared to have about the same protein level which did not show

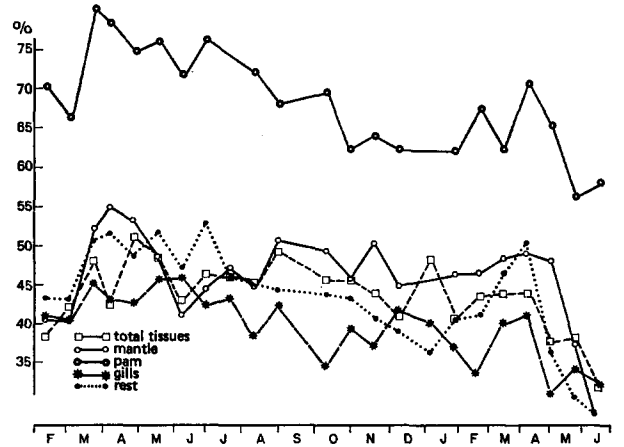

Fig. 8. Seasonal variation in relative amounts of protein (% of dry weight) in total tissues and different organs.

much seasonal variation. In the first period of our investigation the growing mussels generally demonstrated a relative increase of protein levels.

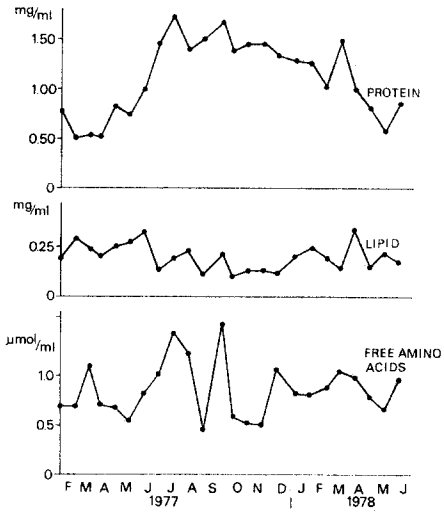

Fig. 9. Seasonal variation in haemolymph constituents; protein and lipid levels (mg per ml haemolymph) and free amino acids (µmol per ml).

The seasonal variation in biochemical constituents of the haemolymph is presented in Fig. 9.

The seasonal variation in concentrations of free amino acids in the different organs and total tissues is shown in Fig. 10. The curves

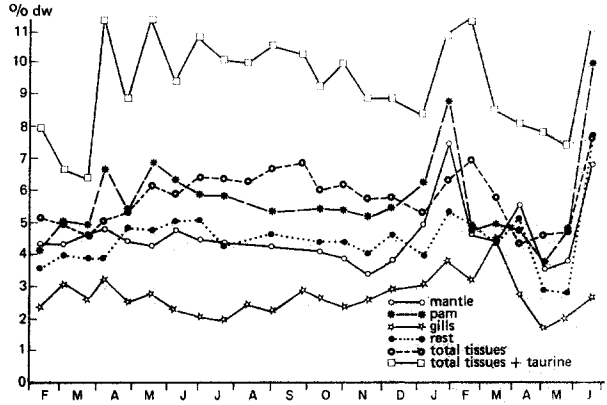

Fig. 10. Seasonal variation in total free amino acids (% of dry weight) of total tissues and different organs; for the total tissues data are also given with taurine included.

represent the sum of the most abundant free amino acids: glycine, alanine, aspartate, asparagine, glutamate, glutamine, proline, serine, threonine. Taurine is only included for the total tissue fraction. The free amino acids formed 2 to 9% of dry weight without taurine and 5 to 15% taurine included. There were only little seasonal changes in the total free amino acid levels.

In the levels of the individual amino acids (Fig. 11), clear annual variations were observed in the total tissues. Alanine, serine and glutamate (glutamine included) behaved more or less the same while glycine and aspartate showed an inverse pattern.

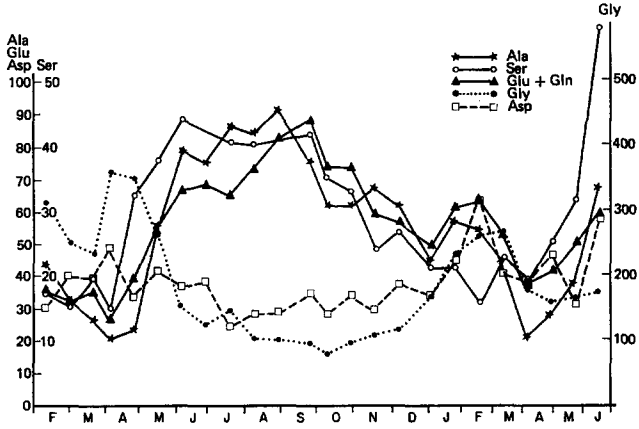

Fig. 11. Seasonal variation in the level of alanine, serine, glutamate (glutamine included), glycine and aspartate (μmol per g dry weight) in total tissues.

For the different organs seasonal variations in amino acid composition are illustrated in Fig. 12. A more or less double peak for glycine during spring 1978 was only observed in mantle and rest tissues. In the posterior adductor muscle the alanine, glutamate (including glutamine) and serine curves are almost the same as in mantle and rest, but at lower levels. In the gills a seasonal variation is more pronounced for aspartate than for glutamate and serine. The glycine levels in the gills were low in contrast with the high levels in the posterior adductor muscle. Fig. 13 shows the percentage composition of the haemolymph free amino acids whereas Fig. 14 presents the taurine levels during different seasons in different tissue fractions. On the whole the highest taurine levels correspond with high levels of alanine, glutamate and serine in the different organs.

Fig. 15 presents the seasonal variation in lipid level of the total tissues and different organs. Most variation was found in mantle and rest tissue. Hardly any variation was observed in gills and posterior

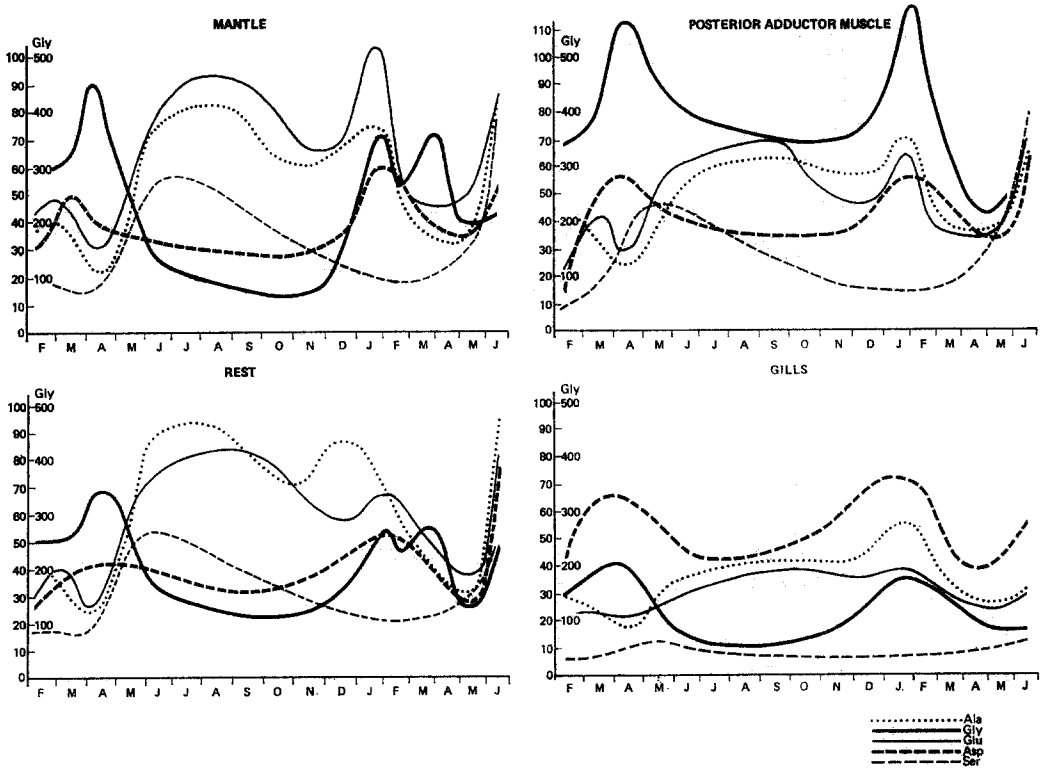

Fig. 12. Seasonal variation in the level of alanine, serine, glutamate (glutamine included), glycine (separate scale), and aspartate (μmol per g dry weight) in different organs and rest tissues.

adductor muscle. The latter contained as little as 3% of the dry weight in contrast with the other tissues which contained about 8% or more (10 to 14% in mantle). The distribution of the total lipid fractions in neutral lipids and phospholipids is shown in Fig. 16. Mantle and rest tissues (including the digestive gland) contained the highest amount of storage lipids. The fatty acid composition of an August sample is shown in Table II. The relative fatty acid composition of both the neutral and phospholipids of the total lipid fractions (Fig. 17) can be summarized as follows. The saturated fatty acids (mainly 16:0 and 18:0) of the neutral lipids decreased somewhat from April 1977 to January 1978. The polyunsaturated fatty acids increased a little from April to September while the fatty acid composition from the phospholipid fractions showed only little variations during the whole experimental period.

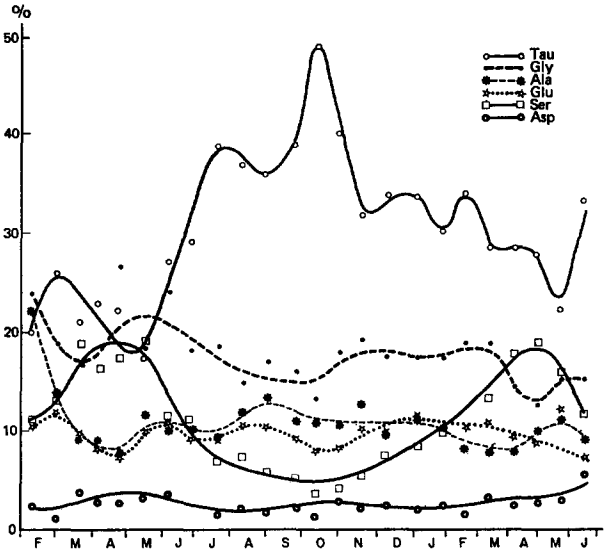

Fig. 13. Seasonal variation in relative amounts (%) of the haemolymph free amino acids.

4. END PRODUCTS OF ANAEROBIC METABOLISM

Fig. 18 shows the accumulation of succinate in *Mytilus edulis* exposed to air during 48 h. It is clear that aerobic succinate level was always low and displayed no seasonal fluctuations. After 2 days of anoxia the succinate concentration was considerably higher. Seasonal changes in succinate concentration are not very clear, though they are present in the

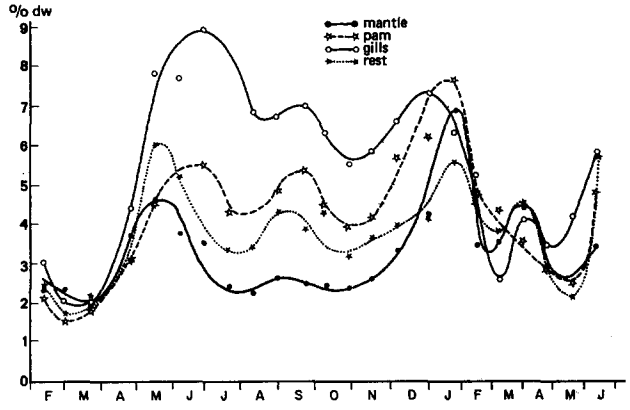

Fig. 14. Seasonal variation in taurine levels (% of dry weight) of different organs.

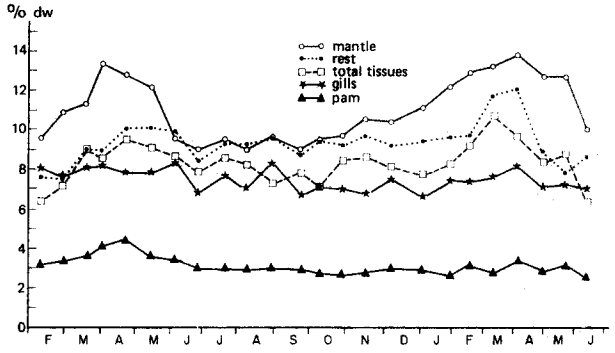

Fig. 15. Seasonal variation in lipid level (% of dry weight) of total tissues and different organs.

total amount synthesized, as appears from the conversion of succinate to propionate which is strongly dependent on seasonal variations. While aerobic levels were always low, anaerobic propionate levels fluctuated strongly and were high in summer and early autumn and virtually zero during winter.

While aerobic acetate concentrations were notably above those for propionate under the same conditions the anaerobic increases were

TABLE II

The percentage composition of fatty acids of mantle and non-mantle tissue of an August sample of *Mytilus edulis*; indicated (br) are branched iso or anteiso-methyl fatty acids.

Fatty acids	*Mantle (%)*	*Non-mantle (%)*	*Fatty acids*	*Mantle (%)*	*Non-mantle (%)*
14 : 0	2.3	2.2	20 : 0	2.2	2.5
15 : 0 br	0.1	0.1	20 : 1	4.0	4.4
15 : 0	0.6	0.5	20 : 2	2.5	3.3
16 : 0 br	0.3	0.2	20 : 3	0.4	0.2
16 : 0	16.3	13.9	20 : 4	2.2	3.6
16 : 1	10.3	8.7	20 : 5	20.5	20.0
17 : 0 br	1.5	0.6	21 : 0	0.5	0.1
17 : 0	0.8	1.0	21 : 2	1.0	0.2
17 : 1	1.0	1.0	22 : 0	0.4	0.7
18 : 0 br	2.0	1.5	22 : 1	0.2	0.2
18 : 0	2.9	3.1	22 : 2	2.8	4.1
18 : 1	5.3	4.4	22 : 4	2.0	2.0
18 : 2	0.2	1.0	22 : 5	0.5	0.5
18 : 3	0.2	0.2	22 : 6	12.4	14.0
18 : 4	1.9	1.7	23 : 0	0.3	0.3
19 : 0 br	0.3	0.3	24 : 1	1.0	1.1
19 : 1	0.4	0.3			

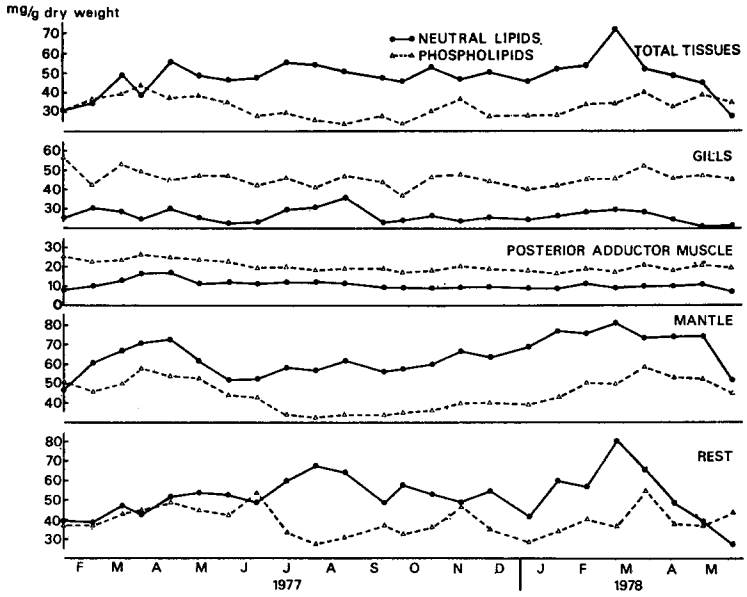

Fig. 16. Seasonal variation in neutral lipids and phospholipids (mg per g dry weight) isolated from total tissues and different organs.

generally small and never approximated the high levels observed for propionate at some periods. Moreover, seasonal changes were relatively small although there is some indication for an increased anaerobic acetate production during summer.

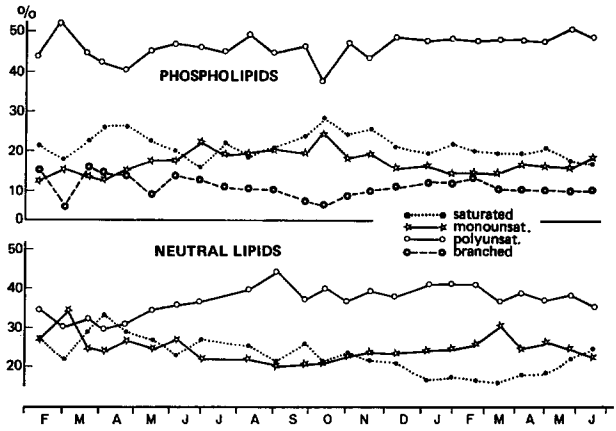

Fig. 17. Seasonal variation in relative composition (% of total) of saturated, monounsaturated, polyunsaturated and branched fatty acids of the neutral lipids and phospholipids from the total tissues.

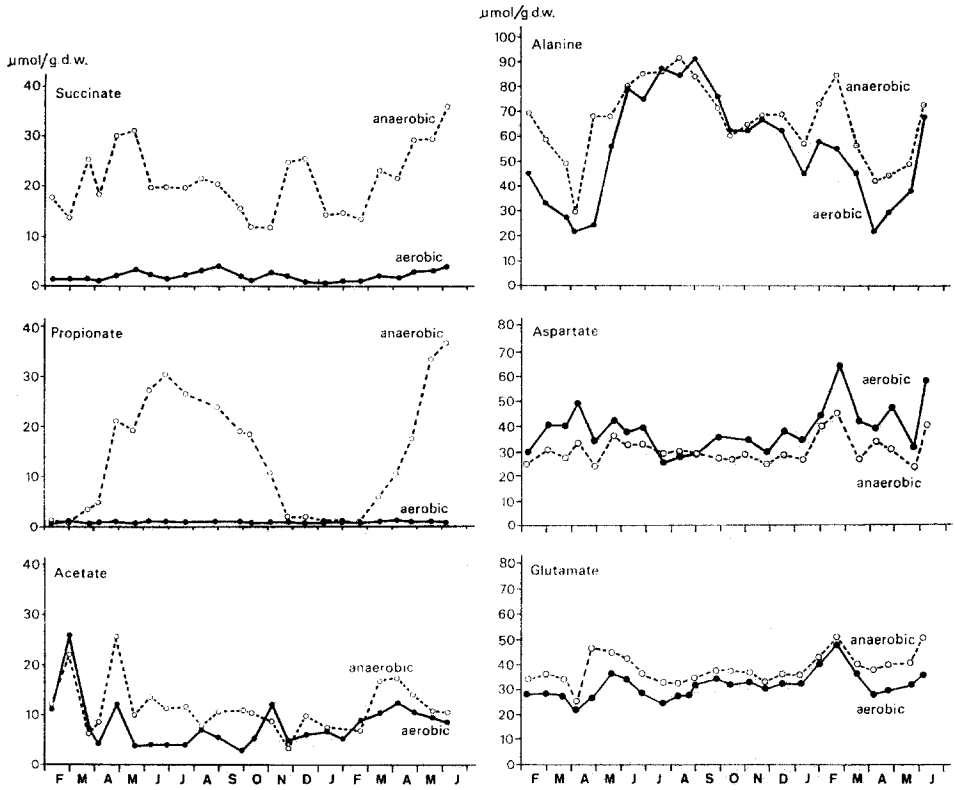

Fig. 18. Seasonal variation in the endogenous concentrations (µmol per g dry weight) of succinate, propionate, acetate, alanine, aspartate and glutamate in aerobic and anaerobic (48 h) groups of *Mytilus edulis*.

In *Mytilus edulis* at least 3 free amino acids are involved in anaerobic metabolism, *viz.* alanine, glutamate and aspartate. Alanine (Fig. 18) showed distinct seasonal changes. The fluctuations both in aerobic and anaerobic animals resembled those observed in anaerobic propionate concentration, with the highest values in summer but winter values remaining always considerably above zero. Anaerobic increases in alanine were higher in the winter periods than in summer and autumn. Seasonal changes in aspartate were rather inconspicuous although there are some indications for higher aerobic aspartate levels during the spawning periods. Anaerobic metabolism led in virtually all experiments to a decrease in aspartate concentration, suggesting a function as a substrate in anaerobiosis. Contrary to aspartate there was a small increase in glutamate levels during anoxia in all experiments which suggests that glutamate is an end product in the blue

mussel. However, as with aspartate no distinct fluctuation neither in aerobic nor in anaerobic conditions was evident, contrasting clearly with the closely related amino acids glutamine and asparagine. Both these amino acids displayed rather clear seasonal concentration changes similar to those observed for alanine (KLUYTMANS *et al.*, 1980).

IV. DISCUSSION

1. GROWTH AND CONDITION INDEX OF MYTILUS EDULIS IN RELATION TO ENVIRONMENTAL CHANGES

When considering seasonal changes in biochemical composition in growing animals it is useful to express the results in terms of the composition in weight per animal as well as in percentage of dry weight. The disadvantage of expressing the results only as a percentage is that changes in one constituent reflect changes in other constituents reciprocally.

The shell length and wet weight (Fig. 3) in relation to dry weight and water content (Fig. 4) determinations illustrate the growth of the mussels during the experimental period. Although the wet body weight stayed at the same level from October through spring, a strong winter loss of proteins and glycogen (Fig. 6) was observed. These losses are not so evident when expressed in percentages of dry weight (Figs 7 and 8 for total tissues and different organs). The loss of proteins and glycogen must be attributed to food scarcity (*cf.* ANSELL & TREVALLION, 1967; ANSELL, 1972; HANCOCK & FRANKLIN, 1972) and the metabolic demands for gametogenesis (*cf.* WIDDOWS & BAYNE, 1971). In addition the shell cavity volume was measured to calculate the condition index (Table I) (PIETERS, 1978).

The highest values of the condition index were found between June and next January with a maximum in September. The calorific content has been calculated from the protein, glycogen and lipid values. The highest calorific content was found in October, *viz.* about 60 kJ per mussel (Table III) or about 20 kJ per g dry weight. Free sugars, amino acids and other valuable organic constituents were neglected. Therefore, the food values were in reality somewhat higher. The average calorific value of the Wadden Sea mussels during the 1977–1978 season was 17.7 kJ per g (= 4.2 kcal per g), based on protein, glycogen and lipid content, which agrees with values found for Conwy mussels (4.9 kcal or 20.6 kJ per g total dry flesh weight) by DARE & EDWARDS (1975).

Both the condition index and food values make it possible to conclude that during the period from June-July until February the mussels,

TABLE III

Calorific content of proteins, glycogen and lipids in *Mytilus edulis*.

Sampling date	*Protein (kJ·100 mussels^{-1})*	*Glycogen (kJ·100 mussels^{-1})*	*Lipid (kJ·100 mussels^{-1})*	*Food value (kJ·mussel^{-1})*
1977 Feb 04	686	89	196	9.7
24	615	54	196	8.6
Mar 17	887	34	284	12.0
31	780	35	270	10.8
Apr 21	1182	115	378	16.7
May 12	1206	158	347	17.1
Jun 02	1300	536	448	22.8
23	1490	650	447	25.8
Jul 14	1987	977	624	35.9
Aug 04	1608	996	646	32.5
25	2814	1371	764	49.5
Sep 22	2175	1512	848	45.4
Oct 06	3287	1758	952	60.0
27	3334	1881	1060	62.8
Nov 17	2767	1248	892	49.0
Dec 08	2436	1213	892	45.4
1978 Jan 05	2996	1054	820	48.7
26	2400	1090	832	43.2
Feb 16	2223	856	792	38.7
Mar 09	2400	540	964	39.0
30	2247	210	892	33.5
Apr 20	1631	176	628	24.4
May 11	1182	278	540	20.0
Jun 01	946	325	365	16.4

as far as biochemical data are concerned, were suitable for consumption. This agrees with the results obtained by PIETERS (1978) who demonstrated that in the same period mussels can resist at least 3 days outside the water, *i.e.* under anaerobic conditions at a temperature of 12° C.

2. BIOCHEMICAL COMPOSITION IN RELATION TO ENERGY METABOLISM AND GAMETOGENESIS

In *Mytilus edulis* mantle and rest tissues (including the digestive gland) showed the highest glycogen and lipid levels (Figs 7 and 15) which is closely related with the specific function of these parts of the body. The annual changes in storage and utilization of these biochemical components are linked to the annual reproductive cycle (GABBOTT, 1976). The stages of gonad development after CHIPPERFIELD (1953) modified by LUBET (1959) are indicated in Fig. 7. In summer when abundant food is available in plankton, mussels are spent (stage O), which is

illustrated by a strong increase of the glycogen content. The initiation of gametogenesis (stage I) in October is followed by a period of oogenesis and vitellogenesis (stage II) which is characterized by a strong loss of carbohydrate from October through March. In March-April 1978 maturation and spawning (stage III) occurred (*cf.* PIETERS *et al.*, 1980). Comparing the Figs 7 and 15, the seasonal changes in lipid level in mantle and rest tissues showed an inverse correlation with the changes of glycogen level, which was also found by LUBET & LE FERON DE LONGCAMP (1969) and WILLIAMS (1969). The increase of lipid level during stage III could be the result of a conversion of pre-stored glycogen into lipid material for the maturating eggs. In marine bivalves the egg reserves for a fatty yolk, consisting mainly of neutral lipids (BAYNE, GABBOTT & WIDDOWS, 1975). These findings are in good agreement with those of LUBET & LE FERON DE LONGCAMP (1969) and the ideas of GABBOTT (1976) with respect to a glycogen storage cycle. In addition, an increase of products of *de novo* synthesis (16:0, 16:1, 18:0 and 18:1 fatty acids) which was observed especially in the 1977 season (Fig. 17), suggests that before spawning time glycogen will be converted to lipid material (*cf.* PIETERS *et al.*, 1979; WALDOCK, 1979).

The analyses of the fatty acid mixtures from mantle and non-mantle tissue (Table II) and from total tissues (Fig. 17) showed percentage compositions which agree fairly well with those found by GARDNER & RILEY (1972) and WALDOCK (1979). Conspicuous in our samples were the high quantities of 20:5 and 22:6 fatty acids in contrast to the results of mentioned authors.

GABBOTT & BAYNE (1973) concluded from their stress experiments with *Mytilus edulis* that in the summer all of the energy loss is accounted for by the breakdown of carbohydrate but in autumn, during more prolonged starvation, they observed a marked increase in the utilization of lipid reserves. Then, in the winter, there is a change-over to protein as the main respiratory substrate (GABBOTT, 1976). However, ZANDEE, HOLWERDA & DE ZWAAN (1980) suggested that during summer lipids are the main source of energy production in growing mussels. The relative amount of saturated fatty acids (mainly 16:0 and 18:0) of the neutral lipids decreased from April 1977 until January 1978 concomitantly with an increase of polyunsaturated fatty acids through September. The fatty acid composition of the phospholipid fraction remained nearly constant during the same period. Presumably from autumn until spring lipids are saved for gametogenesis whereas proteins and glycogen are used for both energy production and gametogenesis. A decrease of proteins, which was observed from autumn through winter, agrees with the findings of BAYNE & THOMPSON (1970), THOMPSON (1972) and GABBOTT & BAYNE (1973).

The levels of the total free amino acids in the different organs and total tissues (Fig. 10) showed little seasonal variations. The most abundant free amino acids of the different organs (Figs 9, 10, 11, 13 and 14) and total tissues, however, fluctuated seasonally. In most organs alanine, glutamate (glutamine included) and serine showed the highest concentrations in summer, whereas lowest concentrations were found for aspartate and glycine during that period. The glycine maxima observed in total tissues (Fig. 11) and in the different organs during April 1977 and February 1978 coincided with the lowest taurine, alanine, glutamate and serine levels in these organs.

Taurine and glycine, the main amino acids with highest concentrations, are generally considered as the main contributors in osmotic adaptation (*e.g.* LIVINGSTONE, WIDDOWS & FIETH, 1979). From these results it must be concluded that in early spring glycine replaces taurine in the tissues, which is in contrast with the haemolymph pattern in which serine, instead of glycine, is contributing to balance the seasonal variation in taurine (Fig. 13).

High taurine levels, combined with high alanine, glutamate and serine levels in the tissues might be the result of an active amino acid metabolism during a period of a positive nitrogen balance. The periods with a negative nitrogen balance (in winter 1977 and 1978) corresponded with high glycine levels. Glycine could be formed from carbohydrate via serine in the tissues.

3. ANAEROBIC ENERGY SUPPLY

It is clear that in the anaerobic metabolism of *Mytilus edulis*, succinate and propionate are, on a quantitative basis, much more important than alanine and acetate, except in the winter period when anaerobic increases in succinate and propionate are about equal to those in alanine and acetate (Fig. 19).

In the summer periods when succinate synthesis is strongly stimulated, the combined anaerobic acetate and alanine formation decreases, indicating an increased inhibition of pyruvate kinase which probably is the rate limiting step in their formation (HOLWERDA & DE ZWAAN, 1973). This could be caused by the increased alanine level during that period together with a more rapid internal acidification by the higher metabolic rate, since alanine and pH are well-known inhibitors of pyruvate kinase, while at the same time conversion of phosphoenolpyruvate via phosphoenolpyruvate carboxykinase to oxaloacetate and finally to succinate and propionate, is stimulated (DE ZWAAN, 1977). As phosphoenolpyruvate carboxykinase activity is not increased by alanine, the stimulation of this route could, in addition to

the rapid fall in pH by which the pH optimum for phosphoenolpyruvate carboxykinase is reached sooner, partly be mediated by an increased phosphoenolpyruvate level, changing the phosphoenolpyruvate-oxaloacetate equilibrium.

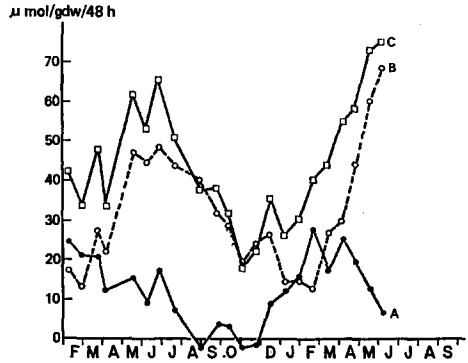

Fig. 19. Seasonal variation in the accumulation of end products of anaerobic metabolism (µmol per g dry weight in 48 h) at ambient temperatures in *Mytilus edulis*; figured are alanine plus acetate (curve A), succinate plus propionate (curve B), and sum of all end products (curve C).

The enhanced flux via phosphoenolpyruvate carboxykinase, however, does not lead to an increased succinate accumulation during 48 h of anoxia in these experiments, but to increases in the propionate production. A relation between a seasonal variable and succinate concentration is not evident, in contrast to changes in propionate which are clearly season dependent (Fig. 18). The seasonal changes in propionate show a strong resemblance to the changes in temperature and salinity of the sea water (Fig. 2). While little is known on a possible relation between salinity and anaerobic metabolism, it is known that temperature affects succinate formation, and especially its conversion to propionate (KLUYTMANS *et al.*, 1978). It therefore seems likely that the observed circannual changes in propionate synthesis are induced by changes in the environmental temperature, rather than by salinity changes. The statistical evaluation of our experimental results (Fig. 20) shows a strong correlation of anaerobic propionate synthesis with temperature which, however, is absent for succinate accumulation.

These results therefore suggest that succinate production is stimulated by elevated temperatures, but above a certain level, which seems to be rather temperature independent, no further accumulation of succinate occurs and the surplus evidently is converted to propionate.

While alanine shows a seasonal pattern somewhat similar to the

changes in the anaerobic propionate level (Fig. 18), this applies here to both aerobic and anaerobic alanine concentrations, whereas in fact the anaerobic contribution to the observed alanine level shows an inverse effect. This can be seen from curve A in Fig. 19. As the acetate

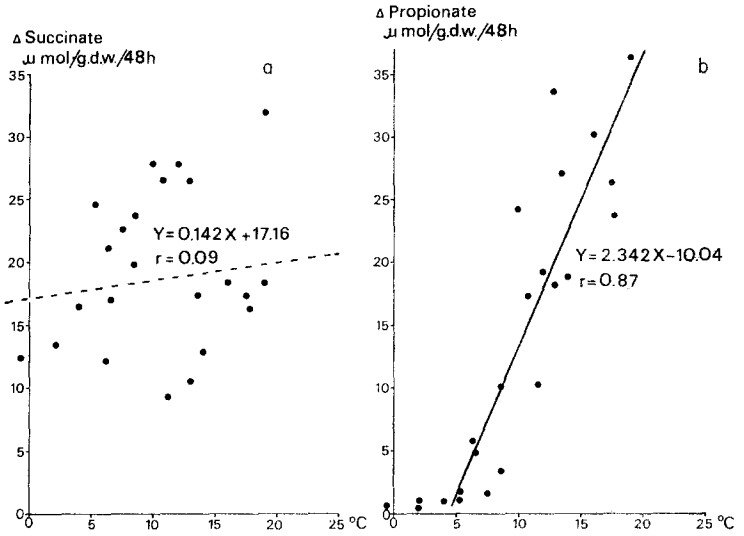

Fig. 20. Relation of the accumulation of succinate (a) and propionate (b) in total tissues of *Mytilus edulis* (μmol per g dry weight per 48 h) with temperature (°C).

contribution to this curve (Fig. 18) is more or less constant and shows virtually no seasonal changes, its form reflects the changes in the anaerobic alanine formation. Although a temperature effect on the observed seasonal alanine changes is certainly conceivable, we are nevertheless inclined to regard salinity changes as the major causative factor in this respect, in view of the well-known role of free amino acids in osmoregulation in marine invertebrates. Since in these experiments temperature and salinity changes occurred concomitantly, no cues as to their relative importance could be obtained from our data.

Fig. 21b shows the statistical relation of alanine concentration with salinity. From these data a correlation coefficient of 0.64 was calculated, which is not as high as one would have expected. The reason for this is not clear but can perhaps be attributed to considerable fluctuations in alanine level which we observed frequently in earlier experiments performed with otherwise comparable groups of animals (KLUYTMANS *et al.*, 1977).

The inverse relationship of anaerobic alanine formation to the observed seasonal changes in the total free alanine pool, indicates a feed-

back inhibition mechanism. Whether this is a direct product inhibition of either alanine dehydrogenase or glutamate-pyruvate transaminase, or functions indirectly by inhibiting pyruvate kinase activity, cannot be inferred with certainty from these experiments but the absence of a

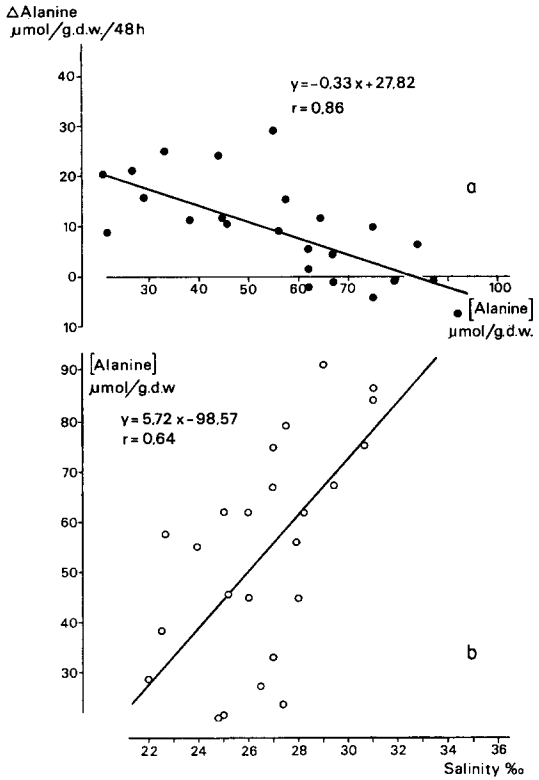

Fig. 21. a. Relation of the anaerobic alanine formation (µmol per g dry weight per 48 h) with its aerobic concentration (µmol per g dry weight). b. Relation of the aerobic alanine concentration (µmol per g dry weight) with the salinity of the sea water (‰ S).

similar seasonal change in acetate points to direct product inhibition. This conclusion is more quantitatively expressed in Fig. 21a which shows a strong correlation (0.86) between the absolute alanine level and its anaerobic formation rate. On the other hand the correlation (0.42) between anaerobic acetate accumulation and temperature which parallels changes in alanine, is much weaker.

Of the free amino acid pool two other members except alanine change in relation with anaerobiosis, *viz.* glutamate and aspartate. In

all 24 experiments except 2, aspartate decreased during 48 h of anoxia, with an average of -7.7 $\mu mol \cdot g^{-1}$ dry weight. A similar effect was recently observed by ZURBURG & EBBERINK (1980) during shorter periods of anoxia. These authors argued that *Mytilus edulis* used aspartate, which can easily be converted to oxaloacetate, as a substrate for anaerobic energy production in the early stages of anaerobiosis, before pyruvate kinase is sufficiently inhibited and phosphoenolpyruvate carboxykinase is sufficiently activated. Our experiments confirm their observations and, moreover, show that after 48 h no replenishment of the aspartate pool has taken place.

The simultaneous increase in glutamate (average 6.4 $\mu mol \cdot g^{-1}$ dry weight) could indicate that α-oxoglutarate functions as an acceptor for the amino groups of aspartate. At the onset of anoxia α-oxoglutarate and pyruvate could be available for transamination reactions from aspartate, concomitantly releasing oxaloacetate for anaerobic energy supply during the time when the switch in the phosphoenolpyruvate branchpoint must be effectuated.

In a certain sense therefore glutamate can be considered as an end product of anaerobic metabolism in *Mytilus edulis*, although it does not contribute directly to the generation of ATP or the maintenance of redox balance.

In conclusion we can say that seasonal factors influence anaerobic energy metabolism mainly in a quantitative way, leading to changes in alanine formation due to changes in salinity and/or temperature and to changes in succinate and propionate due to temperature fluctuations. Qualitative changes occur only in so far as the biosynthesis of propionate is completely switched off at temperatures below 5° C, while anaerobic alanine formation is absent in summer.

V. SUMMARY

1. Seasonal changes in biochemical composition in relation to energy metabolism and to gametogenesis were studied in *Mytilus edulis* for nearly one and a half year.
2. During the whole experimental period animals were selected from samples of the same musselbed in the Dutch Wadden Sea at intervals of about three weeks.
3. The biochemical composition of total tissues and different organs was analyzed. In growing mussels a gradual increase of protein, glycogen and lipid contents was observed from spring to autumn. From November to next April the protein and glycogen content declined, whereas the lipid content remained rather constant until spawning. The observed changes are discussed in relation to environ-

mental parameters as temperature, salinity and nutrient levels, as well as to energy production and gametogenesis.

4. Hardly no seasonal variations were found in the total free amino acid concentrations from both the total tissues and different organs. However, the individual amino acids showed clear seasonal changes in all tissues investigated. The greatest fluctuations were found for taurine and glycine. These amino acids showed an inverse relation to each other.
5. Seasonal changes in the accumulation of end products of anaerobic metabolism and in the composition of the free amino acid pool were found in groups of mussels exposed to air for 48 h. The observed variations in propionate and alanine accumulation as well as other compounds involved in anaerobic metabolism are discussed in relation to temperature and salinity.

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