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Differential sensitivities to hypoxia by two anoxia-tolerant marine molluscs: a biochemical analysis *

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Abstract. The metabolic responses to a series of low oxygen tensions were compared for two species of Mediterranaean bivalves, *Mytilus galloprovincialis* and *Scapharca inaequivalvis*. Whereas both species have well-developed and similar tolerances of anoxia, the metabolic responses of *S. inaequivalvis* to low oxygen tensions indicate a substantially greater tolerance of hypoxia. Compared with *M. galloprovincialis*, the responses of *S. inaequivalvis* included the ability to maintain a constant oxygen consumption down to a much lower pO_2 value (ca. 1.7 vs 3.4 ppm), and a lower critical pO_2 for the recruitment of fermentative pathways of ATP production (ca. 1 vs 3 ppm). Furthermore, a graded increase in the output of anaerobic products (succinate, alanine) occured at oxygen tensions below 3 ppm in *M. galloprovincialis* and reached a maximum at 1.6 ppm whereas in *S. inaequivalvis* the net accumulation of anaerobic products at the lowest oxygen tension tested (0.5 ppm) was still substantially less than the level of production output in complete anoxia. This suggests that fermentative pathways are maximally activated at all oxygen tensions below 1.6 ppm in *M. galloprovincialis* whereas rates of anaerobic pathways are still less than maximum at 0.5 ppm in *S. inaequivalvis*. These results indicate that in situations of declining oxygen tensions, such as occur due to eutrophication, *M. galloprovincialis* would not only begin to experience metabolic stress at higher oxygen tensions than *S. inaequivalvis* but would experience greater stress at any given pO_2. Such differences in hypoxia tolerances may explain the success of the recently introduced *S. inaequivalvis* in out-competing the native *M. galloprovincialis* in the Adriatic Sea.

Introduction

In the Adriatic Sea populations of two species of bivalve molluscs of great commercial importance, the blue mussel *Mytilus galloprovincialis* and the "vongole" *Venus gallina*, are in steep decline whereas populations of the recently introduced blood clam *Scapharca inaequivalvis*, a native of the Indo-Pacific region, are rapidly growing and out-competing the two native species (Ghisotti and Rinaldi 1976). The demise of the native species is related to the eutrophication of the Adriatic waters causing seasonal cycles of severe lack of oxygen in the bottom sediments, the habitat of these benthic species (Marchetti et al. 1989). The success of *S. inaequivalvis* also appears to be related to oxygen availability, including the greater tolerance of the species to hypoxia and anoxia, as well as related to the presence of hemoglobin-containing erythrocytes (Weber et al. 1990, Brooks et al. 1991, de Zwaan et al. 1991). These erythrocytes have an O_2 transport function, and possibly also an O_2 storage function since the extracellular body fluid volume is large and the hematocrit is 9% (Weber et al. 1990). Due to the presence of erythrocytes, the blood of *S. inaequivalvis* can contain as much as 1 m*M* oxygen compared with oxygen concentrations of about 0.2 m*M* for other marine bivalves that these cells miss (Chiancone et al. 1985).

Over the last few years we have been carrying out an extensive analysis of the physiological and biochemical responses to low oxygen and heavy metal exposure by the three species above (Isani et al. 1989, Weber 1990, Weber et al. 1990, Brooks et al. 1991, Cortesi et al. 1991, de Vooys et al. 1991, de Zwaan et al. 1991). Although all three species strongly reduce metabolic rate in anoxia (to 4.5–13% of the corresponding normoxic rate), *Venus gallina* shows very poor survival of anoxia, with LT_{50} (anoxia exposure time resulting in 50% mortality) values of only 4 d at 17 to 18 °C compared with 17 d for *Scapharca inaequivalvis* (Brooks et al. 1991). The anoxia survival time of *Mytilus galloprovincialis*, by contrast, is nearly as great as that of *S. inaequivalvis* (16.1 vs 19.8 d for individuals compared in May at 20 °C) (de Zwaan et al. 1991).

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Thus, whereas poor anoxia tolerance may explain the success of *S. inaequivalvis* over *V. gallina* populations, it does not appear to be sufficient to explain the demise of *M. galloprovincialis* populations.

However, the stress experienced by these species in the natural environment is, in fact, a seasonal cycle of declining oxygen content that imposes first a hypoxic and then a fully anoxic condition upon the inhabitants of the benthic sediments. The superior survivorship of *Scapharca inaequivalvis*, compared with *Mytilus galloprovincialis*, may be related, therefore, to a greater ability of the former species to endure low ambient oxygen tensions. The present study compares the hypoxia tolerances of the two species, examining both physiological (VO_2) and metabolic responses (accumulation of fermentative end products) to low oxygen availability.

Materials and methods

Individuals

Specimens of *Scapharca inaequivalvis* were dredged in September from ca. 10 m depth at a distance of 7 to 8 km off the Italian Emilia-Romagna coast in the Adriatic Sea near the town of Cesenatico at ambient seawater temperature of 22.5 °C. The individuals were transported to the Cesenatico lab; their shells were cleaned of algae, and 360 clams were kept for 2 wk in 600 l of running seawater at 20 °C before use. Specimens of *Mytilus galloprovincialis* were obtained at the same time from a fish farm at Goro (province of Ferrara). In the lab 600 mussels were held for 2 wk in 1500 l running seawater at 20 °C before use. Intact *S. inaequivalvis* measured 4.8 ± 0.5 cm and weighed 5.8 ± 0.4 g whereas mussels measured 6.5 ± 0.6 cm and weighed 5.5 ± 0.3 g. The mean weight of the posterior adductor muscles of the *M. galloprovincialis* used was 0.23 ± 0.03 g. Salinity of the seawater for both species was approximately 36‰. Control individuals in all experiments were sampled directly from the aerated holding tanks.

Tolerance to anoxia

Anoxia was imposed by filling 4.5 l conical flasks with seawater that had been deoxygenated by bubbling with nitrogen gas for 2 h. After transferring the individuals to the flasks (16 to 17 per flask), bubbling was continued for a further 30 min, and then flasks were sealed. The deoxygenated seawater was replaced and the molluses were examined for survival on a daily basis. No significant difference in survivorship was found between the three replicate flasks, and data was pooled.

Oxygen consumption rate

For oxygen measurements at constant oxygen levels a twin-respirometer design was used that was equipped with computerized data-handling [see van den Thillart and Verbeek (1982) for a detailed description]. The two respirometer chambers of 12 l each could be adjusted independently to any desired seawater oxygen tension. The experimental procedure was as follows. Thirty-five animals were placed in each respirometer chamber. Firstly, the oxygen tension was adjusted for 3 h (*Scapharca inaequivalvis*) or 6 h (*Mytilus galloprovincialis*) to ca. 5 mg $O_2\ l^{-1}$ (= 5 ppm; ca. 70% of full saturation) and was subsequently lowered to a specific tension between 0.5 and 5 ppm. The precise oxygen tensions were established by the Winkler method on water samples taken from the respirometer chambers. The oxygen uptake at the end of the 5 ppm exposure and at the start and the end of the 12 h hypoxic incubation was estimated by closing the chamber and registering the decline in oxygen tension against time. Blank consumption rates (chamber without individuals) were also estimated before introducing the individuals and after taking them out of the chambers. The blank values were caused by free-floating microorganisms in the natural seawater, as no difference was noticed when empty shells of the experimental individuals were placed in the chamber. By extrapolation the blanks were established for the periods of experimental measurements and used for correction.

Measurement of 6-phosphofructo-1-kinase (PFK) and glycogen phosphorylase in *Mytilus galloprovincialis* adductor

Posterior adductor muscles from control mussels vs mussels held for 24 h in oxygen-free seawater were rapidly dissected out and cooled on ice. Muscles from four specimens were immediately pooled and placed in a centrifuge tube containing dry phenylmethylsulfonyl fluoride. Five volumes (w/v) of 50 m*M* imidazole-HCl pH 7.0 containing 5 m*M* EDTA, 5 m*M* EGTA, 1 m*M* dithiothreitol and 100 m*M* NaF (Buffer A) were added, and the tissues were homogenized using an Ultra-Turrax at full speed for 15 sec. The homogenate was centrifuged for 3.5 min at 12 000 rpm in a Sigma Model M-202 Centrifuge (Osterode, Germany) equipped with a No. 12041 rotor. The resulting supernatant was desalted by passage through a 2.5 ml column of Sephadex G-25 equilibrated in 20 m*M* imidazole-HCl buffer, pH 7.0 containing 1.5 m*M* EDTA, 1 m*M* EGTA, 0.3 m*M* dithiothreitol and 30 m*M* NaF. The enzyme fraction was eluted with 3.5 ml of the same buffer.

Eluates were assayed directly for PFK activity. Assay conditions were 50 m*M* imidazole-HCl buffer, pH 7.0 with 5 m*M* $MgCl_2$, 0.15 m*M* NADH, 0.5 IU aldolase, 20 IU triosephosphate isomerase, and 2 IU glycerolphosphate dehydrogenase, with varied concentrations of fructose-6-P and Mg.ATP (1:1 mix of ATP and $MgCl_2$). Reaction velocities were monitored by following the NADH oxidation at 340 nm at 25 °C. K_m values (substrate concentration producing half-maximal enzyme velocity) for fructose-6-P were determined at 0.3 m*M* Mg.ATP whereas I_{50} values (inhibitor concentration reducing enzyme velocity by 50%) for Mg.ATP were determined at 1 m*M* fructose-6-P.

For glycogen phosphorylase, tissue samples were homogenized in 9 volumes (w/v) of buffer A and then centrifuged at 8000 rpm for 2 min in a No. 12045 rotor. Samples were not desalted before assay. Enzyme activity was measured by adding an aliquot of the supernatant to 50 m*M* potassium phosphate buffer, pH 7.0 containing 0.25 m*M* EDTA, 10 m*M* $MgCl_2$, 2 mg/ml glycogen, 10 μ*M* glucose-1,6-bisphosphate, 0.4 m*M* NADP, 0.2 IU phosphoglucomutase and 0.9 IU glucose-6-P dehydrogenase. Total phosphorylase vs phosphorylase *a* activities were determined in the presence vs absence of 1.6 m*M* AMP.

Metabolite analysis

After the test individuals were removed from the respirometer they were cooled on a bed of broken ice and then rapidly dissected. For *Mytilus galloprovincialis*, the posterior adductor muscle or the whole body was excised and then squeezed between aluminium blocks that had been pre-cooled in liquid nitrogen. Whole body samples from *Scapharca inaequivalvis* were obtained similarly. To remove blood samples from *S. inaequivalvis*, the shells of other individuals were wedged open with a knife until a small hole was created. The knife was then rotated 45° to open the shell sufficiently to drain the extrapallial seawater. Blood was then removed from the pericardial cavity using a Pasteur pipette. On average 1.75 ml blood was obtained per individual.

To prepare extracts of whole individual soft tissues, the frozen molluscs were first lyophilized for 48 h. The dry tissue of individual

specimens was then powdered, weighed and samples of ca. 300 mg were homogenized in 10 ml of an ethanol-phosphate buffer pH 7.0 (70% ethanol + 30% 33 m*M* KH_2PO_4) using an Ultra-Turrax (4 x 15 sec at maximum speed). After centrifugation as described above, the supernatant was removed and used for metabolite determinations.

To prepare adductor muscle extracts, freeze-clamped adductor muscles (five replicates of three pooled muscles) were pulverized under liquid nitrogen together with 4 ml frozen 6% perchloric acid in 20% ethanol using a mortar and pestle. The frozen adductor muscle-PCA powder was then thawed in ice water, homogenized, and centrifuged at 12 000 rpm for 10 min using a No 12041 rotor. The supernatant was neutralized with 5 *M* K_2CO_3 and after recentrifugation was used immediately for the determination of phosphoarginine and ATP; for the measurement of other metabolites the extract was stored frozen at −20 °C for several days until use.

To prepare blood extracts, blood from five specimens was pooled and mixed, a sample was removed for the estimation of haematocrit, and then 4 ml were transferred to a centrifuge tube containing 1 ml 30% perchloric acid. After sonication for 4 x 15 s, precipitated proteins were removed by centrifugation as above. The supernatant was adjusted to pH 7 to 8 by the addition of 5 *M* K_2CO_3 and the $KClO_4$ formed was removed by a second centrifugation. The supernatant was used for the determination of metabolites. The haematocrit percentage was estimated after centrifugation of blood samples at 2000 rpm for 3 min using a microhaematocrit rotor #11 000 (Sigma 202 M laboratory centrifuge). Plasma does not contain any significant amounts of the metabolites under study here (de Vooys et al. 1991) so utilizing the haematocrit percentage, metabolite concentrations have been expressed as μmol ml^{-1} wet red blood cells.

Metabolites (succinate, L-alanine, L-aspartate, ATP, phosphoarginine, D-octopine, D-lactate, D-strombine + *meso*-alanopine) were measured enzymatically according to standard procedures as described by Bergmeyer (1984) or in previous papers (de Zwaan et al. 1982). Recovery was checked with added standards. Lactate was the D-isomer in *Mytilus galloprovincialis*; lactate was not detected in *Scapharca inaequivalvis*. Alanine, measured here enzymatic by using L-alanine dehydrogenase, was confirmed as the L-isomer for selected samples by measurement using an amino acid analyzer (de Vooys et al. 1991). Due to enzyme cross-reactivity with both substrates, strombine and alanopine data are presented as the sum of the two compounds. Statistical testing of the data was performed using Student's *t*-test.

Results

Survival of anoxia

Fig. 1 shows the survival rates for *Mytilus galloprovincialis* and *Scapharca inaequivalvis* under anoxia. Survivorship was ≥95% for both species up to 11 d of anoxia. Survivorship began to decline at 12 d, and after longer times survival rates for the two species diverged. The calculated LT_{50} value for *M. galloprovincialis* was 15.1 d (14.7 to 15.4 d; 95% confidence) estimated by the Trimmed Spearman-Karber method (Hamilton et al. 1977). Mortality was 100% at 18 d. The calculated LT_{50} for *S. inaequivalvis* was significantly longer at 18.3 d (17.4 to 19.2 d; 95% confidence) with 100% mortality after 22 d of anoxia exposure.

Enzyme responses to anoxia in *Mytilus galloprovincialis*

Table 1 shows the effects of 24 h anoxia exposure on glycogen phosphorylase and 6-phosphofructo-1-kinase (PFK) from the posterior adductor muscle (PAM) of *M. galloprovincialis*. Anoxia exposure did not alter the maximal activities of either enzyme in the muscle. The percentage of phosphorylase in the active *a* form decreased significantly in anoxia, however. To gauge the effect of anoxia on PFK, an assessment of I_{50} values (the inhibitor concentration producing a 50% reduction of enzyme activity) for Mg.ATP was chosen since this parameter is markedly affected by anoxia exposure in other species (Storey 1984, Brooks et al. 1991, Whitwam and Storey 1991). In *M. galloprovincialis* the I_{50} ATP dropped dramatically in anoxia, the value being only 31.6% of the control, aerobic value.

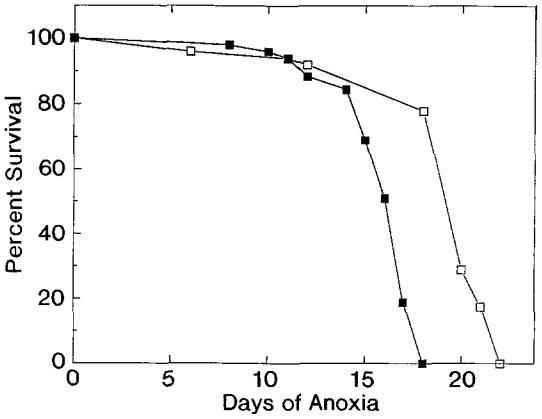

Fig. 1. *Mytilus galloprovincialis* and *Scapharca inaequivalvis*. Survival time of *M. galloprovincialis* (■) and *S. inaequivalvis* (□) in anoxic seawater at 20 °C. Fifty individuals of each species were used

Table 1. *Mytilus galloprovincialis*. Effect of 24 h anoxia exposure at 20 °C on glycogen phosphorylase and phosphofructokinase from *M. galloprovincialis* posterior adductor muscle. Values are means ±SD; $n=4$ samples with posterior adductor muscles from four mussels pooled per sample. All parameters were assayed at 25 °C. *: significantly different from corresponding control value using the Student's *t*-test; $P<0.01$

	Aerobic	Anoxic
Glycogen phosphorylase		
IU g^{-1} wet wt	1.09 ±0.23	0.86 ±0.16
Percentage *a*	74.0 ±8.5	54.7 ±8.5*
Phosphofructokinase		
IU g^{-1} wet wt	5.00 ±0.37	5.05 ±0.27
I_{50} ATP	5.7 ±0.19	1.8 ±0.24*

Oxygen consumption at low oxygen tensions

Fig. 2 shows rates of oxygen consumption for the two species at varying environmental oxygen tensions (note that at 20 °C an oxygen content of 7.6 ppm represents full saturation of the seawater). For *Mytilus galloprovincialis* oxygen consumption declined when oxygen tension dropped below ca. 3.4 ppm. *Scapharca inaequivalvis* showed a maximum oxygen consumption that was only

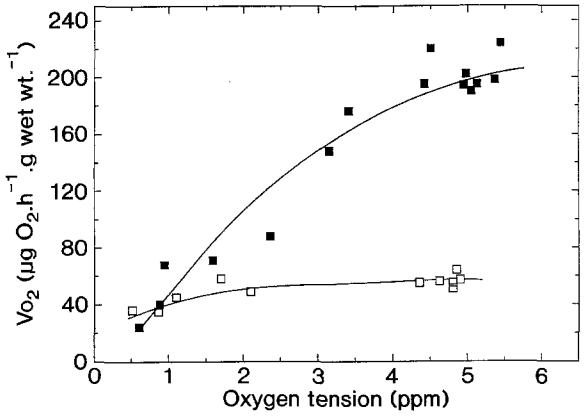

Fig. 2. *Mytilus galloprovincialis* and *Scapharca inaequivalvis*. Oxygen consumption, µg O_2 h^{-1} g^{-1} wet wt (excluding shell) at different pO_2 values at 20 °C. (■): *M. galloprovincialis*; (□): *S. inaequivalvis*. Each data point represent a pool of 35 individuals

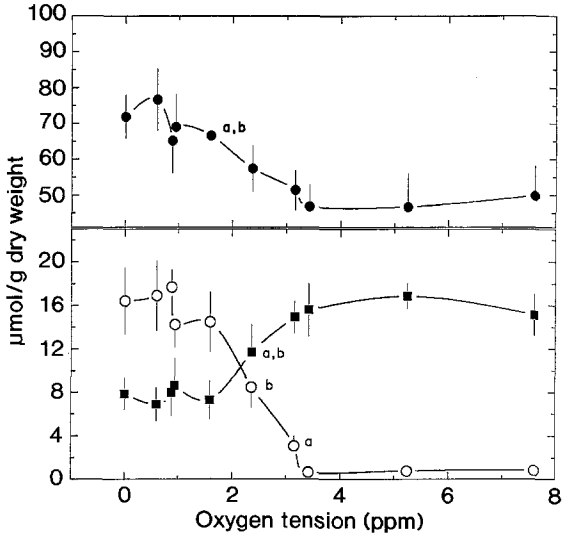

Fig. 3. *Mytilus galloprovincialis*. Whole body concentrations of succinate (o), L-alanine (●) and L-aspartate (■) after 12 h exposure to different oxygen tensions. Data are µmol g^{-1} dry wt; means ± SD; $n=5$ individuals. (a): This value and values at all lower oxygen tensions are significantly different from the aerobic control value (7.6 ppm), $P<0.025$. (b): The value and values at all higher oxygen tensions are significantly different from the value in anoxia (0 ppm), $P<0.01$

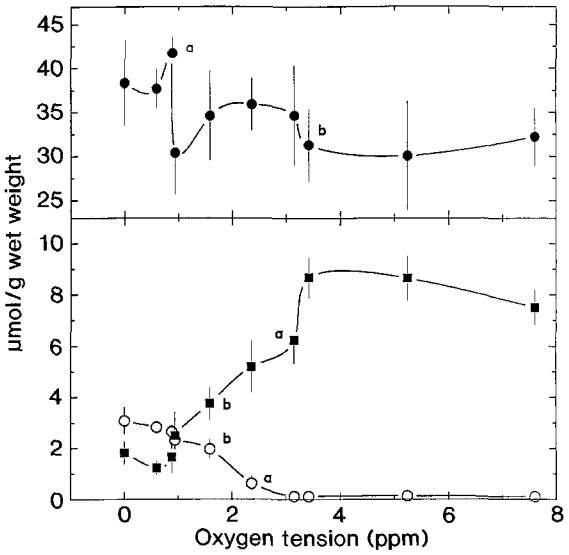

Fig. 4. *Mytilus galloprovincialis*. Concentrations of succinate (o), L-alanine (●), and L-aspartate (■) in the posterior adductor muscle after 12 h exposure to different oxygen tensions. Data are µmol g^{-1} wet wt; means: ± SD; $n=5$ replicates (each of three pooled muscles). (a): This value and values at all lower oxygen tensions are significantly different from the aerobic control value (7.6 ppm), $P<0.025$. (b): The value and values at all higher oxygen tensions are significantly different from the value in anoxia (0 ppm), $P<0.05$

one-third of the value for *M.* galloprovincialis, and consumption remained constant down to ca. 1.7 ppm. Below a critical pO_2, however, oxygen consumption in both species decreased. For *M. galloprovincialis* the overall decrease in oxygen consumption was ca. 9-fold between 3.4 and 0.6 ppm whereas consumption dropped by about one-third in *S. inaequivalvis* to between 1.7 and 0.6 ppm.

Effect of low oxygen contents on metabolite levels in *Mytilus galloprovincialis*

Fig. 3 shows the whole body contents of succinate, L-alanine and L-aspartate in *Mytilus galloprovincialis* exposed for 12 h at 20 °C to selected low oxygen concentrations. Levels of all three metabolites are clearly dependent upon oxygen availability with succinate and alanine levels rising and aspartate content decreasing when mussels were incubated at the lower pO_2 values. Succinate content was unchanged from the control value at 5.24 and 3.41 ppm oxygen but increased sharply when oxygen fell to 3.15 ppm and continued to increased as oxygen fell to 1.59 ppm. Incubations at three lower oxygen contents resulted in no further increase in succinate levels; at oxygen contents of 1.59 ppm and below, succinate contents were 17 to 21-fold higher than the values for aerobic control mussels but were not significantly different from the value for anoxic animals. The results for aspartate showed an inverse pattern to that of succinate. Aspartate levels were not altered by incubation at the higher oxygen contents but were decreased significantly after incubation at 2.36 ppm and were lower still at 1.59 ppm. Aspartate levels at lower oxygen tensions were not significantly different from each other or from the value for anoxic individuals. Alanine content of the mussels was very high (46 to 76 µmol g^{-1} dry wt), and a significant accumulation of alanine, compared to the aerobic controls, was noted only when oxygen content was decreased to 1.59 ppm. Alanine levels in individuals at the three lowest oxygen contents were not significantly different than the value for anoxic mussels.

Effect of low oxygen contents on posterior adductor muscle metabolites in *Mytilus galloprovincialis*

Fig. 4 shows the effects of 12 h exposures to different low oxygen tensions on the levels of succinate, L-alanine and

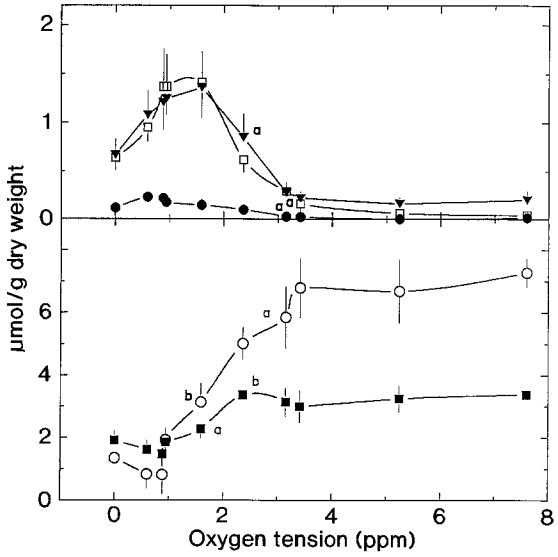

Fig. 5. *Mytilus galloprovincialis.* Concentrations of ATP (■), phosphoarginine (○), D-lactate (●), octopine (□), and strombine and/or alanopine (▼) in the posterior adductor muscle after 12 h exposure to different oxygen tensions. Data are µmol g^{-1} wet wt; means: ±SD; $n=5$ replicates (each of three pooled muscles). (a): This value and values at all lower oxygen tensions are significantly different from the aerobic control value (7.6 ppm), $P<0.05$. (b): The value and values at all higher oxygen tensions are significantly different from the value in anoxia (0 ppm), $P<0.05$

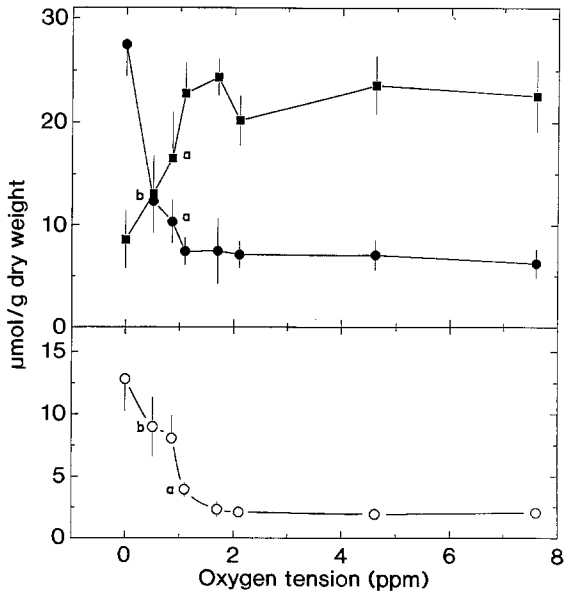

Fig. 6. *Scapharca inaequivalvis.* Whole body concentrations of succinate (○), L-alanine (●), and L-aspartate (■) after 12 h exposure to different oxygen tensions. Data are µmol g^{-1} dry wt; means: ±SD, $n=5$ animals. (a): This value and values at all lower oxygen tension are significantly different from the aerobic control value (7.6 ppm), $P<0.025$. (b): The value and values at all higher oxygen tensions are significantly different from the value in anoxia (0 ppm), $P<0.025$

L-aspartate in the posterior adductor muscle (PAM) of *Mytilus galloprovincialis*. Again, as was seen for the whole individual, metabolite concentrations changed in a graded manner in response to declining oxygen content. A significant rise in succinate content (compared to aerobic controls) was first seen at 2.36 ppm with a progressive increase in succinate noted down to 0.94 ppm. At this and lower oxygen tensions, succinate levels were not significantly different from the value for an anoxic muscle. Changes in aspartate contents of the PAM were also graded with the first significant decrease in the aspartate pool seen at 3.15 ppm followed by a progressive decrease as oxygen fell down to 0.94 ppm. Aspartate levels at this and lower oxygen tensions were not significantly different from the content of an anoxic muscle. Alanine levels in the muscles were high and variable, and significant increases over control levels were noted only at the two lowest oxygen tensions as well as in anoxia.

Changes in ATP content in the muscle occurred within a very narrow range of oxygen tension. Levels of ATP were unaltered down to oxygen values as low as 2.36 ppm (Fig. 5). At 1.59 ppm, however, ATP content decreased sharply to 67% of the control level and to 55% of control at 0.94 ppm. At lower oxygen tensions, as well as in anoxia, the ATP pool remained constant at 43 to 56% of the aerobic control value. Compared with ATP, changes in phosphoarginine occurred over a wider range of oxygen tensions, consistent with the role of phosphoarginine in buffering ATP in the cell. A significant decrease in phosphoarginine content was first noted at 3.15 ppm oxygen and subsequently phosphagen levels decreased progressively down to 0.88 ppm.

Fig. 5 shows the effects of reduced oxygen tensions on the levels of D-lactate, octopine, and strombine and/or alanopine in the PAM of *Mytilus galloprovincialis*. D-Lactate content of the muscle was very low (<0.23 µmol g^{-1} wet wt) in all cases but rose progressively over the range between 3.15 and 0.60 ppm. The first significant increase in octopine content occurred at 3.41 ppm with the highest levels reached between 0.88 and 1.59 ppm. The increase in octopine paralleled the decrease in phosphoarginine although net octopine accumulation (1.4 µmol g^{-1} at maximum compared with control) was substantially less than net phosphoarginine depletion (ca. 6 µmol g^{-1}) indicating that a rise in the free arginine pool must also have occurred. Strombine and/or alanopine [strombine is typically the major product in PAM of *Mytilus edulis* (de Zwaan et al. 1983)] were also accumulated by the PAM, again rising progressively with decreasing oxygen tensions. Interestingly, the net accumulation of all of these compounds was lower for mussels exposed to anoxia than for those exposed to intermediate low oxygen tensions.

Effect of low oxygen contents on metabolite levels in *Scapharca inaequivalvis*

Fig. 6 shows the effect of 12 h exposures to low oxygen tensions on the production of succinate and L-alanine and the utilization of L-aspartate in *Scapharca inaequivalvis*. Whole body contents of succinate remained equivalent to control values at oxygen tensions as low as 1.70 ppm whereas alanine and aspartate levels were unaf-

fected down to 1.10 ppm. At lower oxygen tensions succinate and alanine contents increased progressively whereas aspartate content decreased. For all three compounds, there was a marked and significant increase in concentrations between the lowest oxygen tension tested (0.51 ppm) and the complete anoxia condition. The net increase in succinate was 6.9 μmol g^{-1} (a 4.4-fold rise) at 0.51 ppm vs 10.8 μmol g^{-1} (a 6.25-fold rise) in complete anoxia. For alanine the increase was 2-fold at 0.51 ppm vs 4.4-fold in anoxia and the aspartate pool decreased to 58% of the original at 0.51 ppm vs 38% of the control value in anoxia.

Effect of low oxygen exposures on metabolite levels in *Scapharca inaequivalvis* red blood cells

To further assess the responses to low oxygen by *Scapharca inaequivalvis*, we examined the changes in metabolite levels in red blood cells of the clam in response to exposure to low oxygen tensions. Small but significant increases in succinate (from 0.15 ± 0.05 to 0.46 ± 0.10 μmol ml^{-1} red blood cells) and L-alanine (from 1.45 ± 0.35 to 2.71 ± 0.56 μmol ml^{-1} red blood cells) levels in the cells were first seen when clams were exposed to 2.1 ppm oxygen whereas levels of both compounds were sharply increased (by 10-fold for succinate, 2.5-fold for alanine) at 0.86 ppm oxygen. The first decline in L-aspartate content occurred when cells were incubated at 1.7 ppm oxygen (Fig. 7). As also occurred in the whole individual, there were significant differences in the levels of all three metabolites between the lowest oxygen tension (0.51 ppm) and the anoxic condition. Levels of strombine and/or alanopine in red blood cells were extremely low and variable (<0.02 μmol ml^{-1} red blood cells) at the higher oxygen tensions, increased slightly (0.028 to 0.042 μmol ml^{-1} at the two lowest oxygen tensions, and rose to 0.057 ± 0.047 μmol ml^{-1} in anoxic clams. However, the contributions of these compounds to anaerobic ATP production were obviously minor compared with net increases of 3.35 and 4.64 μmol ml^{-1} red blood cells for succinate and alanine, respectively. No D-lactate or octopine was detected in the red blood cells nor was phosphoarginine found. ATP content of the red blood cells was maintained at a high level with exposures to oxygen tensions as low as 1.70 ppm (Fig. 7). Below this value, however, ATP content declined progressively reaching 58% of the control value in anoxia. The ATP concentration at 0.51 ppm was significantly higher than the value in full anoxia.

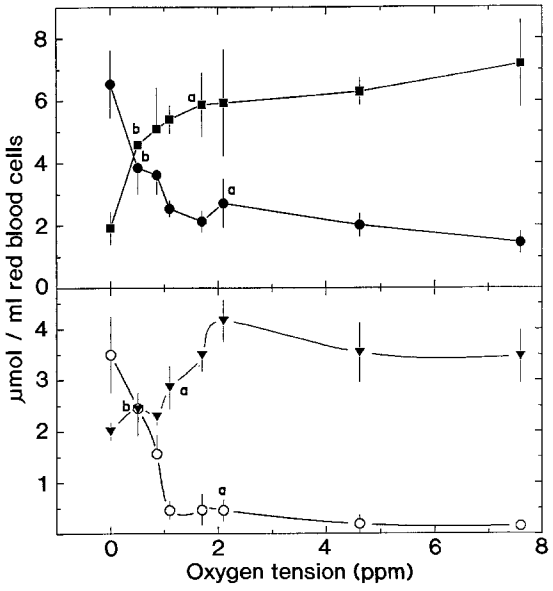

Fig. 7. *Scapharca inaequivalvis*. Concentrations of succinate (○), L-alanine (●), L-aspartate (■), and ATP (▼) in red blood cells after 12 h exposure to different oxygen tensions. Data are μmol ml^{-1} wet red blood cells; means: ±SD, $n=5$ individuals. Mean haematocrit volume was 8.06 ± 1.61% of total blood volume and was not significantly altered by hypoxia or anoxia exposures. (a): This value and values at all lower oxygen tensions are significantly different from the aerobic control value (7.6 ppm), $P<0.05$. (b): The value and values at all higher oxygen tensions are significantly different from the value in anoxia (0 ppm), $P<0.025$

Discussion

Both *Mytilus galloprovincialis* and *Scapharca inaequivalvis* have high tolerances of anoxia showing 100% survival of 11 d of anoxia exposure and high LT_{50} values of 15.1 and 18.3 d (at 20 °C), respectively. Net accumulations of succinate and alanine over 12 h of anoxia were also similar for the two species (Fig. 3 vs 6) suggesting similar metabolic responses to the complete absence of oxygen. The anoxia tolerance of both species contrasts dramatically with the very limited anoxia survival of *Venus gallina* which showed LT_{50} values of only 4 to 5.2 d at 18 to 20 °C (Brooks et al. 1991, de Zwaan et al. 1991).

The impressive anoxia survival of *Mytilus galloprovincialis* and *Scapharca inaequivalvis* compared with *Venus gallina* probably derives largely from differences between the species in their capacity for metabolic arrest. In a previous experiment we established that anoxic metabolic rates in *S. inaequivalvis* and *V. gallina* were 4.5 and 13% of the corresponding aerobic rates, respectively (Brooks et al. 1991). Data in the present study allow us to make a rough calculation of metabolic arrest for *M. galloprovincialis* provided that two assumptions are made. We assume that the anaerobic ATP and phosphoarginine contributions to ATP provision that are established for the adductor muscle can also be applied to the whole body (perhaps a slight overestimate) and that negligible conversion of succinate to proprionate occurred during the 12 h incubation [proprionate production occurs only after a lag period; for *M. edulis* this is about 10 h at 20 °C (Kluytmans et al. 1978, Ho and Zubkoff 1982)]. From the data in Fig. 3 and 4 an anaerobic ATP turnover rate of ca. 9 μmol (g dry wt)$^{-1}$ h^{-1} can be derived [calculated as in Brooks et al. (1991) from the increase in succinate × 2.75, the accumulation of alanine in excess of aspartate × 1.5, and the decrease in ATP and phosphoarginine]. The data in Fig. 2 provide an aerobic oxygen consumption of ca. 200 μg (g wet wt)$^{-1}$ h^{-1} which can be converted to an aerobic ATP turnover rate of 195 μmol

(g dry wt)$^{-1}$ h^{-1} (wet/dry weight ratio = 5.2). The anoxic metabolic rate of *M. galloprovincialis* appears, therefore, to be about 4.5% of the normoxic resting rate. Thus, *M. galloprovincialis* and *S. inaequivalvis*, both with anoxic metabolic rates about 4.5% of aerobic metabolic rate are clearly better prepared for long term anoxia survival than is *V. gallina* with its corresponding value of 13%. Furthermore, only *M. galloprovincialis* and *S. inaequivalvis* showed evidence of the use of specific molecular mechanisms for the coordinated reduction of the rates of various metabolic processes in anoxia. Glycolysis is the central pathway for anaerobic ATP production, and as such, control over its regulatory enzymes is a key element of metabolic arrest. Anoxia-induced changes in the properties of glycogen phosphorylase, PFK and pyruvate kinase, indicative of post-translational modifications of these enzymes to produce less active enzyme forms in the anoxic state, were found in *M. galloprovincialis* (Table 1) and *S. inaequivalvis* but not (or far less pronounced) in *V. gallina* (Isani 1987, Brooks et al. 1991). Thus, anoxia exposure led to a significant decrease in the percentage of phosphorylase in the active *a* form in the PAM of *M. galloprovincialis* as well as a 3.2-fold decrease in the I_{50} for Mg.ATP of PFK (Table 1). In *S. inaequivalvis* foot muscles 5 d of anoxia exposure also resulted in a significant drop in the percent phosphorylase *a* (from 60.4± 2.9% in control to 49.4±2.2% in anoxic clams) and a 6.8-fold decrease in the I_{50} Mg.ATP of PFK (from 9.6±1.2 m*M* to 1.4±0.04 m*M*) (Brooks et al. 1991). By contrast, these parameters were unaltered by anoxia exposure in *V. gallina* foot muscles (Brooks et al. 1991). Anoxia-induced changes to PFK properties have also been reported for organs of various other marine molluscs (Storey 1984, Michaelidis and Storey 1990, 1991, Whitwam and Storey 1991), although the response of PFK from PAM of *Mytilus edulis* was the opposite of that seen here, with I_{50} ATP increasing by 2-fold after 24 h anoxia at 23 °C (Michaelidis and Storey 1991). Nonetheless, the stable modification of PFK properties in anoxia in all of these species except *V. gallina* suggests that such enzyme modification, producing less active enzyme forms, are an integral part of the anoxia-induced metabolic arrest survival strategy of good facultative anaerobes.

Whereas *Mytilus galloprovincialis* and *Scapharca inaequivalvis* appear to be similarly equipped for anoxia survival, the two species had substantially different sensitivities to hypoxia. Oxygen consumption decreased when pO_2 values fell below ca. 3.4 ppm for *M. galloprovincialis* whereas constant oxygen consumption was maintained down to 1.7 ppm for *S. inaequivalvis*. Furthermore, elevated whole body amounts of succinate and alanine, indicative of the recruitment of fermentative pathways of ATP production, were encountered at 3.15 and 1.59 ppm, respectively, in *M. galloprovincialis* but at much lower oxygen tensions in *S. inaequivalvis*, 1.10 and 0.86 ppm, respectively (Fig. 3, 6). Studies on the marine worms *Arenicola marina* and *Scoloplos armiger* found threshold oxygen tensions for succinate synthesis much the same as those reported here for *M. galloprovincialis* (Schöttler et al. 1983, Schöttler and Grieshaber 1988). In addition, whereas the metabolite responses to oxygen tensions of 1.59 ppm and below were all indistinguishable from the response to full anoxia by *M. galloprovincialis*, there was a major difference between net metabolite accumulations in *S. inaequivalvis* even at the lowest (0.51 ppm) oxygen tension compared with full anoxia. This indicates that, even at this very low pO_2 value, fermentative pathways of ATP production are still not fully recruited, and this implies that there is still a substantial aerobic component to metabolism in *S. inaequivalvis* at this low pO_2. By contrast, *M. galloprovincialis* showed maximum recruitment of fermentative pathways of ATP output at pO_2 values of 1.59 ppm and in terms of these metabolite responses appears to be behaving as if it were functionally anoxic (even though low rates of oxygen consumption continued). In addition, as viewed from the metabolite data, the graded transition from aerobic to hypoxic to anoxic metabolism occurred between 3.15 ppm and 1.59 ppm for *M. galloprovincialis* (Fig. 3) whereas for *S. inaequivalvis* it began at 1.10 ppm and extended well below 0.51 ppm (Fig. 6). Thus, it is obvious that in situations of declining oxygen availability, such as during progressive eutrophication of the benthic waters of the Adriatic Sea, *M. galloprovincialis* will experience hypoxia, and any negative effects of hypoxia on metabolism, long before low oxygen conditions become severe enough to affect *S. inaequivalvis*. *For* any given oxygen tension below 3.4 ppm, *M. galloprovincialis* would experience a limitation on its maximal metabolic rate which could affect numerous functions such as rates of food assimilation, growth and reproductive development. For *S. inaequivalvis*, such stress would only be experienced below 1.7 ppm. Thus, at any given low oxygen tension the degree of stress experienced by *M. galloprovincialis* would be substantially higher than that felt by *S. inaequivalvis* and, ultimately, continued low oxygen stress could result in a higher mortality of the mussel species.

The data also provide evidence that individual organs within each species can show different sensitivities to hypoxia. From Fig. 3 vs 4 it is evident that the adductor muscle of *Mytilus galloprovincialis* was less sensitive to declining oxygen tension than was the mussel as a whole. Thus, a significant increase in succinate occurred only at 2.36 ppm oxygen in the PAM (Fig. 4) compared with 3.15 ppm for the intact individual (Fig. 3). Alanine accumulation was similarly first evident at a lower oxygen tension in the PAM than in the whole body. This suggests that individual organs of *M. galloprovincialis* have different threshold oxygen concentrations below which aerobic pathways are supplemented by fermentative ATP production. The opposite situation was seen in *Scapharca inaequivalvis* for the comparison between red blood cells vs the whole individual (Fig. 6, 7). Red blood cells showed significantly increased levels of succinate and alanine at 2.10 ppm oxygen (Fig. 7) whereas the average succinate and alanine contents of the whole individual were increased only with exposure to oxygen tensions of 1.10 ppm or lower (Fig. 6).

The data for *Mytilus galloprovincialis* PAM also allow some interesting comments to be made on the role of pyruvate-utilizing dehydrogenases in metabolism. In the

PAM, the highest net accumulation of lactate + octopine + strombine/alanopine (2.66 μmol g^{-1}) occurred in the mussels exposed to oxygen tensions between 0.88 and 1.59 ppm. The net accumulation of glycolytic end products decreased at both higher and lower oxygen tensions and, in complete anoxia, the total was only 40% of the value at 1.59 ppm. These results suggest that the involvement of imino acids and lactate as products of fermentative metabolism is greatest in hypoxia. In complete anoxia and at very low oxygen tensions that are functionally anoxic to the individual, these products are of much less importance to overall ATP-generation. It appears, then, that these compounds are produced when aerobic ATP production needs to be supplemented in an attempt to maintain high and/or constant levels of ATP use. This may indicate, therefore, that lactate and imino acids are products of hypoxia stress, not of anoxia. This also agrees with their role in the recovery from anoxia, for levels of these compounds rise rapidly when anoxic individuals are first re-exposed to aerobic conditions (de Zwaan et al. 1983). Notably, maximal accumulation of these compounds occurred at the same oxygen tension (1.59 ppm) that first caused a drop in cellular ATP levels (Fig. 5). At lower pO_2 values, however, at which both ATP and phosphoarginine are depleted, lower amounts of these products were produced, probably because of the overriding influence of metabolic arrest strategies in both reducing overall ATP requirements and, via phosphorylation inactivation of pyruvate kinase in channeling carbon flow into the succinate pathway.

Mangum and Mauro (1985) found that the red blood cells from other marine invertebrates lysed instantly when pO_2 was lowered to zero. Our present in vivo studies with *Scapharca inaequivalvis* red blood cells, however, show that these have as high a capacity for anaerobic survival as would be expected of the species. Isolated cells equilibrated with a constant stream of water saturated nitrogen gas for 6 h neither lysed nor coagulated and accumulated succinate and alanine while depleting aspartate (Cortesi et al. 1991). Similar metabolic responses by red blood cells were observed during 24 h anoxia exposure in vivo (de Vooys et al. 1991). The metabolism of these cells is somewhat different from that of other organs of the clam, however. Phosphoarginine was missing, glycogen was very low and phosphorylase was undetectable (de Vooys et al. 1991). The production of end products from pyruvate was also very poor. Fermentative ATP production in the red blood cells probably relies strictly on the coupled catabolism of glucose (taken up from plasma) and aspartate.

In conclusion, whereas both *Scapharca inaequivalvis* and *Mytilus galloprovincialis* are good facultative anaerobes with similarly well-developed biochemical adaptions for metabolic arrest and fermentative ATP production in anoxia, *S. inaequivalvis* shows a superior ability for dealing with hypoxia. Both the recruitment of fermentative pathways to supplement aerobic ATP production as well as the maximal activation of these pathways occur at substantially lower pO_2 values for *S. inaequivalvis* than for *M. galloprovincialis*. It follows, therefore, that when faced with equivalent environmental low oxygen conditions that populations of *S. inaequivalvis* would find seasonal cycles of hypoxia to be less stressful than *M. galloprovincialis* populations. It is clear, then, that the sensitivities of different species to hypoxia can be a valuable indicator of the potential for population survival in environments experiencing eutrophication.

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