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Salt and water balance in the oligohaline clam, Rangia cuneata

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Salt and Water Balance in the Oligohaline Clam *Rangia cuneata*

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I: ANISOSMOTIC EXTRACELLULAR REGULATION

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ABSTRACT

The phenomenon of hyperosmotic regulation of the blood of Rangia cuneata at low salinity is examined. The species maintains blood sodium above levels found in the ambient medium, presumably by an active uptake process which involves Na^+/NH_4^+ exchange. The mantle is suggested as the primary site of this process. At high salinity blood Na^+ is hypo-ionic to the medium and may be actively extruded. The intracellular free amino acid pool of the tissues is adjusted to compensate internally for changes in blood osmolality. During prolonged periods of anaerobiosis erosion of the shell prevents a drop in pH in the mantle fluid and blood.

INTRODUCTION

The problem of osmotic and ionic adaptation is especially great in the bivalve molluscs, which are found in habitats ranging from marine to freshwater. Krogh (1939) demonstrated the osmotic conformity of body fluids and the environment in polyhaline (18-30°/oo salinity) species, a relationship which has been confirmed many times (Robertson, 1964; Potts, 1968). In recent years osmoconformity has also been shown in species that have invaded the more dilute or mesohaline (5-18°/oo salinity) waters of estuaries (Robertson, 1964; Pierce, 1970). Although the blood of bivalves is slightly hyperosmotic, the change in osmolality parallels that of the external medium throughout the range of salinity tolerance. Although they do not regulate their blood osmolality, these species appear to regulate cell volume in large part by extrusion from intracellular fluids of non-essential free amino acids (Pierce and Greenberg, 1972). However, none of the osmoconforming bivalves can successfully inhabit the most dilute or the oligohaline zone of an estuary (0.5-5°/oo salinity), which represents the transition between estuarine and freshwater habitats. This zone has been described as a "marginal habitat" of markedly low species diversity (Odum, 1967; Castagna and Chanley, 1973), which is particularly evident in the molluscan fauna (Gainey and

Greenberg, 1977).

One of the few bivalves that successfully inhabits the oligohaline zone is the mactrid clam Rangia cuneata. Found only in the Gulf of Mexico until the mid nineteen fifties, R. cuneata is now in the midst of a population explosion along the Atlantic coast from Florida north to Maryland (Hopkins and Andrews, 1970). Throughout its geographic range R. cuneata is the dominant macrobenthic species in the oligohaline and, although its ecological range extends from 0 to 15°/oo (Castagna and Chanley, 1973), it is always most abundant and largest in size between 2 and 5°/oo (Gunter, 1961; Godwin, 1967). The key to its success in this sparsely populated habitat is twofold: 1) at 5°/oo and below anisosmotic regulation maintains the blood 55-85 mOsM above the external medium (Bedford and Anderson, 1972). The mechanism of hyperosmotic regulation has not been investigated, but it is unlikely that a shift from osmoconformity to hyperosmotic regulation at 5°/oo would result from a passive mechanism (Fyhn, 1976). 2) The species is also notable for its utilization of anaerobic pathways of metabolism, which differ from those found in vertebrate skeletal muscle in a higher yield of ATP and in end products that do not require oxidation or excretion but instead may be funnelled into other biosynthetic pathways. Perhaps most pertinent to the present investigation, Chen and Awapara (1969) reported that the anaerobic pathway terminating in alanine and succinate is

operative under normoxic as well as hypoxic conditions.

We have investigated the osmotic and respiratory adaptations that permit this unusual bivalve to exploit areas of low and fluctuating salinity. In this contribution we report the effect of the osmotic gradient between blood and water on the composition of solutes in body fluid, nitrogen excretion, and oxygen uptake. The relationship of modes of metabolism to regulation of the intracellular free amino acid pool following a salinity change is reported elsewhere (Henry and Mangum, 1978 b.c.).

MATERIALS AND METHODS

Animals were collected from the sand and mud flats of the James River estuary (0.2-2°/oo) at College Creek, near Williamsburg, Virginia, with a clam rake. The shells were cleaned of fouling organisms with a wire brush, and the animals kept in filtered, aerated aquaria in natural water at room temperature (22°C) for a period of 7 days. At that time individuals were either sacrificed for the low salinity measurements, or transferred to aquaria of York River estuary water (20-24°/oo) for 14 days.

The measurements of ions were made on animals held in James River flood waters (0.2°/oo), James River estuary water that had been concentrated by aeration and evaporation (4°/oo) and York River estuary water (24°/oo).

Blood was obtained either by draining the sinuses in the foot and adductor muscle into centrifuge tubes (Pierce, 1970), or by anaerobic syringe sampling through the hinge (Fyhn and Costlow, 1975). After centrifugation blood and water pH were measured with Radiometer G298 or GK2391C electrodes. Inorganic ions were determined with a Fisher Accumet Model 520 digital pH/ion meter and ion selective electrodes (Mangum et al., 1978). PCO_2 was determined with a Radiometer Blood Gas Apparatus (BMSI), using the pH module as the amplifier. Bicarbonate levels were calculated from

the Henderson-Hasselbach equation dissociation and solubility constants given by Horne (1969).

Ammonia excretion was determined by the phenolhypochlorite method of Solórzano (1969). Individuals were placed in 400 ml of water, and the ammonia level determined from 5 ml samples taken initially and after one hour of ventilation. 0.5 ml blood samples were diluted to 5.0 ml with ammonia-free water, and blood ammonia determined by the same procedure. Ammonia-free water was made by a double distillation using 20 g/l of potassium persulfate and 200 g/l of permanganate (K.L. Webb, pers. comm.) During the same experiments total ninhydrin positive substances (TNPS) were determined according to the method used by Clark (1964). Blood (0.3 ml) was deproteinized in 1.2 ml of 80% ethyl alcohol and centrifuged; 1.0 ml of the supernatant was then removed and assayed. Medium (1 ml) was added to 1 ml 80% ethyl alcohol; 1 ml being removed and used in the determination. A leucine standard was used as a reference. The amount of free amino acids (FAA) excreted by the animal and in the body fluid was calculated by subtracting the amount of ammonia from the TNPS value. At the end of each experiment animals were removed from their shells and dried at 60°C to a constant weight.

Ammonia excretion in the presence of ouabain was carried out according to Mangum et al. (1978). Animals were incubated in 100 ml of various concentrations of ouabain for 2 hours, and the excretion rate determined. Heartbeat and gill

cilia activity were monitored simultaneously by direct observation. Excretion values for both experiments are reported as a percent of the control excretion rates.

Intracellular free amino acids (FAA) of the adductor muscle were determined on a Technicon Auto Amino Acid Analyser (Du Paul and Webb, 1970). The tissue was excised, blotted dry, extracted with 10 ml of 80% ethyl alcohol for 48 hours, removed and dried at 60°C to a constant weight.

Oxygen uptake of intact animals was measured by a Clark type polarographic electrode (Yellow Springs Instrument Company model 5420) inserted into a sealed respiratory vessel. Oxygen uptake of excised tissue was determined in a Gilson differential respirometer.

RESULTS

At 0.2°/oo the blood of R. cuneata is hyperionic to the medium with respect to all four major cations (Table 1). Sodium and chloride, both 24mM above the medium, are the most strongly regulated ions. At 4°/oo, the cations are slightly hyperionic, and Cl^- is hypoionic, suggesting that the transition from osmoregulation to conformity takes place near the upper salinity range of the oligohaline zone. The observed enrichment of blood Mg^{+2} at 4°/oo is surprising; even though it was found in two successive sets of samples at this salinity and not in the others, contamination with intracellular fluids must be regarded as a possible explanation.

At high salinity (24°/oo) the blood ion profile is altered further, with both Na^+ and Cl^- hypoionic to the medium (Table 1), and only Ca^{+2} hyperionic. In addition, the anion deficit disappears at high salinity and the Na:Cl ratio rises from unity to 0.8, the characteristic value of seawater.

After 24 hours with valves C-clamped, blood Ca^{+2} in animals acclimated to 24°/oo doubles. At the same time mantle fluid Ca^{+2} increases almost as much to 14.52 (± 0.49 S.E.) mM/l, and mantle HCO_3^- rises from a value approximately the same as that in ambient water (1.01mM) 11.77 (± 2.77 S.E.). In 6 sets of paired observations the ratio of the Ca^{+2}:HCO_3^- increase was 1.7:1, strongly implicating the shell as a

source of buffer under anaerobic conditions. Mantle fluid pH actually increased from 7.32 to 7.42 while blood pH in a set of animals treated similarly fell by only 0.11 pH units (Fyhn and Costlow, 1975).

At both high (20°/oo) and low (2°/oo) salinity, the contribution of FAA to blood osmolality is more than 10 times that of ammonia (Table 2). The FAA, which have a net negative charge at physiological pH, are present in the blood at high salinity in a concentration equal to that of the Cl^- ion deficit. Ammonium is the only ion that is found in a lower concentration in the blood at the higher salinity. However, the rate of ammonia excretion at both salinities is approximately eight times the output of FAA. The excretion rates of both ammonia and FAA are inversely related to acclimation salinity.

The osmotic concentration of intracellular fluids of R. cuneata is controlled primarily by the intracellular FAA pool. Calculations from the data of Fyhn (1976) on tissue water and solute concentrations reveal that at a salinity of 14°/oo the adductor muscle TNPS (FAA plus NH_3) make up approximately 59% of total intracellular osmolality. The total FAA pool increases about six fold from 2°/oo to 20°/oo; the change in osmolality of the pool is about 75% of that in the ambient medium (Table 3). Alanine is by far the predominant amino acid; other amino acids of some importance include glutamic acid and glycine at low salinity, and glutamic, glycine, and

proline at high salinity. The present data on the composition of the FAA pool in R. cuneata acclimated to low and high salinity essentially agree with previous findings on acclimated animals (Allen, 1961; Fyhn, 1976), although lower values of alanine reported in the former work could be explained by differences in methodology.

At 2°/oo ammonia excretion is reduced by the presence of ouabain (Table 4). Surprisingly, however, at 20°/oo and 10^{-6} or 10^{-5}M ouabain, there is a 2-6 fold increase in ammonia excretion. Neither of these ouabain concentrations proved fatal over a period of two days.

Earlier we reported that at 2°/oo R. cuneata excretes ammonia against a molecular concentration gradient, suggesting that NH_4^+ output is coupled to an active process (Mangum et al., 1978). No evidence of ammonia excretion against a molecular gradient was found at the higher salinity.

Whole animal oxygen uptake is significantly ($P<.05$ Student's t test) higher at 2°/oo than at 20°/oo both in intact animals (Table 5) and excised gill and mantle tissue (Table 6). The salinity induced change in gill oxygen uptake (38%) is much greater than that of mantle tissue (11%), while that of adductor muscle does not change ($P>0.5$, Student's t test). The values for adductor muscle, unlike those of the other tissues, were obtained with glucose (4mM) in the medium. Without the addition of exogenous substrate, no oxygen uptake could be detected, indicating an extremely low aerobic

capacity of this tissue. At 20°/oo ouabain has no significant effect ($P>0.05$, F test) on oxygen uptake of either gill or adductor muscle (Table 6). And although mantle oxygen uptake was reduced (11.6^{-5} and 18.4%) by ouabain, the effect at high salinity is not significant ($P>.05$ F test). At 2°/oo however, there is clearly a significant ($P<.05$ F test) decrease in mantle oxygen uptake at both 10^{-5}M (14%) and 10^{-3}M (20%) ouabain. Oxygen uptake in gill and adductor muscle remained unchanged by the addition of ouabain ($P>.05$ F test).

DISCUSSION

At low salinity Rangia cuneata maintains a higher concentration of free Na^+ and Cl^- in its blood than in the ambient medium. At this salinity the animal excretes ammonia against a molecular gradient, and ammonia excretion is sensitive to ouabain, which also inhibits the activity of its $Na^+ + K^+$ ATPase (Saintsing and Towle, 1978). At 20°/oo, however, the blood is low in Na^+ and Cl^- relative to the medium, ammonia excretion against a molecular gradient cannot be detected and the process is not inhibited by ouabain. As discussed by Mangum and Towle (1977), however, the enzymatic basis of this relationship is not yet clear, and it may not entail a simple exchange of NH_4^+ for Na^+, involving no other cation. The physiological coupling of ammonia excretion and hyperosmotic regulation, however, is clear.

The energetic cost of ion transport across biological membranes has been estimated to lie in the range 20-35% of total metabolism (Bernstein, 1953; Lang et al., 1977). An increase in the oxidative metabolism of a transporting epithelial tissue such as the crustacean gill, however, is proportional to the decrease in ambient salinity and not to the osmotic gradient between blood and water (Engel and Eggert, 1974). Activity of the $Na^+ + K^+$ ATPase in this tissue is relatively high at salinities to which the blood osmotically

conforms, even though a large increase accompanies the onset of hyperosmotic regulation (Towle, Palmer and Harris, 1976). Thus a finding of ouabain sensitivity of oxygen uptake in R. cuneata mantle tissue at 20°/oo, would not be unexpected even if it were clear. More importantly, ouabain sensitivity of mantle tissue oxygen uptake at 2°/oo is quite clear and significant, while the effects on gill and muscle tissue are not (Table 6). This result implicates the mantle as a primary site of Na^+ uptake, which is strongly supported by the relative $Na^+ + K^+$ ATPase activities of various tissues (Saintsing and Towle, 1978).

That the mantle should be a major site of active ion uptake in R. cuneata is not unexpected. The mantle is highly vascularized, perfused with both blood and external medium, and its secretory activity of divalent cations has been known for many years (Wilbur, 1964).

Although the depression of gill oxygen uptake by ouabain is not significant, the absolute rate is much higher than that of either mantle or adductor muscle (Table 7), and the difference increases from 20% at high salinity to 44% at low salinity. This indicates that the gill is performing a highly oxidative function at 2°/oo which the mantle and the adductor muscle do not, and which the gill itself does not perform at 20°/oo. The difference at 20°/oo may reflect the activity of gill cilia, but the increase at low salinity is unlikely to be related to ciliary activity, which either decreases or

remains unchanged at low salt levels (Ghiretti, 1966). It is suggested that the salinity-induced increase in gill oxygen uptake may reflect its role in reducing the size of the free amino acid pool, which is examined more thoroughly by Henry and Mangum (1978c).

The lack of detectable oxygen uptake by adductor muscle in the absence of glucose, and the small value obtained in the presence of 4mM glucose provides additional evidence of the low aerobic capacity of deep tissue in bivalves, which extracts very little oxygen from the blood (Booth and Mangum, 1978). Although glycogen, the primary source of energy for bivalves, occurs in high concentrations in gill and mantle tissues (de Zwaan and Wijsman, 1976), the levels in muscle tissue have not been measured. Moreover, glycogen is utilized as substrate for anaerobic as well as aerobic metabolism. The role of anaerobic pathways in salt and water balance of R. cuneata is considered in more detail elsewhere (Henry and Mangum, 1978b).

The pattern of osmoconformity in R. cuneata acclimated to high salinity is very much like that in other phyla. While the blood is slightly hyperosmotic to the external medium, the two major ions, Na^+ and Cl^- are significantly hypoionic. It is not known whether the hyposaline condition of the blood at high salinity, which is also true of annelids (Oglesby, 1969) and crustaceans (Weiland and Mangum, 1975) is maintained by active extrusion of Na^+ and Cl^-. The simple and direct proportionality

of blood and ambient osmolality above 4°/oo does not implicate an active process. Further investigation of the possibility of active Na^+ extrusion would be warranted, however, by the following results: 1) the ouabain-induced increase in net ammonia output at high salinity, 2) the ouabain-induced decrease in mantle oxygen uptake, and, 3) the disappearance of net ammonia output against a concentration gradient at high salinity.

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Table 1: The concentration of free ions in the blood of Rangia cuneata acclimated to low and high salinity. mM/l (mean ± S.E.) N=4-6

	Na^+	K^+	Ca^{+2}	Mg^{+2}	Cl^-
blood	26.5± 1.2	0.65± 0.14	2.74± 0.22	2.68± 1.01	26.2± 1.4
medium (0.2°/oo)	2.68	0.05	0.75	0.41	2.08
blood	61.3± 0.4	2.31± 0.02	2.74± 0.30	6.16±	62.3± 1.6
medium (4°/oo)	60.8	1.21	1.87	2.53	71.8
blood	309.0± 3.8	6.78± 0.05	7.71± 0.10	28.72±	376.5± 5.9
medium (24°/oo)	316.3	6.72	6.00	29.00	391.8

Table 2: Nitrogen excretion and blood nitrogen levels in Rangia cuneata acclimated (14 days) to low and high salinity. 22°C (mean ± S.E.) N=10

salinity	blood ammonia μM/l	ammonia excretion μM/gm.dry wt.-hr.	blood FAA mM/l	FAA excretion μM/gm.dry wt.-hr.
2°/oo	192± 16	33.4± 2.8	2.64± 0.25	4.1± 1.3
20°/oo	151± 13	23.9± 3.7	14.5± 1.2	3.0± 0.68

Table 3: Adductor muscle FAA of Rangia cuneata acclimated to low and high salinity. μ moles/gm. dry wt. (mean ± S.E.) N= 5

Amino Acid	2°/oo	% total	20°/oo	% total
Cysteic acid	--	0	--	0
Taurine	--	0	--	0
Aspartic acid	0.96 ± 0.3	1.6	2.5 ± 0.8	0.7
Threonine	1.5 ± 0.1	2.4	5.7 ± 0.9	1.5
Serine	2.0 ± 0.3	3.4	5.3 ± 1.0	1.4
Glutamic acid	11.9 ± 0.7	19.7	30.0 ± 2.3	8.1
Proline	0.2	0.3	22.1 ± 1.2	6.0
Glycine	6.3 ± 1.4	10.4	26.7 ± 2.7	7.2
Alanine	27.2 ± 2.3	44.7	258.3 ± 14	69.7
Valine	0.5 ± 0.1	0.8	1.7 ± 0.1	0.5
Cystine	--	0	--	0
Methionine	<0.1	<0.1	1.3 ± 0.1	0.4
Isoleucine	0.5 ± 0.1	0.8	1.5 ± 0.1	0.4
Leucine	0.4 ± 0.1	0.7	1.2 ± 0.1	0.3
Tyrosine	0.4 ± 0.1	0.6	2.5 ± 0.1	0.7
Phenylalanine	0.5 ± 0.1	0.8	4.6 ± 0.2	1.2
Ornithine	3.9 ± 0.6	6.6	1.2 ± 0.1	0.3
Lysine	1.4 ± 0.1	2.3	4.0 ± 0.4	1.1
Tryptophan	0.8 ± 0.2	1.3	--	0
Histidine	2.3 ± 0.4	3.8	2.2 ± 0.1	0.6
Arginine	0	0	0.7	0.2
TOTAL	60.7 ± 3.3	100	370.8 ± 14	100

Table 4: Sensitivity of ammonia excretion to various concentrations of ouabain for Rangia cuneata acclimated 6 weeks to low and high salinity - values expressed as percent of control excretion rate for paired observations on same individuals. (mean ± S.E.) N=6

	control	percent inhibition by ouabain		
salinity	μmoles/gm.dry wt.-hr.	10^{-6}M	10^{-5}M	10^{-4}M
2°/oo	15.7 ± 2.7	33.3 ± 13	37.6 ± 6.9	41.9 ± 0.8
20°/oo	6.3 ± 1.6	-129.3 ± 28	-367.6 ± 106	———

Table 5: Oxygen uptake on intact Rangia cuneata acclimated for 14 days to low and high salinity at 22°C. Mean ± S.E. for readings between 100% and 70% saturation made on the same individual. $\mu l/O_2/gm.$ dry wt.-hr. N=6

	salinity		
clam	2°/oo	20°/oo	% increase at low salinity
1	716 ± 63	631 ± 59	13.5
2	575 ± 48	447 ± 82	28.6
3	588 ± 20	489 ± 28	20.0
mean	626 ± 45	522 ± 56	20.7 ± 4.4

Table 6: Oxygen uptake ($\mu l/O_2$/gm. dry wt.-hr.) by exised gill, mantle, and adductor muscle, of Rangia cuneata after acclimation for 3 weeks to high and low salinity and in the presence of various concentrations of ouabain at 22°C. Mean ± S.E. N=6

		ouabain		
2°/oo	control	10^{-5}M	10^{-4}M	10^{-3}M
gill	1093.6 ± 152	1288.9 ± 109	1290.3 ± 96	1017.4 ± 57
mantle	613.2 ± 30	527.9 ± 25	———	488.6 ± 22
adductor muscle	168.5 ± 12	———	———	155.0 ± 6.7
20°/oo				
gill	679.7 ± 22	731.4 ± 25	695.9 ± 47	800.9 ± 75
mantle	546.3 ± 45	483.1 ± 50	———	445.7 ± 33
adductor muscle	157.2 ± 14	———	———	186.6 ± 19

II: ACCUMULATION OF INTRACELLULAR FREE AMINO ACIDS DURING HIGH SALINITY ADAPTATION

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ABSTRACT

During adaptation to an increase in ambient salinity *Rangia cuneata* synthesizes free amino acids *de novo* from keto acids produced from a modified Embden-meyerhof glycolytic pathway and free ammonia. It is suggested that the pathway for this is direct amination of pyruvate to alanine. Oxygen is not necessary for the production of FAA but is critical to the survival of the species during high salinity adjustment.

INTRODUCTION

For many years it has been known that aquatic invertebrates possess significant amounts of nonessential free amino acids (FAA) in their tissues. Large quantities of alanine, glycine, glutamate and taurine exist in members of the major phyla. These amino acids and also proline and aspartic acid are responsible for the majority of the FAA pool in various species (Simpson et al., 1959; Allen, 1961; Awapara, 1962; Lange, 1963; Lynch and Wood, 1966; Gilles and Schoffeniels, 1969; Florkin and Schoffeniels, 1969; Virkar and Webb, 1970; Du Paul and Webb, 1970, 1974; de Zwaan and Zandee, 1972). Because the size of the pool varies directly with environmental salinity (Allen, 1961; Lynch and Wood, 1966; Florkin and Schoffeniels, 1969; Du Paul and Webb, 1970; Pierce, 1971a) the FAA are believed to function as intracellular osmotic effectors, by maintaining the isosmotic condition of intracellular and extracellular fluids (Florkin and Schoffeniels, 1969). Indeed, Pierce (1971a) has suggested that the limits of euryhalinity in osmoconformers such as bivalve molluscs depend upon the ability of the cells to withstand changes in volume in the face of changing external salinity, which in turn depends upon the degree to which the size of the FAA pool can be regulated.

The mechanisms that control the size of the labile FAA pool are not fully understood. An increase in FAA during

adaptation to an increase in ambient salinity, for example, has been attributed to 1) active uptake of FAA from the medium (Wright and Stephens, 1977), 2) catabolism of protein either intracellular (reviewed by Bishop, 1977), or extracellular (Siebers et al., 1972) and 4) de novo synthesis (see review by Schoffeniels, 1976). In crustaceans, the third hypothesis is supported by evidence of the activity in various tissues of glutamate dehydrogenase (GDH), which performs reductive amination, a necessary precedent of the series of transamination reactions. This enzyme fixes ammonia onto ∝ ketoglutarate (∝KGA) forming glutamate, which is then transaminated to any number of Tricarboxylic Acid Cycle (TCA) or glycolytic intermediates, thus building up the FAA pool (Florkin and Schoffeniels, 1969; Gilles, 1974a, 1975; Schoffeniels, 1976). However, significant activity of GDH is not found in molluscs (Campbell and Bishop, 1970; Addink and Veenhof, 1975; Wickes and Morgan, 1976; Reiss et al., 1977) and, in a recent review Bishop (1977) concludes that the enzyme responsible for initial ammonia fixation has yet to be described.

Another finding in recent years is the notable anaerobic capacity of many of the bivalve molluscs, especially the euryhaline species. The glycolytic Embden-Meyerhof pathway terminating in lactate (eg. mammals) is modified in these animals to yield a variety of end products, including the free amino acids alanine, glutamate, and proline (Stokes and Awapara, 1968; de Zwaan and Van Marrewijk, 1973). This mode of anaerobic

metabolism operates under normoxic conditions, concomitant with notably high rates of ventilation and seemingly inefficient oxygen extraction from the ample supply and with rates of oxygen uptake that are not conspicuously low, either in whole animals or in isolated gill and mantle tissue (Chen and Awapara, 1969b; Mangum and Burnett, 1975; Henry and Mangum, 1978a.). It has been suggested that this coupling of physiological characters, which appears paradoxical from an energetic point of view, may be understood in terms of the importance of the end products in cell volume regulation (Mangum and Burnett, 1975) In support of the idea that anaerobic pathways are critical to the process, Baginski and Pierce (1975) showed that oxygen uptake of isolated heart muscle decreases while the FAA pool is being enlarged during high salinity adaptation.

The oligohaline clam Rangia cuneata is particularly tolerant of hypoxia; at room temperature it survives at least three weeks in deoxygenated water, however, anaerobic metabolism is carried out in aerated water as well (Chen and Awapara, 1969b). The reactions involved in anaerobic glycolysis are known in detail (Allen and Awapara, 1960; Awapara and Campbell, 1964; Simpson and Awapara, 1964, 1965, 1966; Stokes and Awapara, 1968; Chen and Awapara, 1969a,b) as are the changes in extracellular osmolality (Bedford and Anderson, 1972). This report deals with the relationship between ambient PO_2, anaerobiosis, and intracellular FAA accumulation during adjustment to an increase in salinity.

MATERIALS AND METHODS

Collection and Storage of Animals

Animals were collected and stored in the manner described by Henry and Mangum (1978a).

Ammonia Excretion and Blood Ammonia Levels

Ammonia levels in the blood and rates of excretion were determined as previously described (Henry and Mangum, 1978a) at intervals following transfer from 2°/oo to 20°/oo water.

FAA Excretion and Blood FAA Levels

Total ninhydrin positive substances (TNPS) in the body fluid and external medium were measured simultaneously with the ammonia determinations, as reported in Henry and Mangum (1978a).

Whole Animal Oxygen Uptake

Oxygen uptake was determined by monitoring the decline in O_2 in a closed container with a Yellow Springs Instrument Co. Model 54 oxygen electrode and Model 80 recorder. Rates were determined immediately before and after exposing a 2°/oo acclimated animal to 20°/oo seawater, both under normal conditions and after 24 hours of exposure to 10^{-5}M iodoacetate.

Adductor Muscle Intracellular FAA

Intracellular FAA of the anterior and posterior adductor

muscles of R. cuneata were determined on a Technicon Automated Amino Acid Analyzer (Du Paul and Webb, 1970; Henry and Mangum, 1978a). The FAA were determined at intervals following transfer from 2°/oo to 20°/oo under normoxic (aerated) and hypoxic ($PO_2 < 0.1$ ppm) N_2-flushed water. The experiments in normoxic water were repeated after the addition to the acclimation medium (2°/oo) of 10^{-5}M iodoacetate, a known glycolytic inhibitor, 24 hours prior to transfer to 20°/oo seawater plus 10^{-5}M iodoacetate.

RESULTS

Changes in the Intracellular FAA Pool

The composition of the intracellular FAA pool in R. cuneata adductor muscle (Table 1) is typical of many bivalve molluscs, but markedly different from others. In animals acclimated to 2°/oo, alanine comprises about half of the total FAA pool, with glutamate and glycine also being found in appreciable amounts. Aspartic acid, serine, threonine, ornithine, lysine, tryptophan, and histidine together account for 21.3% of the total intracellular FAA, although none of them alone make up more than 7%. Cysteic acid, taurine, cystine, and arginine were not detected, and proline and methionine were found in only one of the five samples.

Upon transfer from 2°/oo to 20°/oo the animals close their valves. A slight opening appears from 4 to 12 hours, and then progressively enlarges until, by 24-48 hours, all animals are ventilating through protruded siphons.

After clams are transferred from low to high salinity the FAA pool increases dramatically (3x) during the first 6 hours (Table 1). There is no further accumulation until 24 hours, when the FAA pool again begins to build up. By nine days exposure to 20°/oo the total pool size is approximately six times that in animals acclimated to 2°/oo, and twice the plateau value seen at 6-24 hours. The complex time course is similar

to that in Mya arenaria (Du Paul and Webb, 1970), while it appears to be unlike that in Modiolus demissus (Baginski and Pierce, 1975, 1977). A plateau could have gone unnoticed during the longer initial interval used in their experiments.

The individual free amino acids display unique patterns of accumulation (Table 1). 74% of the total increase during the first 6 hours is due to the changes in glycine and alanine. From 6 to 12 hours glycine remains virtually unchanged, but then it begins to decline to only half the quantity (at 24 hours) that had been present at 6 and 12 hours. Between 24 and 48 hours there is a small secondary rise in glycine, but by 9 days glycine has again declined to a minor component of the intracellular FAA pool. In contrast, alanine continued to accumulate throughout the entire experiment. By 12 hours it had risen to essentially the same level as glycine, and it continued to increase as glycine declined. By 9 days alanine levels were almost three quarters of the total pool.

Of the other amino acids, none showed as interesting or as clear a pattern of accumulation as alanine and glycine. Glutamate levels oscillated during the initial phases of the experiment, and then slowly increased during the remainder. Proline was detectable only in trace amounts until 12 hours when it began to accumulate to an appreciable fraction of the total pool. In notable contrast to several other bivalves, taurine was also found only in small amounts, and it never became important. The remaining FAA showed only slight increases

except for ornithine, which declined, and tryptophan, which disappeared altogether.

Changes in Blood Amines and Nitrogen Excretion

During the period of tight valve closure following the transfer to high salinity ammonia excretion virtually ceased, which is hardly surprising, and it remained negligible until 48 hours (Table 2). At that time a decrease in the level of ammonia in the medium raises the possibility of net uptake. By 4 days ammonia excretion was again detectable; at 7 days the rate had risen sharply, and at 14 days, long after blood osmolality had stabilized, it appeared to level off. The rate at $20^{o}/oo$ is significantly ($P<0.05$, F test) lower than that for animals acclimated to $2^{o}/oo$. In contrast, when ammonia excretion at $2^{o}/oo$ was measured at intervals during a 3 week period with no salinity change, the rate did not decrease ($P>0.05$, F test).

During the initial period of tight valve closure and no ammonia excretion (0-4 hours after the high salinity shock), the accumulation of ammonia in the blood is significant ($P<0.05$, Student-Newman-Keuls test); from 4-12 hours when the valve opening and ammonia excretion are both small, blood ammonia drops significantly ($P<0.05$, SNK test). There is a significant increase in blood ammonia at 16 hours, but this value was still significantly lower than that recorded at 4 hours ($P<0.05$, SNK test). From 16 hours to 4 days, the level of ammonia in the blood gradually declined ($P<0.05$, SNK test), and, as acclima-

tion progresses,,blood ammonia rises and stabilizes at a level which is significantly ($P<0.05$, SNK test) lower than in animals acclimated to 2°/oo (Table 2). The transient peak in blood ammonia that accompanies the large increase in FAA from 0 to 4 hours is probably a result of accumulation of normal protein metabolism, producing ammonia that is simply prevented from leaving the animal by the shell closure.

In contrast to the changes in ammonia output free amino acid excretion exhibits no clear trend (Table 2); the values fluctuate over the first 24 hours of the experiment, and then they remain fairly constant. Blood FAA do not change significantly ($P>0.05$, SNK test) until 24 hours after the salinity change, and then the level increases, first by threefold and later by almost sixfold.

Changes in Oxidative Metabolism

Oxygen uptake of intact animals remains fairly constant over a wide range of environmental PO_2, until ceasing abruptly at a critical low value of 18-21 mm Hg (Figure 1). After transfer from 2°/oo to 20°/oo oxygen uptake ceases entirely for 16-22 hours, regardless of environmental oxygen conditions (Table 3). When oxygen uptake was resumed, four of six animals showed a transient increase. By the completion of acclimation, however, the rate of oxygen uptake in three of the four surviving animals had fallen to a lower rate than at 2°/oo.

In animals incubated in 10^{-5}M iodoacetate for 24 hours prior to the salinity change the return to oxidative meta-

bolism was much more rapid (Table 4), occurring in less than half the time than in the controls. At the time of resumption of ventilation oxygen uptake is, significantly ($P<0.05$, Student's t test) lower than that at 2°/oo.

Effect of Anaerobic Conditions on High Salinity Adaptation

Under anaerobic conditions, both the quantitative and qualitative aspects of FAA accumulation differ dramatically from the normoxic trends (Table 5). The threefold increase in FAA during the initial period of 0-6 hours disappears, the FAA pool size remains significantly lower ($P<0.05$, F test) until 24 hours after the salinity change, and the plateau between 6 and 24 hours disappears. Nonetheless at 48 hours, the size of the FAA pool is essentially the same, regardless of PO_2 ($P>0.05$, F test). In contrast to the absence of mortality in normoxic water, the combination of hypoxia and hypersaline shock resulted in 25% mortality at 48 hours and 100% mortality at 72 hours.

The changes in composition of the FAA pool were also somewhat different under anaerobic conditions. No transient peaks occurred in any of the three major amino acids (Table 5). Glycine decreased during the initial 6 hours and then remained low in both absolute quantity and percentage of the total pool. Alanine increased more slowly during the first 12 hours, but after 24 hours the levels were nearly the same under aerobic and anaerobic conditions. There was also no difference in the percentage of the total FAA pool made up by alanine at 48 hours. Glutamate accumulated gradually over the duration of

the experiment; by 48 hours the level was significantly higher ($P<0.05$, F test) under anaerobic conditions (Tables 1 and 5).

Effects of Inhibition of Anaerobic Metabolism

As expected, the total FAA pool increases significantly less in the presence of oxygen and 10^{-5}M iodoacetate (Table 6). During the first 6 hours there is an actual reduction in the size of the pool of about 25%. At the same time intervals the values for total FAA in Table 6 are 27-65% lower than those for control conditions (Table 1), and 34% lower than for anaerobic conditions (Table 5). The differences tend to decrease with time. In addition, alanine buildup is blocked by iodoacetate for approximately 24 hours, after which it increases at a significantly lower rate ($P<0.05$, F test). At 48 hours the level remains somewhat lower than in control or anaerobic animals.

The pattern of glutamate increase was very similar to that of alanine, but glycine failed to increase at any time. Once again, the remaining FAA did not contribute significantly to the total pool, and did not vary significantly throughout the experiment. No other amino acid compensated for the suppression of alanine accumulation. The net result was a pool of FAA only 73% as great after 48 hours without fully operative anaerobic pathways as after normal adaptation to high salinity (Tables 1 and 6).

DISCUSSION

Role of the FAA Pool

R. cuneata is typical of many bivalves in utilizing intracellular FAA as osmotic effectors. Moreover, the major component of the FAA pool, alanine, is synthesized by non-oxidative pathways of metabolism (Stokes and Awapara, 1968). This amino acid is also among the chief osmotic effectors in several other bivalves: *Mya arenaria* (Virkar and Webb, 1970), *Modiolus demissus granosissimus* (Pierce, 1971a), and *Spisula solidissima* (Du Paul and Webb, 1974). Thus it has been suggested that the conspicuous anaerobic capacity of these animals may be related to the fueling of osmotic adaptation (Mangum and Burnett, 1975; Baginski and Pierce, 1975).

There is, however, a group of bivalves in which different amino acids dominate the pool. Taurine comprises 70% of the total in at least three species: *Mytilus edulis* (Lange, 1963), *Modiolus squamosus* (Pierce, 1971a), and *Mercenaria mercenaria* (Du Paul and Webb, 1974). Although the biochemical diversity suggests considerable plasticity in the mode of adaptation to a common environmental problem, the significance of the difference is certainly not clear. Neither taxonomic affinity, degree of euryhalinity nor anaerobic capacity are clearly correlated with the pattern. Two closely related species belonging to the mussel genus *Modiolus* utilize different amino acids (Pierce, 1971a), and species belonging to different euryhaline

genera such as Rangia and Mya have similar FAA profiles as the stenohaline S. solidissima. Thus the selection of a component such as taurine may be more closely related to other physiological functions such as digestion (de Zwaan and Van Marrewijk, 1973).

Mechanism of Enlargement of the FAA Pool

The present data are not easily explained by several of the mechanisms postulated to control the size of the FAA pool. Firstly during the initial period of tight valve closure immediately following the salinity change, the only FAA available for active uptake from the ambient medium are those in mantle fluid, a total of approximately 0.02 μmoles. And yet the increase during this period is an absulute value of 24 μmoles in the adductor muscles alone. Thus this mechanism can only be possible in the latter stages of adaptation. Moreover, an increase in FAA in deep tissue such as the adductor muscle would have to involve FAA transport in the blood from the site of uptake to the target organ, and yet no change in blood FAA can be detected (Table 2). In addition, Bishop (1977) has calculated that, at measured rates of FAA uptake, the process would require a minimum of 12 days and in some species as much as 113 days to reach the level which is actually achieved in only 7 days. Finally, the initial FAA buildup is blocked by iodoacetate (Table 6), a specific glycolytic inhibitor which should not interfere with active amino acid uptake. The data for R. cuneata do not exclude the possibility of an exogenous

source of FAA after ventilation is resumed. The apparent uptake of ammonia from the medium at 24-48 hours and the concomitant sharp rise in blood FAA (Table 2) could be interpreted as support for this suggestion, although an equally likely source of the FAA in blood is leakage from the cell into the blood. Animal cells both concentrate amino acids against a gradient, and leak them out again into the surrounding medium (reviewed by Heinz, 1972). The delayed appearance of FAA in the blood may result from the establishment of an equilibrium between the two processes in which leakage at high salinity exceeds uptake.

Secondly, if an increase in FAA were achieved by a uniform hydrolysis of protein, it would not result in the striking changes in the percent composition of the pool (Schoffeniels, 1976). Changes in the pool size are always biased in favor of a few particular amino acid species (eg. Table 1), and there is no evidence of peptide stores consisting primarily of these residues. Moreover, the relationship between body fluid osmolality, FAA pool size, and total protein content is actually direct (Fyhn, 1976), providing no evidence of protein hydrolysis as a control mechanism.

The hypothesis which is most consistent with the present findings is the de novo synthesis of free amino acids, involving the fixation of ammonia onto the keto acids produced in carbohydrate metabolism. Like other bivalves, the very large glycogen stores (10-25% of dry weight) in R. cuneata, together with the algal diet, indicate a carbohydrate based metabolism

(de Zwaan and Van Marrewijk, 1973; de Zwaan and Wijsman, 1976). The major gaps in this hypothesis have been an unidentifiable source of ammonia for the initial reductive amination, and an unknown pathway by which the ammonia fixation takes place. In both crustaceans (Mangum and Towle, 1977) and in R. cuneata (Henry and Mangum, 1978a), however, it is very clear that the relationship between ammonia production and ammonia excretion is not simple and linear. In R. cuneata blood ammonia levels rise during the initial period of FAA accumulation (Table 2) and then drop off precipitously; net ammonia output remains virtually undetectable throughout the period of first high and then low levels of ammonia in the blood. It is not unreasonable to suggest that reversal of the increase in blood ammonia results from its utilization in amination of keto acids.

Modes of Metabolism and the Production of Alanine

After valve closure the oxygen remaining in the mantle fluid, body fluid, or tissue of most bivalves is reduced in less than one half hour to levels that do not support oxidative metabolism (de Zwaan et al., 1976), and thus it is likely that the threefold increase in the total FAA pool (Table 1) took place anaerobically, although perhaps not exclusively by anaerobic pathways. R. cuneata has a fully operational TCA cycle (Awapara and Campbell, 1964) which, in other species, still operates under anaerobic conditions (Livingstone and Bayne, 1974; de Zwaan et al., 1976). Thus both modes of metabolism

could have contributed to the initial increase in FAA. Conversely, the second increases in FAA, after oxygen uptake had resumed (Tables 1 and 3) could involve anaerobic pathways, which continue to operate in the presence of oxygen (Chen and Awapara, 1969b).

Alanine, the most important amino acid in R. cuneata in both initial and final stages of high salinity adaptation, is produced from pyruvate by nonoxidative reactions (Stokes and Awapara, 1968). The mechanism is believed to be transamination from glutamate, but this hypothesis is difficult to reconcile with the present findings, for several reasons. The simplest pattern expected of a metabolic intermediate would be an initial rise as glutamate is first produced, then a stabilization as production and conversion to the end product proceed simultaneously, and finally a decline to previous levels as it is used up. The simple pattern described above does not occur (Table 1). It might not be seen if transamination proceeded as fast as production, but glutamate showed no increase (6-12 hours in Table 1) when alanine remained constant, yielding no evidence of transamination. Under anaerobic conditions (Table 5) glutamate continuously increased, along with alanine. This buildup of glutamate occurred under conditions in which the activity of the TCA cycle, and hence the levels of αKGA, must have been depressed, and yet alanine production remained high, it suggests if anything, a transamination from alanine to αKGA. Moreover,

when alanine production, and thus the postulated transamination from glutamate, was blocked by iodoacetate, the precursor glutamate did not accumulate (Table 6). In fact, no other amino acid increased during the period in which alanine accumulation was suppressed, yielding no indication of an amino acid precursor, which donates its amino group to pyruvate, suggesting instead that alanine is formed from the direct reductive amination of pyruvate. Also, as pointed out by Hochachka and Somero (1977) the high turnover rate of αKGA required for energy production during anaerobic metabolism in molluscs would deplete the supplies available for amination to glutamate.

The transamination of aspartate to pyruvate has also been postulated to account for alanine synthesis in bivalves (Du Preez and Webb, 1971). This sequence is equally inconsistent with the results for R. cuneata. The suggestion that alanine in bivalves is formed by direct reductive amination of pyruvate was introduced by de Zwaan and Van Marrewijk (1973). Although the reaction is known only the bacteria (Meister, 1965), these investigators point out that the well established anaerobic pathways in bivalves are very similar. Under normoxic conditions R. cuneata produces alanine in a ratio of 7:1 over succinate (Chen and Awapara, 1969b), thus at the moment of salinity change and the accompanying onset of anaerobiosis, metabolism is highly geared for alanine production, and hence the initial rise (Table 1). As NAD^+ is reduced to NADH, succinate production increases

to maintain redox balance, diverting phosphoenolpyruvate (PEP) from pyruvate production, which results in a decline in the ratio of alanine to succinate to unity after 6 hours of anaerobiosis (Stokes and Awapara, 1968). In Table 1 the slowdown in alanine synthesis appears at 6-12 hours while metabolism remains anaerobic (Table 3). Between 12 and 24 hours alanine accumulation is accompanied by a reduction in glycine (Table 1), which raises the possibility of a transamination of glycine to alanine during the time when alanine amination declines. When the animal resumes oxidative metabolism at 24 hours (Table 3), the role of succinate as an electron acceptor is superseded, and alanine production is again favored, and the level rises (Table 1).

Regardless of oxygen level or mode of metabolism, alanine appears to be synthesized de novo (Tables 1, 5 and 6). The hypothesis of de Zwaan and Van Marrewijk (1973) of reductive amination of pyruvate requires an alanine dehydrogenase, which is unknown in most animal tissues. However, an N-(1-carboxyethyl)-alanine dehydrogenase has recently been found in the cockle Clinocardium nuttali (Fields, 1977).

Although blocked by iodoacetate for 24 hours, alanine accumulation eventually occurs (Table 6). The delayed accumulation is probably due to the competitive action of the inhibitor on glycolysis and the release of inhibition after buildup of the substrate, D-glyceraldehyde-3-phosphate to threshold concentrations. Alternatively, it is possible that the drug simply wore

off or was metabolized by the animal.

Alanine is regarded as a transient product of anaerobic metabolism in bivalves, with propionate and CO_2 being the final metabolites (de Zwaan et al., 1976). Pyruvate kinase (PK), the enzyme that channels PEP toward alanine production, is inhibited by the product alanine, the process being enhanced at lowered pH. Under anaerobic conditions the conversion of neutral carbohydrates into organic acids lowers the pH in Mytilus edulis from between 7.3 and 7.5 to between 6.7 and 6.5 (de Zwaan et al., 1976), which not only inhibits PK, but activates phosphoenolpyruvate carboxykinase (PEP-CK), and directs PEP from alanine and towards succinate production. In R. cuneata, erosion of the shell during anaerobic conditions prevents an appreciable drop in pH (Fyhn and Costlow, 1975; Henry and Mangum, 1978a), and thus product inhibition would remain minimal. In addition, PK and PEP-CK operate simultaneously in R. cuneata with almost identical activities (Chen and Awapara, 1969a) thus resulting in both alanine and succinate production, not an "either/or" situation. Unlike other bivalves, in which alanine declines to undetectable levels after prolonged anaerobiosis (de Zwaan et al, 1976) alanine persists in R. cuneata, although its synthesis is slowed down (Tables 1 and 5).

Origin of Glycine

The origin of the transient glycine peak (Table 1) is

unclear. A transient increase in serine, which would support the hypothesis of synthesis from glycolytic intermediates (ie. the serine cycle), was not found. If the amino group of glycine were derived from alanine via transamination, then the lower rate of alanine accumulation relative to glycine accumulation would not be surprising (0-6 hours, Table 1). It is conceivable that early glycine production opposes product inhibition of PK during the initial stages of anaerobiosis by drawing off alanine. As anaerobiosis progresses transamination back to alanine would also be advantageous if the deamination of glycine to acetate were accompanied by the formation of ATP. This additional phosphorylation, however, is known only in algae, and not in bivalves (de Zwaan et al., 1976). Du Paul and Webb (1970) suggest that during high salinity acclimation of Mya arenaria glycine may not be synthesized de novo but rather released from an osmotically inactive form. In R. cuneata, however, the glycine peak is labile, and it disappears under anaerobic conditions (Table 5). It is not clear that the release of free glycine would be oxygen dependent.

Role of Aerobic Metabolism

The striking change in mortality when salinity shock is combined with anoxia is especially interesting in viewof the ease with which the animal adjusts to either stress alone. The increase in ATP production that occurs from the modification

in the Embden-Meyerhof pathway may not suffice to fuel the increased metabolic demands of high salinity adaptation, requiring the aerobic generation of metabolic energy. We suggest that the duration of shell closure during high salinity adjustment is determined by energetic and not osmotic demands. The animal opens its valves and flushes its tissues with the new medium much sooner when its anaerobic supply of ATP is blocked by iodoacetate (Table 4); and when the intracellular FAA pool is still very small (Tables 1 and 6).

Conclusions

In conclusion, the present results together with those of previous findings show that non-oxidative reactions occurring in a modified form of glycolysis are capable of effecting the accumulation of intracellular FAA during high salinity adaptation. However, oxidative metabolism is critical to the overall success of the process, although not to the production of osmolytes. Total oxidative metabolism is reduced when acclimation to high salinity is complete. It will be interesting to learn whether this decrease results from 1) a lower demand for energy production from oxidative pathways due to the ATP yield of an increased rate of anaerobic metabolism, or 2) a rate-limiting effect on the electron transport system due to redirection

of reducing equivalents into reductive amination.

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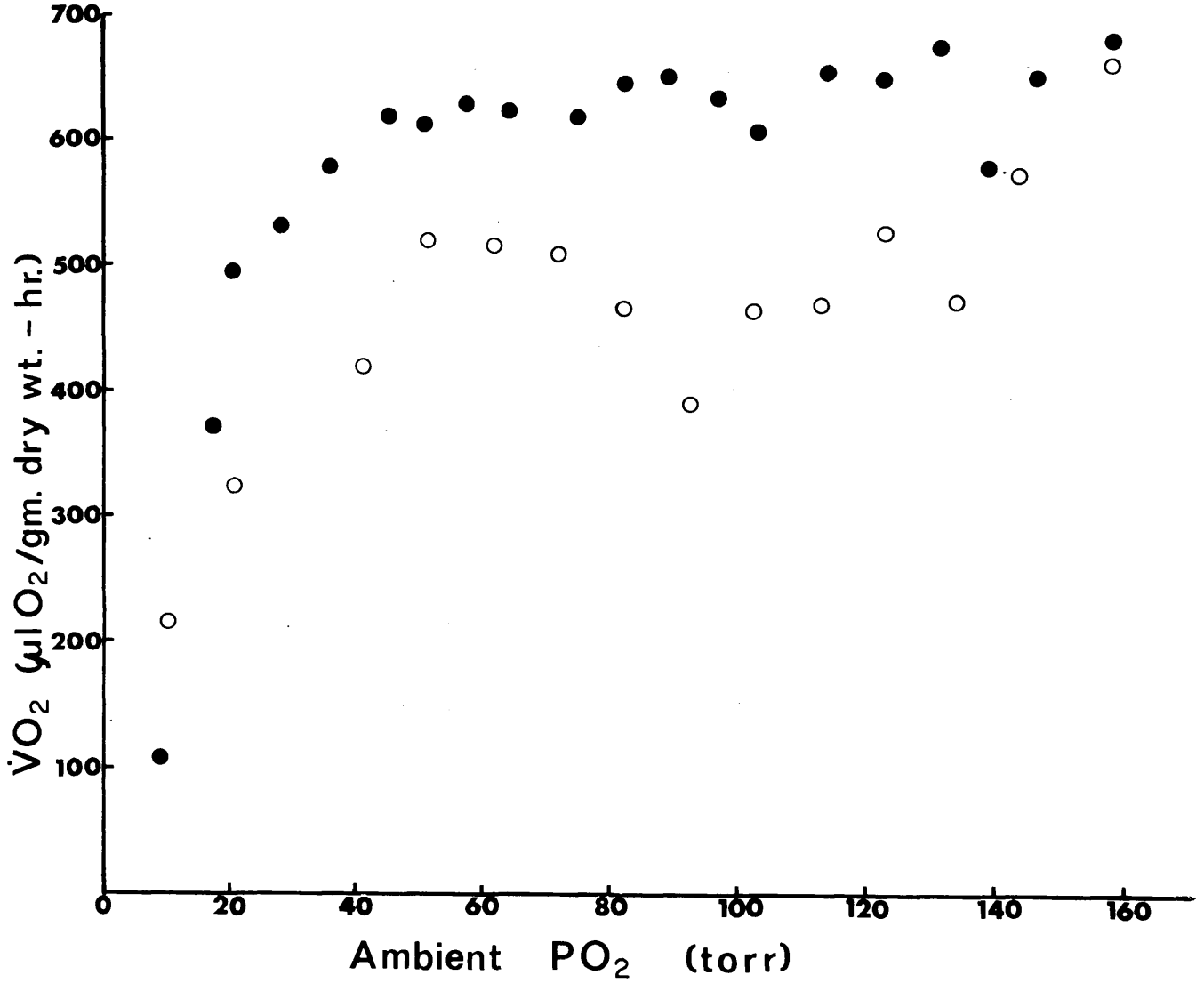
Table 1: Accumulation of adductor muscle intracellular FAA under normoxic (air saturation) conditions after transfer of Rangia cuneata from $2^{o}/_{oo}$ to $20^{o}/_{oo}$ salinity. μ moles/gm. dry wt. tissue (mean ± S.E.) 22^{o} N= 4-5

Amino Acid	0 hr	%	6 hr	%	12 hr	%	24 hr	%	48 hr	%	9 da.	%
Cysteic acid	--	0	3.6± 0.3	1.9	2.1± 0.5	1.1	1.9± 0.2	1.0	--	0	--	0
Taurine	--	0	2.9± 0.8	1.5	1.9± 0.3	1.0	3.0± 0.4	1.6	--	0	--	0
Aspartic acid	0.9± 0.3	1.6	2.2± 0.4	1.2	1.9± 0.3	1.0	1.1± 0.4	0.6	1.8± 0.7	0.7	2.5± 0.8	0.7
Threonine	1.5± 0.2	2.4	1.4± 0.7	0.7	1.1± 0.2	0.6	2.0± 0.3	1.1	3.4± 0.4	1.3	5.7± 0.9	1.5
Serine	2.0± 0.3	3.4	4.8± 0.6	2.6	4.3± 0.5	2.3	3.0± 0.5	1.6	5.7± 1.0	2.4	5.3± 1.0	1.4
Glutamate	11.9±0.7	19.7	21.8±1.5	11.8	15.1±1.5	8.1	20.7±2.7	11.0	28.1±3.0	10.5	30.0±2.3	8.0
Proline	0.3	0.4	0.5	0.9	1.6± 0.3	0.8	4.4± 0.6	2.3	7.0± 0.8	2.6	22.1±1.2	5.9
Glycine	6.3± 1.4	10.3	76.8± 16	41.6	78.1± 10	42.1	38.1±6.5	20.2	51.5± 10	19.3	26.7±2.7	7.1
Alanine	27.2±2.3	44.7	60.3±2.6	32.7	72.7±13.5	39.2	99.1±1.2	52.3	144.9±12.3	54.4	258.3±14.3	68.5
Valine	0.5± 0.1	0.7	0.6± 0.2	0.3	0.5± 0.1	0.2	1.1± 0.2	0.6	1.9± 0.2	0.7	1.7± 0.1	0.4
Cystine	--	0	--	0	--	0	--	0	--	0	0	--
Methionine	< 0.1	<0.1	0.2± 0.1	0.1	0.1± 0.1	0.1	0.5± 0.1	0.3	0.9± 0.1	0.3	1.3± 0.1	0.4
Isoleucine	0.5± 0.1	0.8	0.4± 0.1	0.2	0.3± 0.1	0.1	0.8± 0.2	0.4	2.0± 0.2	0.7	1.5± 0.1	0.4
Leucine	0.4± 0.1	0.7	0.5± 0.1	0.3	0.4± 0.1	0.2	1.1± 0.4	0.6	3.0± 0.4	1.1	1.2± 0.1	0.3
Tyrosine	0.4± 0.1	0.7	0.8± 0.3	0.4	0.5± 0.1	0.3	0.8± 0.3	0.4	1.8± 0.4	0.7	2.5± 0.1	0.6
Phenylalanine	0.5± 0.1	0.8	0.5± 0.1	0.3	0.6± 0.1	0.3	2.1± 0.7	1.1	4.9± 0.9	1.8	4.6± 0.2	1.2
Ornithine	4.0± 0.6	6.5	4.2± 0.3	2.3	2.8± 0.4	1.3	6.0± 1.1	3.2	6.8± 1.8	2.5	1.1± 0.1	0.3
Lysine	1.4± 0.1	2.3	1.6± 0.2	0.9	1.3± 0.2	1.5	1.3± 0.2	0.7	2.6± 0.5	1.0	4.0± 0.4	1.1
Tryptophan	0.8± 0.2	1.2	0.6± 0.1	0.3	0.9± 0.1	0.5	1.2± 0.4	0.6	0.3	0.1	--	0
Histidine	2.3± 0.3	3.8	2.8± 0.2	0.5	1.6± 0.3	0.9	1.5± 0.3	0.8	1.2± 0.2	0.5	2.2± 0.1	0.6
Arginine	--	0	--	0	--	0	1.2	0.6	2.6± 0.6	1.0	1.1	0.3
TOTAL	60.7±3.2	100	184.6±19	100	185.4±21	100	188.7±7.3	100	266.4±12.4	100	370.6±14.7	98.7

Table 2: Time course of nitrogen excretion and blood nitrogen levels in Rangia cuneata during normoxic (air saturation) conditions after transfer from 2°/oo to 20°/oo salinity. 22°C (mean ± S.E.) N=10

time (hours)	ammonia excretion μmoles/gm. dry wt.-hr.	FAA excretion μmoles/gm. dry wt.-hr.	blood ammonia μM/l	blood FAA mM/l
0	33.4± 2.8	4.1± 1.3	192± 16	2.6± 0.3
4	0.4± 0.2	4.1± 1.5	263± 18	2.9± 0.2
8	———	———	———	———
12	3.2± 0.7	0.5± 0.4	145± 15	3.1± 0.1
16	6.2± 0.8	5.1± 2.1	211± 9	3.9± 0.3
20	2.3± 0.4	0.8± 0.5	167± 13	3.0± 0.3
24	1.1± 0.3	2.7± 1.3	159± 11	2.5± 0.1
48	< 0	———	143± 7	7.1± 0.2
4 da.	4.8± 0.7	2.8± 0.7	91± 5	7.2± 0.2
7 da.	20.5± 2.0	———	129± 13	———
14 da.	23.9± 3.7	3.0± 0.7	151± 3	14.5± 1.2

Figure 1: Oxygen uptake vs. ambient oxygen tension for intact Rangia cuneata acclimated (14 days) to 2°/oo (closed circles) and 20°/oo (open circles) salinity at 22°C.


700
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120
140
160
$\dot{V}O_2$ (µl O_2/gm. dry wt. - hr.)
Ambient PO_2 (torr)

Table 3: Time course of oxygen uptake in intact Rangia cuneata during acclimation to a salinity increase from 2°/oo to 20°/oo at 22°C. Mean± S.E. of values obtained in PO_2 range of 100% to 70% saturation. N=6

clam	2°/oo $\mu l/O_2/gm.dry\ wt.-hr.$	Time of resumption of VO_2 after 20°/oo shock hours	$\dot{V}O_2$ at time of resumption $\mu l/O_2/gm.dry\ wt.-hr.$	$\dot{V}O_2$ 14 days after transfer $\mu l\ O_2/gm.dry\ wt.-hr.$
1	716± 82	22	818± 97	631± 71
2	575± 62	16	766± 82	447± 56
3	792± 69	22	1029± 121	931± 101
4	588± 71	21	637± 79	490± 57
5	620± 68	18	569± 63	——
6	686± 74	20	624± 71	——

Table 4: Time course of oxygen uptake in intact Rangia cuneata exposed to 10^{-5}M iodoacetate during acclimation under normoxic conditions to a salinity increase from 2°/oo to 20°/oo. Mean ± S.E. of values obtained in P_{O_2} range 100% to 70% saturation.

clam	2°/oo $\dot{V}_{O_2}$ $\mu l/O_2/gm.dry\ wt.-hr.$	Time of resumption of $\dot{V}_{O_2}$ after 20°/oo shock hours	$\dot{V}_{O_2}$ at time of resumption $\mu l/O_2/gm.dry\ wt.-hr.$
1	556 ± 36	9	122 ± 22
2	448 ± 52	10	103 ± 19
3	539 ± 41	7	93 ± 12
4	716 ± 0	3	143 ± 31

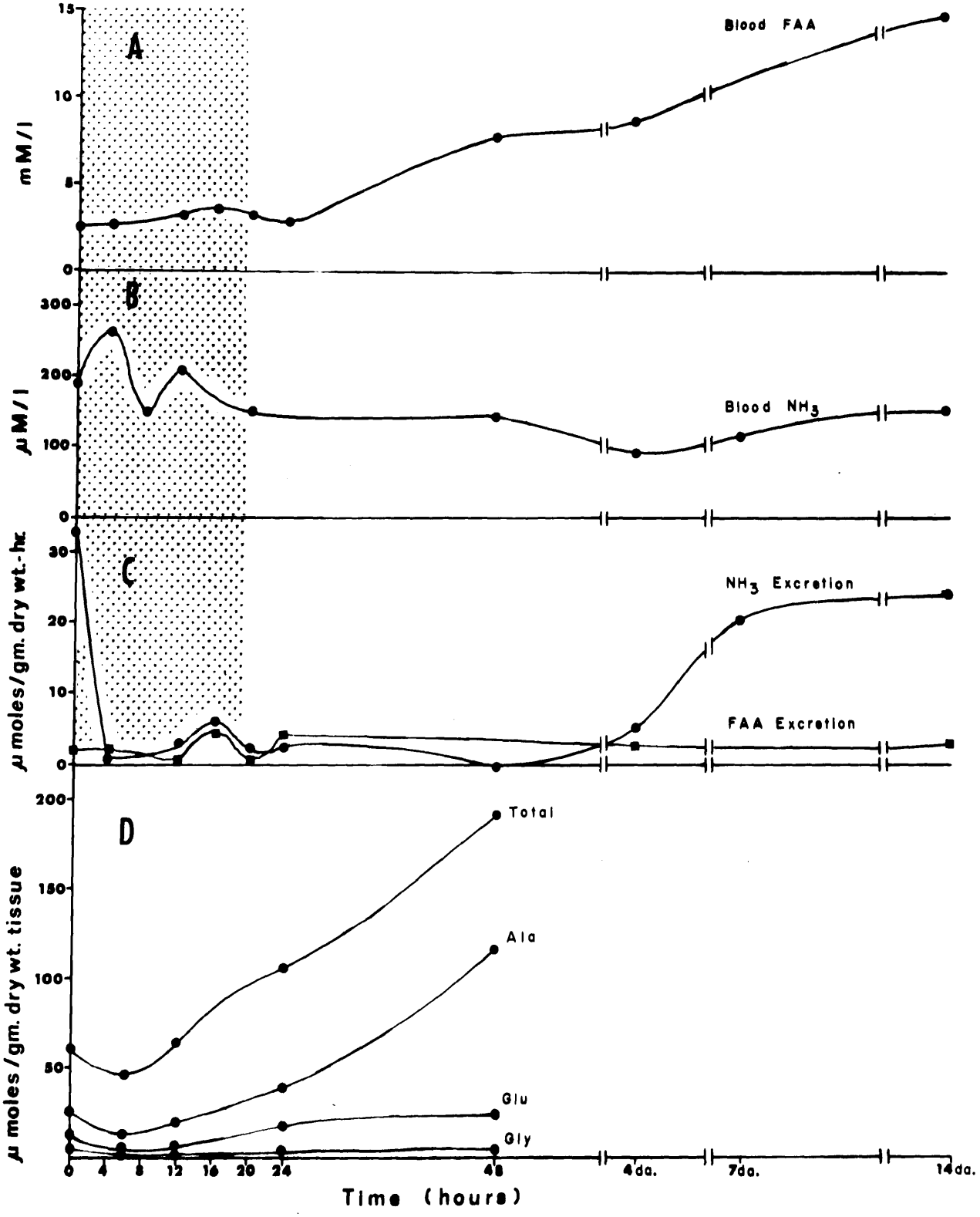
Table 5: Accumulation of adductor muscle intracellular FAA under anaerobic (PO_2 < 0.1 ppm) conditions after transfer of Rangia cuneata from $2^{o}/_{oo}$ to $20^{o}/_{oo}$ salinity-- 22° C (mean ± S.E.) μ moles/gm. dry wt. tissue N= 3-5

Amino Acid	0 hr	%	6 hr	%	12 hr	%	24 hr	%	48 hr	%
Cysteic acid	--	0	4.7 ± 0.3	6.7	4.3 ± 0.7	4.3	2.3 ± 0.4	1.3	4.3 ± 1.4	1.8
Taurine	--	0	2.5 ± 0.2	3.6	4.0 ± 0.8	4.0	4,3 ± 1.7	2.5	11.2 ± 2.6	4.6
Aspartic acid	0.9 ± 0.3	1.6	1.4 ± 0.3	2.0	0.9 ± 0.1	0.9	1.1 ± 0.1	0.6	4.7 ± 1.6	2.0
Threonine	1.5 ± 0.2	2.4	1.2 ± 0.3	1.7	1.8 ± 0.1	1.8	3.1 ± 0.3	1.8	1.7 ± 0.5	0.7
Serine	2.0 ± 0.3	3.4	1.5 ± 0.1	2.1	0.9 ± 0.1	0.9	2.0 ± 0.2	1.1	1.3 ± 0.1	0.5
Glutamate	11.9 ± 0.7	19.7	10.7 ± 0.7	15.1	13.6 ± 1.9	13.7	24.5 ± 4.4	14.1	42.4 ± 5.2	17.5
Proline	0.3	0.4	2.4 ± 0.7	3.4	10.2 ± 3.6	10.2	12.8 ± 2.6	7.4	15.9 ± 3.2	6.6
Glycine	6.3 ± 1.4	10.3	2.0 ± 0.3	2.8	3.4 ± 0.3	3.4	4.7 ± 0.6	2.7	6.0 ± 0.9	2.5
Alanine	27.2 ± 2.3	44.7	30.3 ± 2.7	42.8	40.7 ± 3.8	40.9	84.1 ± 10.1	48.4	133.3 ± 19.8	55.2
Valine	0.5 ± 0.1	0.7	0.7 ± 0.1	1.1	1.3 ± 0.3	1.3	2.9 ± 0.5	1.7	2.2 ± 0.2	0.9
Cystine	--	0	--	0	--	0	--	0	--	0
Methionine	<0.1	<0.1	0.3 ± 0.1	0.4	0.4 ± 0.1	0.4	0.5 ± 0.1	0.3	--	0
Isoleucine	0.5 ± 0.1	0.8	0.5 ± 0.1	0.7	1.1 ± 0.3	1.1	2.4 ± 0.5	1.4	1.5 ± 0.2	0.6
Leucine	0.4 ± 0.1	0.7	0.7 ± 0.1	1.0	1.5 ± 0.2	1.5	3.3 ± 0.5	1.9	1.9 ± 0.5	0.8
Tyrosine	0.4 ± 0.1	0.7	0.4 ± 0.1	0.5	0.4 ± 0.1	0.4	1.7 ± 0.1	1.0	1.4 ± 0.3	0.6
Phenylalanien	0.5 ± 0.1	0.8	0.7 ± 0.1	1.0	1.8 ± 0.8	1.8	5.5 ± 1.2	3.2	2.7 ± 0.2	1.1
Ornithine	4.0 ± 0.6	6.5	5.4 ± 0.2	7.6	9.5 ± 2.7	9.5	15.0 ± 3.1	8.7	9.5 ± 1.0	3.9
Lysine	1.4 ± 0.1	2.3	1.4 ± 0.1	1.9	2.0 ± 0.2	2.0	1.7 ± 0.1	1.0	1.6 ± 0.4	0.7
Tryptophan	0.8 ± 0.2	1.2	0.9 ± 0.1	1.2	0.9 ± 0.3	0.9	1.3 ± 0.1	0.7	--	0
Histidine	2.3 ± 0.3	3.8	3.0 ± 0.4	4.2	1.7 ± 0.9	1.7	2.1 ± 0.2	1.2	--	0
Arginine	--	0	--	0	--	0	--	0	--	0
TOTAL	60.7 ± 3.2	100	70.9 ± 3.6	100	99.6 ± 6.8	100	173.6 ± 22	100	241.7 ± 31	100

Table 6: Accumulation of adductor muscle intracellular FAA under normoxic (air saturation) conditions and in the presence of 10^{-5} M iodoacetate after transfer of Rangia cuneata from $2^{o}/_{oo}$ to $20^{o}/_{oo}$ salinity. (mean ± S.E.)
u moles/gm. dry wt. tissue 22° C N=4-5

Amino Acid	0 hr	%	6 hr	%	12 hr	%	24 hr	%	48 hr	%
Cysteic acid	--	0	4.4 ± 0.6	9.6	4.3 ± 0.2	6.6	3.8 ± 0.7	3.6	3.2 ± 0.3	1.7
Taurine	--	0	1.9 ± 0.1	4.2	3.4 ± 0.3	5.2	3.4 ± 0.3	3.2	6.3 ± 0.7	3.3
Aspartic acid	0.9 ± 0.3	1.6	0.2 ± 0.1	0.4	0.4 ± 0.1	0.6	0.5 ± 0.1	0.4	1.1 ± 0.2	0.6
Treonine	1.5 ± 0.2	2.4	0.8 ± 0.1	1.8	1.2 ± 0.1	1.8	1.2 ± 0.3	1.1	2.3 ± 0.3	1.2
Serine	2.0 ± 0.3	3.4	1.2 ± 0.1	2.7	1.3 ± 0.2	2.0	0.9 ± 0.3	0.9	1.2 ± 0.4	0.6
Glutamate	11.9 ± 0.7	19.7	4.5 ± 0.5	9.7	7.7 ± 2.3	11.8	18.0 ± 3.5	17.1	25.3 ± 5.6	13.1
Proline	0.3	0.4	1.0 ± 0.4	2.1	3.2 ± 1.1	5.0	5.3 ± 0.2	5.1	14.3 ± 3.0	7.4
Glycine	6.3 ± 1.4	10.3	2.2 ± 0.2	4.9	2.9 ± 1.1	4.4	2.8 ± 0.5	2.6	4.0 ± 0.4	2.0
Alanine	27.2 ± 2.3	44.7	14.0 ± 1.9	30.5	21.2 ± 2.8	32.6	38.8 ± 8.4	36.9	117.7 ± 9.2	60.9
Valine	0.5 ± 0.1	0.7	0.7 ± 0.2	1.5	0.9 ± 0.2	1.4	1.1 ± 0.2	1.1	1.8 ± 0.2	1.0
Cystine	--	0	--	0	--	0	--	0	--	0
Methionine	<0.1	<0.1	0.4 ± 0.1	0.8	0.7 ± 0.2	1.1	0.6 ± 0.1	0.5	0.8 ± 0.1	0.4
Isoleucine	0.5 ± 0.1	0.8	0.6 ± 0.1	1.3	0.8 ± 0.2	1.2	1.2 ± 0.2	1.1	1.7 ± 0.3	0.9
Leucine	0.4 ± 0.1	0.7	0.7 ± 0.1	1.6	1.0 ± 0.2	1.6	1.5 ± 0.2	1.5	1.8 ± 0.3	0.9
Tyrosine	0.4 ± 0.1	0.7	0.4 ± 0.1	0.8	0.7 ± 0.2	1.0	0.7 ± 0.1	0.7	1.2 ± 0.1	0.6
Phenylalanine	0.5 ± 0.1	0.8	1.0 ± 0.2	2.2	1.7 ± 0.6	2.5	4.1 ± 0.9	3.9	6.1 ± 0.2	3.2
Ornithine	4.0 ± 0.6	6.5	5.3 ± 1.1	11.5	6.9 ± 2.0	10.6	17.0 ± 3.8	16.2	2.0 ± 0.3	1.0
Lysine	1.4 ± 0.1	2.3	0.9 ± 0.1	2.0	1.1 ± 0.1	1.7	1.3 ± 0.3	1.3	1.1 ± 0.1	0.6
Tryptophan	0.8 ± 0.2	1.2	1.2 ± 0.2	2.6	1.5 ± 0.1	2.4	1.6 ± 0.4	1.5	0.4 ± 0.1	0.2
Histidine	2.3 ± 0.3	3.8	1.7 ± 0.3	3.8	1.2 ± 0.1	1.9	0.8 ± 0.1	0.7	1.4 ± 0.2	0.7
Arginine	--	0	3.5 ± 0.3	7.7	5.3 ± 0.4	8.2	1.8 ± 0.7	1.7	--	0
TOTAL	60.7 ± 3.2	100	45.9 ± 3.3	100	65.1 ± 7.7	100	105.1 ± 17	100	193.4 ± 18	100

Figure 2: Time course of change in nitrogen compounds in Rangia cuneata transferred from 2°/oo to 20°/oo salinity under a variety of conditions at 22°C. A) Blood FAA, normoxic conditions B) blood ammonia, normoxic conditions C) ammonia and FAA excretion, normoxic conditions D) intracellular FAA, normoxic conditions and in the presence of 10^{-5}M iodoacetate E) intracellular FAA under anaerobic (PO_2 < 2mmHg) conditions F) intracellular FAA under normoxic conditions. Shaded areas show the period of anaerobiosis.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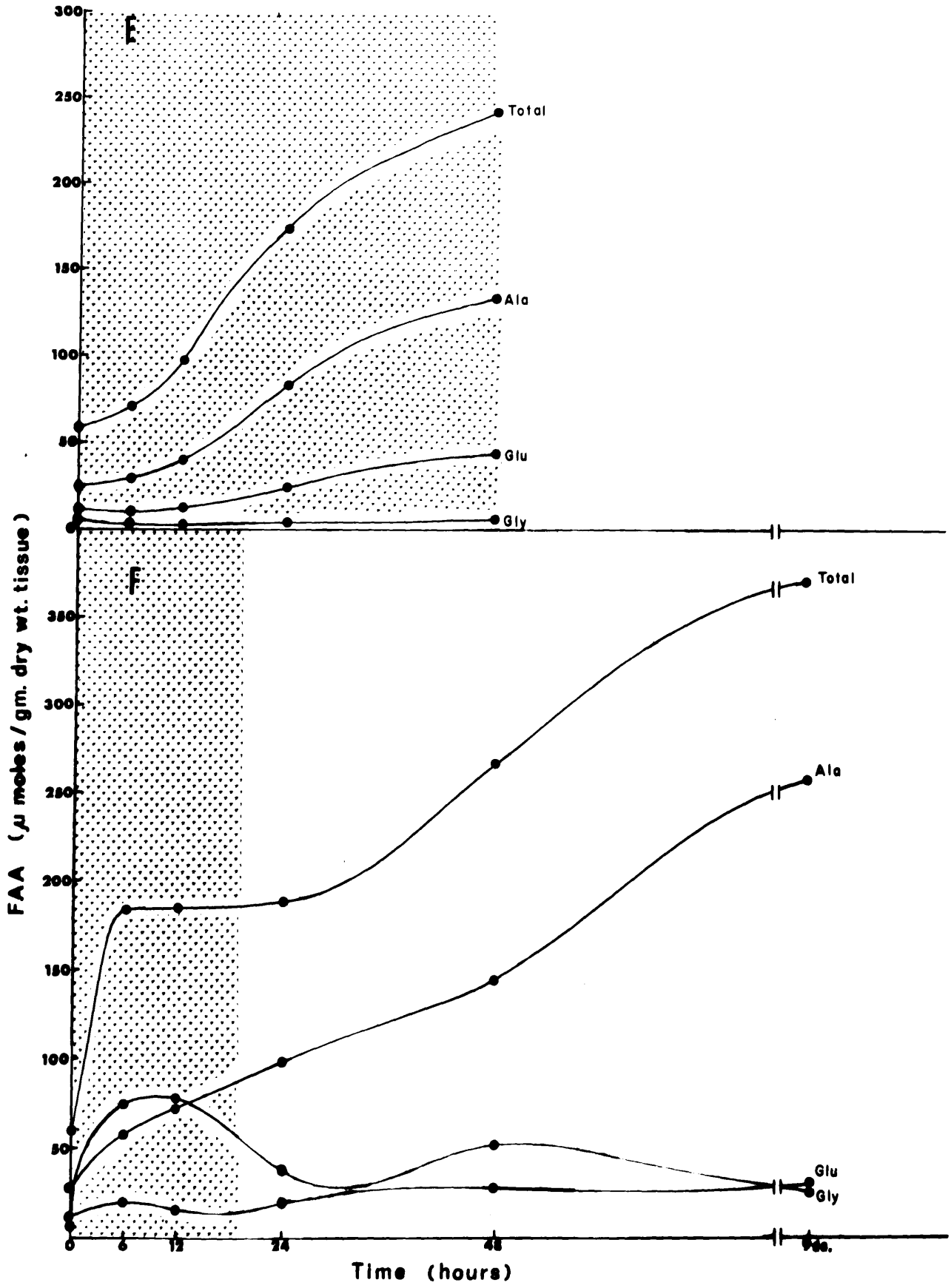
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FAA (μ moles / gm. dry wt. tissue)
Time (hours)

III: REDUCTION OF THE FREE AMINO ACID POOL DURING LOW SALINITY ADAPTATION

LIST OF TABLES AND FIGURES

ABSTRACT

During hypo-osmotic adjustment Rangia cuneata reduces its intracellular FAA pool to maintain cell volume. Free amino acids are extruded intact from the cell into the blood and are deaminated at a centralized site, the gill. In situ deamination appears to play a minor role in the deamination process.

INTRODUCTION

When placed in a dilute medium the total body water content in bivalve molluscs of the genus Modiolus changes considerably less than that of the medium, which has been interpreted as evidence of cell volume regulation (Pierce, 1971a). Intracellular solute is extruded, along with osmotically obligated water, presumably reducing the volume of initially swollen cells. For many years it has been clear that a labile pool of free amino acids (FAA), the size of which varies directly with ambient salinity, plays an important role in this process (Florkin and Schoffeniels, 1969; Pierce, 1971b).

Using isolated tissue, several investigators have demonstrated that free amino acids leave the cell intact during hypo-osmotic stress, leading to the hypothesis that the size of the pool is reduced by FAA release into the blood (Lang and Gainer, 1969; Gilles and Schoffeniels, 1969; Vincent-Marique and Gilles, 1970; Pierce and Greenberg, 1972, 1973). This hypothesis has been somewhat difficult to reconcile with the findings on intact organisms, however. The levels of FAA in the blood are never very great, and excretion of FAA into the ambient medium is always very small, especially in the bivalves (Lum and Hammen, 1964; Potts, 1967; Florkin and Bricteux-Gregoire, 1972; Henry and Mangum, 1978a). The excretion of ammonia, however, is significantly elevated

after low salinity shock (Emerson, 1969; Allen and Garrett, 1971; Bartberger and Pierce, 1976), while urea excretion in this group remains very small and insensitive to the salinity change (Allen and Garrett, 1971). Thus, according to the hypothesis, the FAA originating from many different tissues in the animal, are transported in the blood to some central site, where they are deaminated by oxidative reactions and the amino nitrogen excreted as ammonia. The possibility of simultaneous ammonia production and FAA extrusion, reflecting a different fate of various amino acids within the cells has not been seriously considered. Moreover, the notion of FAA transport followed by centralized deamination lacks the evidence of FAA input into the blood immediately prior to a burst of oxidative metabolism or a pulse of ammonia excretion. Finally an assessment of the quantitative adequacy of the hypothesis of FAA extrusion into and transport by the blood has not been attempted.

We have examined the changes in FAA and ammonia, along with oxidative metabolism, during low salinity adaptation in the oligohaline clam Rangia cuneata (Gray), which is a poor regulator of blood osmolality within its range of salinity tolerance (Bedford and Anderson, 1972). In view of the suggestion that the mantle is the site of deamination in Modiolus demissus (Bartberger and Pierce, 1976), we have focused our attention on two superficial tissues which are highly vascularized, the gill and the mantle, and a single deep tissue

which contains a large blood sinus, the adductor muscle, as well as the intact animal.

MATERIALS AND METHODS

The collection and storage of animals, and the procedures for measuring ammonia excretion, blood ammonia, FAA excretion, blood FAA, and intracellular FAA are given by Henry and Mangum (1978a). The excretion of ammonia and FAA was also measured in isolated tissues before and after a hyposaline shock. Adductor muscle, gill, and mantle tissue were removed from animals acclimated to 20°/oo and washed in 50 ml of 20°/oo seawater for 10 minutes. One aliquot of tissue was then placed in 75 ml of 20°/oo water, and a second aliquot from the same individual placed in an equal volume of 2°/oo water. Ammonia and TNPS in the medium were measured, as above at intervals of 1 hour.

Oxygen uptake was measured by taking paired observations on intact animals immediately before and after transfer from 20°/oo to 2°/oo, as described previously (Henry and Mangum, 1978b).

Heart rates of intact animals were determined with an impedance pneumograph (E and M Instrument Co.) with platinum or silver electrodes (de Fur and Mangum, 1979), implanted into each of two small holes drilled through the shell on either side of the heart, and sealed with dental wax.

RESULTS

Behavior, Nitrogen Excretion and Blood Nitrogen Levels of Intact Animals

When subjected to a hyposaline shock, R. cuneata clamps shut its valves, a transient avoidance response which is common among bivalves (Pierce, 1971b; Gilles, 1972; Hoyaux, Gilles and Jeuniaux, 1976; Shumway, Gabbott and Youngson, 1977). A movement of carmine particles indicated that ventilation had been partly resumed by 4 hours, after the salinity change, although siphons were not extended until 12 hours, and then in only half of the animals. By 16-24 hours all animals had assumed a high velocity pumping posture, with siphons fully protruded, along with full extension of the foot, a response observed only at this time. By 48 hours the foot had been retracted and the normal ventilation posture resumed.

Not surprisingly, reduction in ammonia excretion (~50%) accompanied shell closure during the first 4 hours of exposure to low salinity (Table 1). From 4 to 16 hours the rate increased to 150% of the control, but the variability during that time was very high, due to similar variability in ventilation behavior. At 16-20 hours the rate abruptly increased, and then continued to rise, peaking at 10 times the control rate (48 hours). From then until the completion of the experiment the excretion rate declined steadily to an acclimated value at low salinity which is 140% of the control (Table 1).

Blood ammonia levels (Table 1) showed a similar pattern during low salinity acclimation. From 0 to 16 hours the ammonia concentration rose only slightly (18%), but between 20 and 48 hours the level more than doubled, also reaching a maximum value at the same time as ammonia excretion. By 4 days the blood ammonia level had declined to 120% of the control, where it remained throughout the rest of the experiment.

Free amino acid excretion (Table 1) was extremely variable, both within and between the different time intervals, showing no general trend. Blood FAA levels, however, showed a clear trend (Table 1). A peak in blood FAA was followed by a slight decline; the peak value of 150% of the control, however, occurred at 4 hours, long before the ammonia peak. From 8 to 20 hours the FAA levels remained constant at approximately 100% of control, and then gradually declined to a stable level of 14% after 14 days.

Oxygen Uptake

Oxygen uptake by intact animals ceased in R. cuneata given a low salinity shock (Table 2) but was quickly resumed. At approximately the same time that movement of low salinity water across superficial tissue was inferred from visual observation (see above), the animals showed a reduced but quite detectable rate of oxygen uptake. Aerobic metabolism persisted throughout the experiment, gradually increasing to values that do not differ significantly from those at high salinity (Table 1).

Responses of Isolated Tissues

Ammonia output by isolated adductor muscle, gill, or mantle tissue did not increase significantly ($P>.05$, F test) when subjected to a hyposaline shock (Table 3). However, FAA excretion by these tissues was much higher during low salinity adaptation than at the acclimation salinity (Table 3). Mantle and adductor muscle showed a continuous output of FAA for the entire 4-hour observation period. FAA loss from gill tissue, on the other hand, was complete in 2 hours.

The intracellular FAA pool of the three tissues (Table 4) was markedly reduced after 14 days at 2°/oo. All three had similar FAA profiles with the exception of taurine, phenylalanine, and tyrosine being more abundant in gill tissues of animals acclimated to 20°/oo than in the other tissues.

As reported earlier, gill tissue VO_2 was significantly higher (2x) at low than at high salinity and at low salinity. The rate in gill was also twice as great as that in mantle, and 8 times that in adductor muscle (Henry and Mangum, 1978a).

Heart Rate

Mean heart rate at 2°/oo is 6 beats/minute. Heart volume, estimated from dimensions at observed distension, as described by de Fur and Mangum (1979), is about 50μl.

DISCUSSION

The response of Rangia cuneata to dilution of the medium is quite consistent with that of Modiolus demissus (Pierce, 1971a; Bartberger and Pierce, 1976). After resumption of ventilation, intracellular FAA are released into the blood and blood ammonia levels increase. In the case of R. cuneata, however, FAA in the blood actually decrease at the time when blood ammonia levels begin to rise. This relationship strongly supports the suggestion that deamination in a centralized organ follows FAA transport in the blood from multiple sites of origin. The data for oxygen uptake also support the hypothesis. In contrast to the prolonged period of anaerobiosis following a hyperosmotic shock (Henry and Mangum, 1978b), animals transferred from high to low salinity quickly resume oxygen uptake and then increase oxygen uptake to the high salinity level, with the peak at 10 hours. During hyperosmotic acclimation, enlargement of the FAA pool precedes the return to aerobic metabolism, whereas reduction of the pool during hypo-osmotic adaptation is closely correlated in time with the active resumption of ventilation and oxidative metabolism. This rapid return to aerobiosis after hypo-osmotic shock appears to be a widespread phenomenon in the various phyla that utilize FAA as osmotic effectors (Negus, 1968; Emerson, 1969; Gilles, 1973; Taylor et al., 1977). Pierce and Greenberg (1973, 1976) have sugg-

ested that the increase in oxygen uptake is due not only to deamination, where oxidative reactions maintain redox balance, but also to a divalent cation-dependent ATP hydrolysis that terminates FAA efflux from the cells.

The suggestion that the mantle is the centralized site of deamination of M. demissus was based on data showing no change in its intracellular FAA pool during the initial period of hypo-osmotic stress; no other tissue was examined (Bartberger and Pierce, 1976). The data for R. cuneata more strongly implicate the gill, for the following reasons: 1) The gill has a smaller pool size at low salinity, however, this relationship might be interpreted alternatively as a smaller role of FAA relative to other osmotic effectors in this tissue than in mantle and adductor muscle. 2) The reduction of FAA in the gill ceases before that of the other two tissues, which could be due to input of FAA from the blood, or it could mean merely that permeability of the gill membrane to FAA and thus release is quicker. 3) The rate of oxygen uptake by branchial tissue at 2°/oo is twice that of mantle and at least 8 times that of adductor muscle, and 4) the increase in oxygen uptake accompanying adaptation to low salinity is more than 4 times as great in branchial than in pallial or muscle tissue. These changes in oxidative metabolism are especially notable in view of the finding that the mantle, has the highest activity of ATPase activity (Henry and Mangum, 1978a, Saintsing and

Towle, 1978), and that hyperosmotic regulation of the blood occurs at 2°/oo but not at 20°/oo. Thus some oxidative process that requires far more energy than cation transport is occurring in the gill and not in the mantle.

The possibility of an appreciable level of *in situ* deamination in various tissues occurring concomitant with FAA extrusion does not seem to be likely during the early phase of low salinity adaptation. On the one hand, all three isolated tissues exposed to a hyposaline medium show almost instantaneous loss of intracellular FAA. On the other hand, a significant increase in blood ammonia levels and ammonia excretion rates in intact animals occurs at 16-20 hours after ventilation had resumed and the blood FAA peak had passed, suggesting that this mechanism becomes more important in the later stages of adaptation.

Quantitative Aspects of Transport in the Blood

We should emphasize that neither the present findings nor those of previous workers provide the basis for a quantitative accounting of the fate of the free amino acid pool. The net fluxes of the various amino acids at epithelia separating blood from ambient water, are not known in molluscs or any other groups.

However, the adequacy of the hypothesis of 1) *in situ* deamination in each tissue, and 2) efflux and transport in the blood to a superficial site of deamination, can be assessed in part on the basis of the performance capability of the cardiovascular system. In the 14 day period of adjustment to low salinity, the

gill, mantle and adductor muscle of R. cuneata lost a total of 935 μM FAA/0.308 gm. dry wt. If the mean FAA change for these three tissues is representative of all tissues in the animal, the total FAA loss was 3.116 mM/1 gm.-animal. At a mean excretion rate of 3.5 μM/gm.-hr., or 1.176 mM/1 gm.-animal-14 days, excretion accounts for about 37.7% of the observed change in intracellular FAA, assuming no addition to the pool from any other source during that period, which is unlikely.

Blood flow is about 6/min. (=heartrate) x 0.05 ml (= ½ of heart volume), or 0.15 ml/min, which is 9 ml/hr in a 1 gm (dry wt) animal. To obtain a conservative estimate of the potential convection of nitrogen metabolites, we have made the following assumptions: 1) stroke volume is only 50% of heart volume, 2) the levels of FAA in Table 1 do not represent an average of those in blood but rather the maximum, which occurs immediately before the blood passes the central deamination site, 3) fully 10% of the FAA remains in the blood after it passes the central site, and 4) all of the FAA excreted were carried by the blood and none pass directly from superficial tissue directly into the ambient medium.

The blood could deliver a maximum of 170 uM FAA/hr (4 hr) and a minimum of 19 uM/hr (14 da.) to a central site. The observed rate of excretion accounts for 0-10% of the amount delivered by blood. Using the same assumptions, the amount of ammonia that could be transported in the blood from a site of

production *in situ* in deep tissue to a site of release into the ambient medium is at least 1.4-3.1 μM/hr. The amount actually excreted is always 8-78 times greater (Table 1). Either 1) 88-99% of nitrogen metabolism is confined to superficial tissue in immediate contact with the ambient medium, which seems excessive, or 2) most of the ammonia excreted originates from precursors transported in the blood from sites of origin in deep tissue to a superficial site of deamination. In either case it is clear that the pulse of ammonia in the blood 20-48 hours after the low salinity shock represents a small fraction of the total amount being produced (<14%).

Thus the performance limits of the cardiovascular system suggest that the major mechanism of reducing the FAA pool is release from the cell and transport in the blood to a superficial site where deamination is followed by excretion of the amino group as ammonia. The hypothesis of *in situ* deamination is deficient by a large margin. Nevertheless, this assessment does not imply that the fate of each of the 21 components of the pool is the same, and it does not exclude the possibility that *in situ* deamination is a subsidiary mechanism of reducing pool size. Even though the 20-48 hour pulse of ammonia in the blood represents a small fraction of the total ammonia,excreted, it is more likely to originate

from deamination in deep than superficial tissue.

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Table 1: Time course of ammonia and FAA excretion, and blood ammonia and FAA levels during acclimation from 20°/oo to 2°/oo salinity. (mean ± S.E.) N=10 22°C

Time (hr.)	ammonia excretion μmoles/gm.dry wt.-hr.	FAA excretion μmoles/g.dry wt.-hr.	blood ammonia μM/l	blood FAA mM/l
0	23.9 ± 3.7	2.9 ± 0.7	151 ± 3	14.4 ± 1.2
4	12.0 ± 1.8	3.9 ± 2.0	157 ± 9	21.0 ± 0.7
8	30.8 ± 11	3.7 ± 1.1	153 ± 14	13.8 ± 1.2
12	37.5 ± 10	1.9 ± 1.0	169 ± 17	15.1 ± 0.5
16	35.8 ± 12	6.3 ± 2.0	178 ± 22	14.0 ± 0.8
20	169.9 ± 12	12.6 ± 3.7	287 ± 22	14.7 ± 0.9
24	177.6 ± 6.8	0	224 ± 10	9.1 ± 0.5
48	243.4 ± 7.6	0	348 ± 30	4.1 ± 0.3
4 da.	117.7 ± 6.8	0	187 ± 11	2.8 ± 0.2
7 da.	73.4 ± 3.6	3.5 ± 1.8	181 ± 7	2.3 ± 0.1
14 da.	33.3 ± 2.8	4.1 ± 1.3	192 ± 16	2.6 ± 0.2

Table 2: Time course of oxygen uptake in intact Rangia cuneata during acclimation to a salinity change from 20°/oo to 2°/oo at 22°C. Data taken and analysed as paired observations on the same individuals.

clam	20‰ $\mu l/O_2/gm.dry$ wt.-hr.	Time of resumption of $\dot{V}O_2$ after 2°/oo shock hours	$\dot{V}O_2$ at time of resumption % control	Time of Peak $\dot{V}O_2$ hours	Peak $\dot{V}O_2$ Value % control	2°/oo % control
1	630	5	-83.8	23	-13.5	+13.5
2	447	1	-69.7	10	+85.7	+28.6
3	931	4	-87.2	7	+1.1	-14.9
4	490	5	-72.5	7	-10.0	+20.6
mean ±S.E.	624.6 ± 109	3.8 ± 1.0	-77.9± 4.6	11.8 ± 3.8	15.8± 23.5	11.8±9.4

Table 3: Intracellular FAA composition of adductor muscle, gill and mantle tissue for Rangia cuneata acclimated to 20°/oo and 2°/oo salinity. μmoles/gm. dry wt. tissue

	adductor muscle N=5				gill N=1				mantle N=1			
Amino acid	20°/oo	%	2°/oo	%	20°/oo	%	2°/oo	%	20°/oo	%	2°/oo	%
cysteic acid	—	0	—	0	—	0	2.1	12.9	—	0	0.5	3.0
taurine	—	0	—	0	10.7	2.5	1.9	11.7	1.4	0.6	0.5	3.0
aspartic acid	2.5±0.8	0.7	1.0±0.3	1.6	2.8	0.6	0.5	3.1	1.7	0.8	0.4	2.4
threonine	5.7±0.9	1.5	1.5±0.1	2.4	1.3	0.3	0.3	1.8	0.9	0.4	0.2	1.2
serine	5.3±1.0	1.4	2.0±0.3	3.4	2.6	0.6	0.5	3.1	1.2	0.5	0.3	1.8
glutamic acid	30.0±2.3	8.1	11.9±0.7	19.7	12.9	3.0	2.1	12.9	6.6	2.9	2.4	14.5
proline	22.1±1.2	6.0	0.2	0.3	14.9	3.4	2.7	16.6	6.6	2.9	0.3	1.8
glycine	26.7±2.7	7.2	6.3±1.4	10.4	75.0	17.3	2.0	12.3	33.2	14.8	1.5	9.1
alanine	258.3±14	69.7	27.2±2.3	44.7	205.5	47.4	1.9	11.7	143.6	64.1	3.6	21.8
valine	1.7±0.1	0.5	0.5±0.1	0.8	1.2	0.3	0.4	2.5	0.3	0.1	0.1	0.6
cystine	—	0	—	0	—	0	—	0	0.4	0.2	0.1	0.6
methronine	1.3±0.1	0.4	<0.1	<0.1	—	0	—	0	0.1	<0.1	0.1	0.6
isoleucine	1.5±0.1	0.4	0.5±0.1	0.8	1.2	0.3	—	0	0.2	0.1	0.1	0.6
leucine	1.2±0.1	0.3	0.4±0.1	0.7	1.4	0.3	0.4	2.5	0.3	0.1	0.1	0.6
tyrosine	2.5±0.1	0.7	0.4±0.1	0.6	23.8	5.5	—	0	0.3	0.1	0.1	0.6
phenylalanine	4.6±0.2	1.2	0.5±0.1	0.8	60.5	14.0	3.3	20.2	7.9	3.5	0.4	2.4
ornithine	1.2±0.1	0.3	3.9±0.6	6.6	8.0	1.8	—	0	4.2	1.9	1.8	10.9
lysine	4.0±0.4	0.1	1.4±0.1	2.3	9.6	2.2	—	0	7.2	3.2	1.1	6.7
tryptophan	—	0	0.8±0.2	1.3	—	0	—	0	3.5	1.6	0.4	2.4
histidine	2.2±0.1	0.6	2.3±0.4	3.8	—	0	—	0	2.1	0.9	1.6	9.7
arginine	0.7	0.2	—	0	2.1	0.5	0.6	3.7	2.4	1.1	0.9	5.5
Total	370.8±14	100	60.7±3.3	100	433.5	100	16.3	100	223.9	100	16.5	100

Vita

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