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Effects of environmental parameters on the oxygen consumption of four marine invertebrates: a comparative factorial study

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Abstract The oxygen consumption curves of two decapod crustaceans (*Palaemon serratus*, *Penaeus monodon*) and two prosobranch molluscs (*Truncularioposis trunculus*, *Nassarius mutabilis*) have been detected in the entire pO_2 interval from 0 to 160 mmHg, under different conditions of temperature and salinity. From the experimental curves, physiological parameters such as the initial oxygen consumption velocity, the Q_{10} values and the oxygen independence indices have been measured. The latter parameters have been obtained using normalised plots which allow their better evaluation. The effects of temperature, salinity and oxygen partial pressure on the oxygen-consumption features have been studied using a factorial experimental plan which allows measurement of the effects of each experimental variable as well as the effects of synergistic interactions between different variables.

Introduction

The adaptation of an organism involves integrated responses to the changes of environmental parameters depending on species-specific homeostatic control mechanisms. Such integrated responses may be exerted at different biochemical, physiological and behavioural levels. The organism itself can be considered an integrator, since the fluctuations of the various environmental parameters are experienced by the animal as an integrated, complex stimulus. In this context, the effects of a single variable are largely overridden by the positive or negative synergism between the various variables of a given environment.

Estuarine and brackish temperate ponds are typically unstable environments, where aquatic organisms are adapted to the marked fluctuations in temperature and salinity, as well as in oxygen partial pressure. The latter is an important environmental parameter that, in aquatic environments, can often undergo large and not easily predictable fluctuations; the low values of the diffusion and solubility coefficients of this gas in water, together with the fact that many organisms are consuming and many others are producing oxygen by photosynthesis during the day, make this environmental parameter a variable strongly affected by the integrated characteristics of the specific environment.

Among the physiological parameters that one can correlate with environmental changes, the respiration rate and its dependence on oxygen partial pressure are very appropriate because they are strictly related to the metabolic work and energy flow of the organism that may be involved in the homeostatic control mechanisms.

In the current paper we present a comparative study of the effects of temperature, salinity and oxygenation level on the rate of oxygen consumption and oxygen dependence of four species of marine invertebrates: two decapod crustaceans, *Palaemon serratus* (Caridea: Palaemonidae) and *Penaeus monodon* (Penaeidea: Penaeidae), and two prosobranch molluscs, *Truncularioposis trunculus* (Muricacea: Muricidae) and *Nassarius mutabilis* (Buccinacea: Nassaridae). With the exception of *P. monodon*, all species live in the lagoon of Venice where the salinity ranges between 27‰ and 34‰ and the temperature spans from 4–5 °C in winter to 25–30 °C in summer time (Di Muro et al. 1988). *P. monodon* is a tropical species; it inhabits an ecosystem in which the general physical conditions tend to be quite stable. It has been recently introduced for aquaculture trials in coastal areas of the northern Adriatic Sea (Lumare et al. 1993) where high growth rates have been achieved within the spring–autumn period, demonstrating that the organism can adapt well to a more variable environment as long as

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temperature does not fall below 10 °C. *P. serratus* is a typical intertidal invertebrate capable of populating shallow sandy bottoms and belts of brown algae, where it usually competes with *Palaemon elegans* (Dalla Via 1985). *P. serratus*, usually considered to inhabit rock pools, can also migrate into the shallow water of lagoons (Guerao and Ribera 2000). Therefore, it can be subjected to rather large variations of temperature, salinity and oxygen partial pressures, especially when it is confined to rock pools in the intertidal zone. The two molluscs are benthic and inhabit areas between the intertidal and the sub-littoral zones; however, they can tolerate hypoxia and evaporation by sealing their shell with an operculum.

In studies on the influence of environmental variables on a given physiological function, it is obviously important to consider the effects brought about by each single pertinent variable; however, as stated above, the effects resulting from the positive or negative synergistic interactions of two or more variables, which are changing simultaneously, can be even more important for a deeper understanding of the complex relationships between an organism and its environment. Thus, the experiments for our comparative study were carried out following a factorial design; this allows evaluation of the interactions of second- and higher order interactions between variables, as well as of the effects assignable to each single variable.

Materials and methods

Collection and maintenance

Specimens of all wild species were caught in the lagoon of Venice. *Penaeus monodon* came from the same area, but it was cultured in the aquaculture plant in the ESAV (Ente di Sviluppo Agricolo del Veneto) area of "Biotopo Bonello".

The wet body weight of the specimens ranged between 10.0 and 18.0 g for *Trunculariopsis trunculus* (shell-free), 0.3 and 0.8 g for *Nassarius mutabilis* (shell-free), 1.5 and 3.0 g for *P. monodon* and 0.4 and 0.8 g for *Palaemon serratus*. Individuals (20–30) of all species were kept in separate aquaria containing 80 l of sea water at 20 °C and 30‰ salinity. Molluscan specimens were kept for no longer than 10 days, without feeding. Shrimps were fed on dried shrimps or chopped mussel meat. Before oxygen consumption measurements, the animals were adapted separately to the desired temperature and salinity in a thermostated and air-saturated 1 l chamber for 24 h. At the end of the adaptation period, the animal was transferred to the respiratory chamber and allowed to overcome the stress of handling under a gentle, air-saturated water flow, at the desired temperature and salinity, for 2 h. The water was filtered with 0.22 μm Millipore filters. Prior to experiments, the surface of the shells of gastropods was sketched, and then cleaned with ethanol to eliminate algae, which could influence the measurements.

The experiment started by stopping the water flow and capping the chamber. Temperature was controlled with a Haake model D3 thermostat, with a precision of ±0.1 °C. The concentration of oxygen, dissolved in the water filling the respiratory chamber, was continuously monitored with a Clarke electrode (YSI; Yellow Spring Instruments). The electrode was equipped with a Teflon membrane of 25 μm thickness. The oxygen consumption by the electrode is so low (0.01 μl O_2 h^{-1}) as to have no effect on the experimental recordings with animal specimens. The oxygen electrode was connected to a 5300 YSI power supply; the output signal was monitored, as a function of time, on a Macintosh LC 630 personal computer with an A/D interface (Vernier Software). A special program for data recording, Data Logger Pro, was used. The measurements were extended to complete anoxia, with exception of those for *P. monodon*, for which concentrations of dissolved oxygen <0.7 mg l^{-1} are lethal (Liao and Murai 1986). In this case the measurements were stopped when dissolved oxygen was about 5% of the initial concentration. When measurements were not completed within 3 h, the water was changed in order to eliminate the produced catabolites, by replacing it with water equilibrated at the same pO_2. Differences in the results of measurements extending up to 5 h, with and without the substitution of water, were found to be non-significant. For each experimental condition, the average value resulting from five measurements, performed on single individuals, was utilised.

At the end of the experiments the prawns were rinsed and weighed; the snails were weighed after removal of the shell. For this purpose, the snails were boiled for 3 min to simplify the extraction of their body from the shells.

From the continuous plots representing the concentration of physically dissolved oxygen as a function of time, the oxygen consumption rate ($\dot{V}O_2$, in ml O_2 g^{-1} wet tissue h^{-1}) was calculated. The plots were also elaborated to obtain the rate of oxygen consumption, $\dot{V}O_2$, as a function of pO_2, in the whole range of oxygen partial pressures covered by the experiment (Bridges and Brand 1980). The pO_2 values are expressed in millimetres of mercury throughout (760 mmHg = 101.308 kPa).

Factorial design of experiments

The experiments were planned according to a factorial design of three variables at two levels (Box et al. 1978). We considered the variables temperature (*T*), salinity (*S*) and oxygen partial pressure (*P*). The factorial plan of the experiments, together with the values assigned to the two levels of variables, are reported in Table 1. The factorial plan with three variables (*T*, *S*, *P*) at two levels (+, −), involves a set of 2^3 experiments resulting from the linear combinations of the levels of the variables, as summarised in Table 2. The symbols $R_1, R_2, \ldots, R_8$ represent the results of experiments $A_1, A_2, \ldots, A_8$, respectively. This approach was applied for the analysis of the effects of the experimental variables (*T*, *S*, *P*) on: (1) the $\dot{V}O_2$, (2) the Q_{10} values and (3) on the parameters of oxyregulation, which were determined from the $\dot{V}O_2 = f(pO_2)$ plots (see below).

Factorial analysis of the experimental results

The treatment of the data for the evaluation of the effect E_J of each variable and of their interactions was performed according to Box et al. (1978). The effects of the single variables ("main effect" of the variable) are obtained by dividing by four the algebraic sum of the R_i values with the signs in the column relative to the specific variable. Therefore, the effect of temperature (E_T) is:

$$(E)_T = \frac{-R_1 + R_2 - R_3 + R_4 - R_5 + R_6 - R_7 + R_8}{4} \qquad (1)$$

Table 1 Summary of experimental parameters used in the factorial design of the oxygen consumption experiments

Levels	Temperature[a] (*T*)	Salinity (*S*)	pO_2 (*P*)
+	20 °C	34‰	159 mmHg
−	10 °C	15‰	75 mmHg

[a] For *Penaeus monodon* the values were 20 °C (−) and 30 °C (+)

Table 2 Summary of the factorial experimental plan. *R* indicates the measured oxygen consumption velocity in the *i*th ($i = 1, 2,..., 8$) experiment, where the levels (+) and (–) of temperature (T) salinity (S) and oxygen partial pressure (P) are as indicated in Table 1

Experimental condition	T	S	P	Result	$T \times S$	$T \times P$	$S \times P$	$T \times S \times P$
A_1	–	–	–	R_1	+	+	+	–
A_2	+	–	–	R_2	–	–	+	+
A_3	–	+	–	R_3	–	+	–	+
A_4	+	+	–	R_4	+	–	–	–
A_5	–	–	+	R_5	+	–	–	+
A_6	+	–	+	R_6	–	+	–	–
A_7	–	+	+	R_7	–	–	+	–
A_8	+	+	+	R_8	+	+	+	+
Effects	E_T	E_S	E_P		$E_{T\times S}$	$E_{T\times P}$	$E_{S\times P}$	$E_{T\times S\times P}$

the effect of salinity (E_S) is:

$$(E)_S = \frac{-R_1 - R_2 + R_3 + R_4 - R_5 - R_6 + R_7 + R_8}{4} \tag{2}$$

and the effect of oxygen partial pressure (E_P) is:

$$(E)_P = \frac{-R_1 - R_2 - R_3 - R_4 + R_5 + R_6 + R_7 + R_8}{4} \tag{3}$$

The second- and third-order effects E_J due to the interactions between the variables are obtained following the same procedure, using for each result R_i the signs obtained by multiplying the signs in the columns of the considered individual variables. Accordingly, as an example, the signs of the column "$T \times S$" are obtained by multiplying the corresponding signs of the columns "T" and "S". In the same way the signs of columns "$T \times P$", "$S \times P$" and "$T \times S \times P$" are obtained (see Table 2).

Significance of the effects E_J was evaluated by comparing the estimated values of the effects E_J with their standard error. The variance relative to each effect, $V_{(\text{effect})}$, was estimated according to Box et al. (1978):

$$V_{(\text{effect})} = \frac{4}{N}\frac{v_1 s_1^2 + v_2 s_2^2 + \cdots + v_8 s_8^2}{v_1 + v_2 + \cdots + v_8} \tag{4}$$

where N is the total number of experiments (in our case a total of 40 runs resulting from five replicates for eight different conditions) and $v_1, v_2,..., v_8$ and $s_1^2, s_2^2,..., s_8^2$ are the degrees of freedom and the variance of the *i*th experimental condition. Thus, the estimated standard error (SE) of an effect is: $\sqrt{V_{(\text{effect})}}$. Significance was determined according to Student's *t*-test for $df = 39$.

Analysis of the oxyregulation curves $VO_2 = f(pO_2)$

The continuous recording of oxygen partial pressure as a function of time allows not only the measurement of the initial oxygen consumption rate $(VO_2)_i$ but also the generation of the oxyregulation plots of $VO_2 = f(pO_2)$. The data were elaborated as follows: initially, the plots were converted by introducing the "reduced variables" $(pO_2)^*$ and $(VO_2)^*$ which result from the normalisation of each pO_2 to 159 mmHg and each VO_2 to the experimental value obtained at 159 mm Hg. In such a plot, completely oxygen-dependent behaviour is expected to yield a straight line, the slope of which is equal to 1. Oxygen independence is manifested as a hyperbolic curve, the curvature of which depends on the degree of independence. The plot of $(VO_2)^*$ versus $(pO_2)^*$ allows quantification of the degree of oxygen independence, as well as the extent of the $(pO_2)^*$ region of oxygen dependence, from the slopes of the lines interpolating the curve $(VO_2)^* = f(pO_2)^*$ in the regions approaching $(pO_2)^* = 1$ and $(pO_2)^* = 0$, respectively. These slopes can be defined as $\tan\alpha_0 = K_0$ and $\tan\alpha_1 = K_1$, where α_0 and α_1 are the angles between the tangent lines to the curve at the points (0, 0) and (1, 1), respectively, and the diagonal connecting the same points. Thus, the values of the oxygen independence indices K_0 and K_1 span between 0 and 1.

Results

Effects of temperature, salinity and oxygen partial pressure on the oxygen consumption rate; factorial analysis of the results

On the basis of an overall comparison of the data reported in Table 3, the initial oxygen consumption rate $(VO_2)_i$ results are quite different between the snail and the shrimp species, while striking similarities can be observed among the two different shrimp taxa and among the two different snail taxa. In the case of the shrimp species, the $(VO_2)_i$ ranges between 0.16 ± 0.01 ml g^{-1} h^{-1} (*Palaemon serratus* condition 1) and 0.91 ± 0.08 ml g^{-1} h^{-1} (*Penaeus monodon* condition 8), while in the case of the molluscan species, values from 0.0043 ± 0.0008 (*Truncilariopsis trunculus*, condition 1) to 0.15 ± 0.02 (*Nassarius mutabilis* condition 8) are obtained.

Table 3 Averages and standard deviations of initial oxygen consumption rates (in ml O_2 g^{-1} wet tissue h^{-1}) of all invertebrate species determined under the different experimental conditions. The conditions indicated for $A_1, A_2,..., A_8$ are as described in Table 2

$R_1, R_2,..., R_8$ [$(VO_2)_i$, in ml g^{-1} h^{-1}] Experimental conditions	*Truncularìopsis trunculus*	*Nassarius mutabilis*	*Penaeus monodon*	*Palaemon serratus*
$A_1(T\!-S\!-P\!-)$	0.0043 ± 8E − 04	0.0212 ± 0.005	0.1950 ± 0.026	0.1615 ± 0.014
$A_2(T\!+S\!-P\!-)$	0.0344 ± 0.004	0.0790 ± 0.006	0.7350 ± 0.045	0.4696 ± 0.052
$A_3(T\!-S\!+P\!-)$	0.0185 ± 0.004	0.0395 ± 0.007	0.2150 ± 0.023	0.1688 ± 0.029
$A_4(T\!+S\!+P\!-)$	0.0790 ± 0.017	0.0951 ± 0.011	0.7390 ± 0.085	0.4890 ± 0.077
$A_5(T\!-S\!-P\!+)$	0.0065 ± 0.001	0.0253 ± 0.004	0.2518 ± 0.034	0.1951 ± 0.011
$A_6(T\!+S\!-P\!+)$	0.0417 ± 0.001	0.0870 ± 0.001	0.9050 ± 0.063	0.5628 ± 0.041
$A_7(T\!-S\!+P\!+)$	0.0208 ± 0.003	0.0549 ± 0.007	0.2810 ± 0.035	0.2035 ± 0.034
$A_8(T\!+S\!+P\!+)$	0.1102 ± 0.018	0.1472 ± 0.018	0.9080 ± 0.082	0.5645 ± 0.087

For all organisms studied, the increase of temperature from the condition $T(-)$ to $T(+)$ results in an increase of $(VO_2)_i$ (Table 3), occurring independently from the variation of the other parameters; namely $(VO_2)_i$ increases from condition $A_1(T-S-P-)$ to condition $A_2(T+S-P-)$ and from condition $A_7(T-S+P+)$ to condition $A_8(T+S+P+)$. Moreover, an increase of $(VO_2)_i$ is also observed upon increasing the temperature when salinity and pO_2 change from the (−) to the (+) condition, either separately [condition $A_1(T-S-P-)$ *versus* $A_4(T+S+P-)$ and condition $A_1(T-S-P-)$ *versus* $A_6(T+S-P+)$; Table 3] or simultaneously [condition $A_1(T-S-P-)$ *versus* $A_8(T+S+P+)$ and condition $A_7(T-S+P+)$ *versus* $A_2(T+S-P-)$; Table 3].

The response of the organisms to the changes in salinity is different for snail and shrimp species. In the case of *T. trunculus* and *N. mutabilis,* the decrease of salinity from 34‰ to 15‰, at constant T and P [$A_7(T-S+P+)$ *versus* $A_5(T-S-P+)$ or $A_8(T+S+P+)$ *versus* $A_6(T+S-P+)$ or $A_4(T+S+P-)$ *versus* $A_2(T+S-P-)$ or $A_3(T-S+P-)$ *versus* $A_1(T-S-P-)$] causes a reduction of $(VO_2)_i$ (Table 3). On the contrary, in the case of shrimps, salinity has no effects on $(VO_2)_i$ under the same conditions. The evaluation of the effects of salinity combined with those of temperature and oxygen pressure are more difficult to evaluate by a pairwise comparison of conditions, given the complexity of the multi-parameter approach. The factorial analysis of the results is, therefore, required to disclose both the main effects of each single variable, as well as the possible synergistic interactions between variables.

The analysis of the results of the factorial experimental plan is reported in Table 4 and Fig. 1; the former reports the numerical values of the average effects [as the average of all $(VO_2)_i$ values for a given species determined under the different conditions], the main effect of each single variable (calculated from Eqs. 1, 2 and 3) and the effects of second- or third-order interactions between the different variables (calculated, as described in the "Materials and methods", on the basis of the sign matrix of Table 2). Figure 1 reports the numerical values of the effects of the different variables in the form of histograms together with their significance. The effect of temperature (E_T) is highly significant ($p < 0.001$) and predominant in all species, compared to the effects of salinity (E_S) and of pO_2 (E_P) (Fig. 1). A significant E_S is characteristic only for mollusc species ($p < 0.001$). Furthermore, for these species, the two-variable interactions have significant effects and are observed for all combinations. For *T. trunculus* the significance decreases in the order $E_{T\times S}$ ($p < 0.001$) > $E_{T\times P}$ ($p < 0.01$) > $E_{S\times P}$ ($p < 0.05$), whereas for *N. mutabilis* high significance is associated with the $E_{S\times P}$ interaction ($p < 0.001$), but $E_{T\times P}$ and $E_{T\times S}$ have lower significance ($p < 0.01$ and $p < 0.025$, respectively). The three-variable interaction has a significant effect ($E_{T\times S\times P}$) for both species, although the level of significance is rather low ($p < 0.05$). In arthropod species significant effects of two-variable interactions are observed only in the case of $E_{T\times P}$ for *P. monodon* ($p < 0.005$).

As far as pO_2 is concerned, E_P is equally significant in the case of both snail and shrimp species ($p < 0.001$). Furthermore, no significant interactions between the variables have been found, with the exception, again, of the interaction $T \times P$ in *P. monodon* ($p < 0.005$) (Fig. 1).

A convenient way to compare the effects of temperature is to consider the Q_{10}, namely the factor by which the oxygen consumption rate increases when the temperature is increased by 10 °C. If we consider the experiments performed in water at a salinity of 34‰ and a pO_2 of 159 mm Hg, a situation common in the northern Adriatic Sea, the increase of 10 °C in temperature (10–20 °C) results in the expected increase of $(VO_2)_i$ (compare the values of R_7 and R_8 in Table 3). The calculated Q_{10} values are 5.2 for *T. trunculus*, 2.7 for *N. mutabilis*, 2.8 for *P. serratus* and 3.2 for *P. monodon* (in this latter case, the temperature extremes are 20–30 °C). The Q_{10} values have also been calculated for the other conditions of salinity and pO_2 studied here [$(S-P+)$, $(S+P-)$, $(S-P-)$; see Table 2 and the experimental values in Table 3], and the data are summarised in Table 5. Thus, the different Q_{10} values can be taken as "responses" (R_i) of a factorial experimental plan which includes two variables (S, P) at two levels (−, +) and the results of the factorial treatment of the data are reported in Fig. 2. The effects of salinity on Q_{10} are clearly significant ($p < 0.005$) and have a negative

Table 4 Analysis of the results of the factorial experimental plan reported in Table 3: main effects of temperature (E_T), salinity (E_S) and oxygen partial pressure (E_P) on the oxygen consumption velocity of the four marine invertebrate species. Effects of interactions between two (second-order interactions) or three variables (third-order interactions) are also reported

Effect	*Truncularioposis trunculus*	*Nassarius mutabilis*	*Penaeus monodon*	*Palaemon serratus*
Average	0.0394 ± 0.0014	0.0687 ± 0.0014	0.5287 ± 0.0086	0.3519 ± 0.0080
E_T	0.0538 ± 0.0029	0.0669 ± 0.0028	0.5860 ± 0.0172	0.3393 ± 0.0159
E_S	0.0354 ± 0.0029	0.0311 ± 0.0028	0.0140 ± 0.0172	0.0092 ± 0.0159
E_P	0.0108 ± 0.0029	0.0199 ± 0.0028	0.1155 ± 0.0172	0.0593 ± 0.0159
$E_{T\times S}$	0.0212 ± 0.0029	0.0071 ± 0.0028	−0.0105 ± 0.0172	0.0013 ± 0.0159
$E_{T\times P}$	0.0085 ± 0.0029	0.0102 ± 0.0028	0.0540 ± 0.0172	0.0251 ± 0.0159
$E_{S\times P}$	0.0060 ± 0.0029	0.0139 ± 0.0028	0.0020 ± 0.0172	−0.0042 ± 0.0159
$E_{T\times S\times P}$	0.0060 ± 0.0029	0.0082 ± 0.0028	−0.0025 ± 0.0172	−0.0047 ± 0.0159

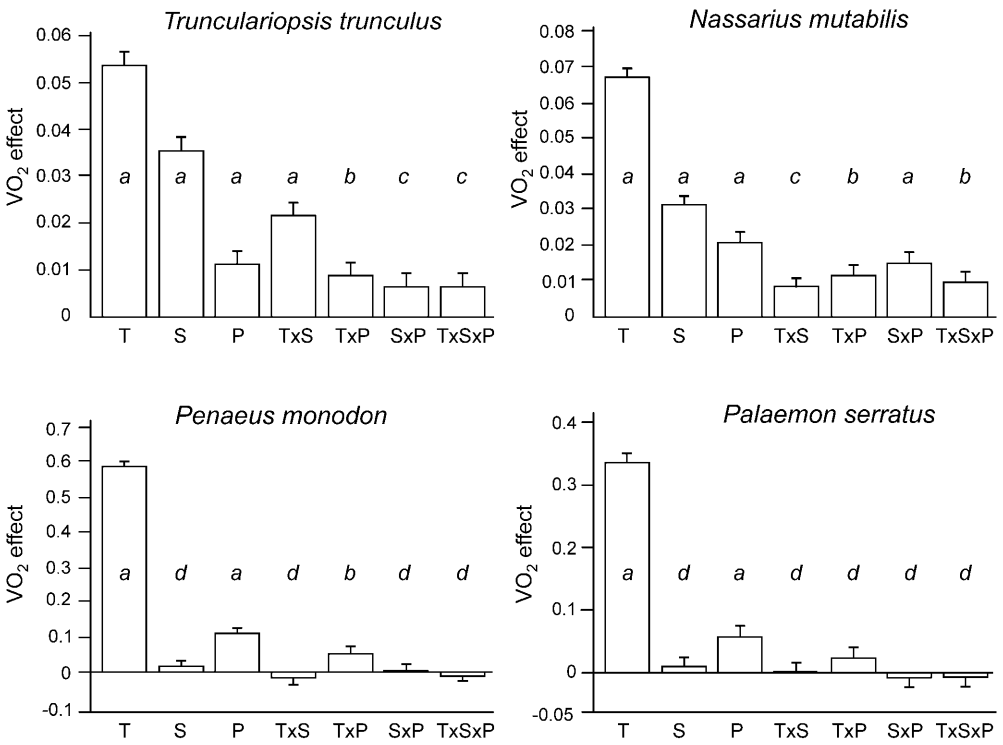


Fig. 1 Effects of the variations of temperature (*T*), salinity (*S*) and oxygen partial pressure (*P*) on the oxygen consumption velocity of *Truncylariopsis trunculus*, *Nassarius mutabilis*, *Penaeus monodon* and *Palaemon serratus*. Effects of second-order ($T \times S$, $T \times P$, $S \times P$) and third-order ($T \times S \times P$) interactions of variables are also shown. Histograms based on the data in Table 4, obtained from factorial analysis of the data reported in Table 3. *Italic letters* represent significance: $p < 0.001$ (*a*); $p < 0.01$ (*b*); $p < 0.025$ or $p < 0.05$ (*c*); not significant (*d*). For details see "Materials and methods". *Error bars*: SE

sign throughout, namely an increase of salinity decreases the Q_{10}. The same conclusion can also be drawn for the effects of oxygen partial pressure, although the results are only significant in the case of the two arthropod species, and the significance level is rather low ($p < 0.01$, $p < 0.05$). The effects of the interaction between salinity and oxygen partial pressure are scarcely significant.

Oxyregulation and effects of environmental parameters on respiratory independence

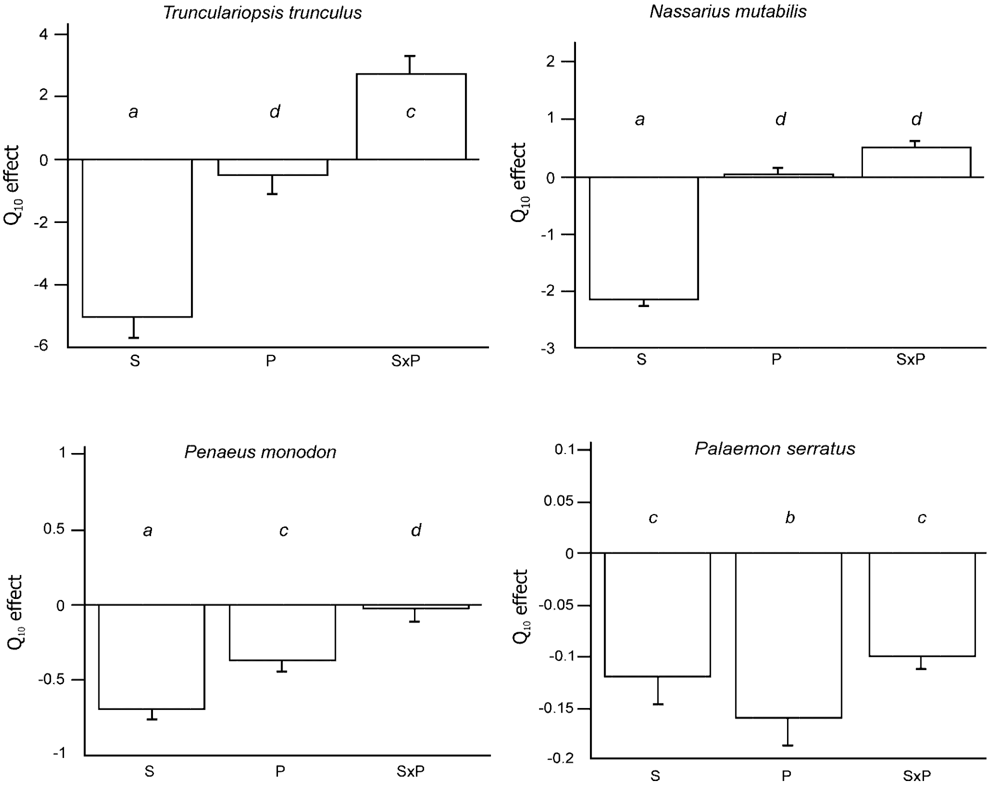
The observation that from the $P(+)$ to the $P(-)$ condition, the $(\dot{V}O_2)_i$ decreases for all species [compare the results of $A_8(T + S + P+)$ *versus* $A_4(T + S + P-)$, $A_5(T - S - P+)$ *versus* $A_1(T - S - P-)$, $A_7(T - S + P+)$ *versus* $A_3(T - S + P-)$ and $A_6(T + S - P+)$ *versus* $A_2(T + S - P-)$; Table 3] prompted us to study the dependence of the oxygen consumption rate on pO_2, in the whole range of pressures, i.e. from 159 mmHg to the lower limit compatible with the survival of the specimens (see "Materials and methods"). The characteristics of oxygen independence, namely the capability to maintain a constant oxygen consumption as pO_2 decreases, can be obtained from plots representing $\dot{V}O_2$ as a function of pO_2 and introducing the "reduced variables" $(pO_2)^*$ and $(\dot{V}O_2)^*$ as described in "Materials and methods". In Fig. 3A, B, the normalised plots of $(\dot{V}O_2)^*$ versus $p(O_2)^*$, representative of two opposite behaviours, are shown. The plot of Fig. 3A (*N. mutabilis* at 20 °C and 15‰ salinity) is representative of almost oxygen-independent behaviour, whereas that of Fig. 3B (*T. trunculus* at 10 °C and 15‰ salinity) can be designated as almost completely oxygen-dependent behaviour.

Table 5 Q_{10} values of invertebrate species determined under different experimental conditions (the experimental values relative to each condition are summarised in Table 1)

$R_1, R_2, \ldots, R_4$ (Q_{10}) Experimental conditions	*Truncylariopsis trunculus*	*Nassarius mutabilis*	*Penaeus monodon*	*Palaemon serratus*
$(S + P+)$	5.23	2.68	3.23	2.77
$(S - P+)$	6.41	3.48	3.59	2.88
$(S + P-)$	4.15	2.40	3.43	2.90
$(S - P-)$	8.00	3.72	3.76	2.91

The values of K_0 and K_1 for all species under the different experimental conditions are summarised in Table 6; in order to evaluate the effects of temperature and salinity on the overall respiratory behaviour of the animals, the K_0 and K_1 values were taken as "responses" (R_i) in a factorial plan of two variables (temperature and salinity) at two levels (+, −; see Table 1). The effects of these variables and of their interactions with K_0 and K_1, obtained by the analysis of the factorial experimental plan, are reported in Fig. 4. The experiments with molluscs at 34‰ salinity show that the increase of temperature from 10 °C to 20 °C determines a loss of respiratory independence with respect to both K_0 and K_1 values [row $(T - S+)$ *versus* $(T + S+)$; Table 6]. On the contrary, at low salinity, the respiratory independence is higher at 20 °C than at 10 °C [row $(T + S-)$ *versus* $(T - S-)$; Table 6]. Therefore, the effects of temperature at the two salinities compensate each other, and the data relative to the main effects of temperature

Fig. 2 Effects of variations of salinity (*S*) and oxygen partial pressure (*P*) on the Q_{10} values determined for *Truncularioipsis trunculus*, *Nassarius mutabilis*, *Penaeus monodon* and *Palaemon serratus*. Effects of second-order ($S \times P$) interactions of variables are also shown. Results obtained from the factorial analysis of the data reported in Table 5. *Italic letters* represent significance: $p < 0.001$ (*a*); $p < 0.01$ (*b*); $p < 0.05$ (*c*); not significant (*d*). For details see "Materials and methods". *Error bars*: SE



resulting from the factorial analysis are scarcely or not significant (the only exception is represented by *T. trunculus* as far as K_1 is concerned; Fig. 4). On the basis of the same considerations, salinity is found to have the opposite effect on each of the two mollusc species, namely the increase of salinity significantly increases K_1 for *T. trunculus*, but significantly decreases both K_0 and K_1 for *N. mutabilis*. Salinity has a positive effect on K_1 for *P. monodon*, whereas, for *P. serratus*, the K_0 and K_1 values are not found to be dependent on the experimental conditions. Highly significant effects of the $T \times S$ interaction are found with *T. trunculus* (both on K_0 and K_1) and *N. mutabilis* (on K_0).

Discussion and conclusions

Generally in biological systems many variables interact with each other. Several studies have specifically addressed the problem of the effects of temperature and salinity on the oxygen consumption rate of marine invertebrates. These studies were generally focused on estuarine benthic invertebrates, since they inhabit dynamic environments characterised by changes in physical-chemical conditions that occur either on a regular basis (depending on the tidal regimes or seasonal fluctuations in temperature) or as unpredictable events (Richmond and Woodin 1999, and references therein). However, it is likely that the variation of one parameter, e.g. temperature, affects the response of the system to the variation of another variable, e.g. salinity. The factorial plan of experiments introduced in the present study allows detection of not only the effects of single variables, but also the effects due to synergistic interaction between several variables.

In the case of *Truncularioipsis trunculus*, all the considered variables determine significant effects on oxygen consumption, as has been documented for other gastropod species (Lange et al. 1972; Newell et al. 1978; Cheung and Lam 1995; Cheung 1997). However, the approach followed in the present work, allows detection of the synergistic effects, especially due to the interaction of temperature and salinity. It means that increasing both temperature and salinity, the increase of oxygen consumption is higher than that expected simply by the addition of the two effects. A similar situation is observed with *Nassarius mutabilis*. In this case the effect of the interaction between temperature and salinity is lower, while the higher synergistic effect is due to the interaction between salinity and pO_2. The significant interaction between temperature and salinity agrees with the results of Cheung and Lam (1995) on *Nassarius festivus*. In the case of the two crustaceans, the effect of salinity is not relevant, while temperature, pO_2 and their interaction affect oxygen consumption significantly.

The metabolic rate and, consequently, the oxygen consumption are directly related to the temperature in heterothermic organisms. This is reflected in our factorial study by the fact that the main effects of temperature on oxygen consumption rate are largely predominant, in all considered species, with respect to the main effects of

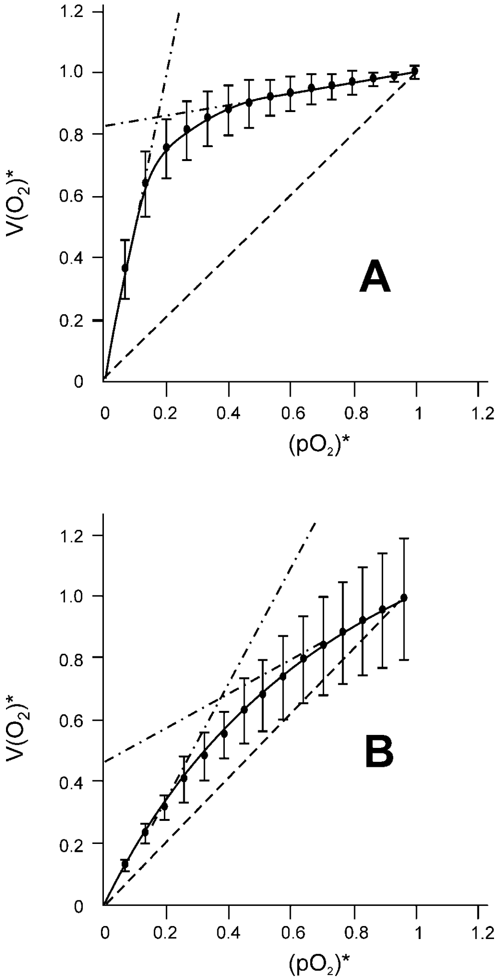


Fig. 3 Oxygen consumption of **A** *Nassarius mutabilis* in sea water at 20 °C and 15‰ salinity and **B** *Truncullariopsis trunculus* in sea water at 10 °C and 15‰ salinity. Data are reported as oxygen consumption velocity as a function of oxygen partial pressure using the reduced variables calculated as described in "Materials and methods". *Error bars*: SE

the other variables. It is worth noting that both the temperatures used in our study, 10 °C and 20 °C, can be considered physiological and within the seasonal thermal excursion of the Adriatic Sea (this consideration is valid also for the temperatures 20 °C and 30 °C, used for the tropical species *Penaeus monodon*).

The Q_{10} values calculated under the different conditions of salinity and pO_2 are rather variable in the case of molluscs, in agreement with the existence of second- and third-order interactions between different variables on the oxygen consumption rate of these species. The higher oxygen consumption rate of *N. mutabilis* could be related to its smaller body size and more intense activity, as compared with *T. trunculus*. In this work, the animals were maintained in air-saturated water at 20 °C and adapted to other conditions for 24 h. The more active species, *N. mutabilis*, is expected to be less affected by the temperature jump from 20 °C to 10 °C, resulting in a lower value of Q_{10}, as compared to *T. trunculus*. The Q_{10}

Table 6 Values of K_0 and K_1 for the four invertebrate species determined from the VO_2 versus pO_2 plots, as described in "Materials and methods" (the experimental values relative to each condition are summarised in Table 1)

Experimental condition	*Truncullariopsis trunculus*		*Nassarius mutabilis*		*Penaeus monodon*		*Palaemon serratus*	
	K_0	K_1	K_0	K_1	K_0	K_1	K_0	K_1
$(T+S+)$	0.332 ± 0.081	0.260 ± 0.009	0.176 ± 0.085	0.266 ± 0.054	0.434 ± 0.089	0.650 ± 0.030	0.543 ± 0.142	0.589 ± 0.080
$(T+S-)$	0.609 ± 0.080	0.482 ± 0.005	0.650 ± 0.048	0.717 ± 0.135	0.412 ± 0.030	0.605 ± 0.004	0.538 ± 0.134	0.525 ± 0.054
$(T-S+)$	0.602 ± 0.089	0.700 ± 0.026	0.503 ± 0.066	0.294 ± 0.005	0.447 ± 0.086	0.655 ± 0.102	0.514 ± 0.080	0.659 ± 0.089
$(T-S-)$	0.266 ± 0.082	0.310 ± 0.098	0.481 ± 0.100	0.624 ± 0.013	0.467 ± 0.074	0.479 ± 0.062	0.509 ± 0.092	0.655 ± 0.018

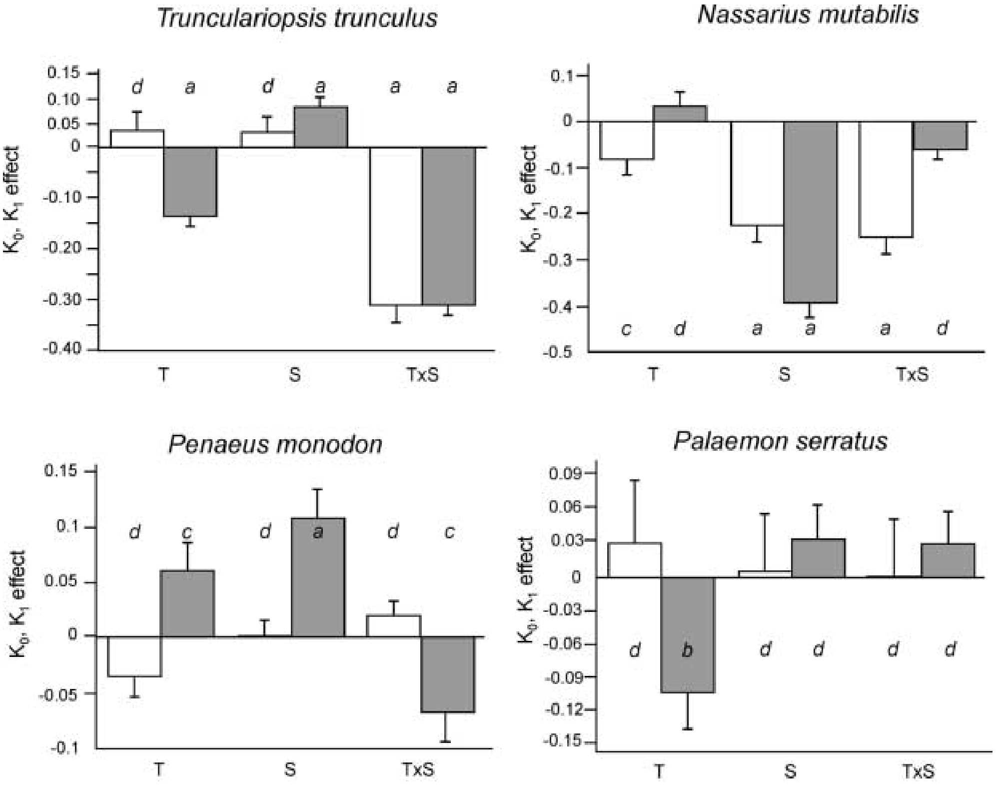


Fig. 4 Effects of variations of temperature (*T*) and salinity (*S*) on the K_0 (*open bars*) and K_1 (*shaded bars*) indices determined for *Trunculariopsis trunculus*, *Nassarius mutabilis*, *Penaeus monodon* and *Palaemon serratus*. Effects of second-order ($T \times S$) interactions of variables are also shown. Histograms obtained from the factorial analysis of the data reported in Table 6. *Italic letters* represent significance: $p < 0.001$ (*a*); $p < 0.01$ (*b*); $p < 0.05$ (*c*); not significant (*d*). For details see "Materials and methods". *Error bars*: SE

values for the decapod species are in agreement with the values given in the literature (Liao and Murai 1986). Furthermore, their Q_{10} values are very constant under all experimental conditions (Table 5), in agreement with the lack of second-order interactions between variables.

The main effects of salinity and oxygen partial pressure, as well as the effects of their interaction resulting from the factorial analysis, are found to be clearly significant on all species only as far as the salinity is concerned (Fig. 2). The negative sign of the effects indicates that the Q_{10} is higher at lower salinity than at higher salinity.

In all the experiments with the two molluscs, the oxygen consumption rate was found to be significantly lower at low salinity in comparison with the high salinity condition. This observation, although it is not a general characteristic of mollusc species, is actually expected for euryhaline, non-osmoregulator molluscs (Hischock 1953; Bielawsky 1961; Negus 1968). Salinity was found to have little or no effect on the oxygen consumption rate in the two shrimps. In general, a decrease in salinity should stimulate an increase in the oxygen consumption rate in crustaceans. However, the metabolic response is often higher than that expected, probably because other factors are involved in addition to the osmotic function (Rao 1958; Kutty et al. 1971). Some contradictory data dealing with the effects of salinity on the oxygen consumption rate in crustaceans have been reported. Gaudy and Sloane (1981) found that *P. monodon* needs at least 3 days to adapt to different salinity conditions. Moreover, the oxygen consumption is quite similar in adapted and non-adapted specimens. Liao and Murai (1986) reported that the same species requires 1 day for adaptation and that oxygen consumption is not affected in a range of salinity between 20‰ and 45‰. In our experiments at 30 °C, upon decreasing salinity from 34‰ to 15‰, we observed no significant differences in oxygen consumption of *P. monodon* [both at higher pO_2, condition $A_8(T + S + P+)$ *versus* $A_6(T + S - P+)$, and at lower pO_2, condition $A_4(T + S + P-)$ *versus* $A_2(T + S - P-)$; Table 3]. In contrast, at 20 °C, a 10% decrease was observed [again, both at higher pO_2, condition $A_7(T - S + P+)$ *versus* $A_5(T + S - P-)$, and at lower pO_2, condition $A_3(T - S + P-)$ *versus* $A_1(T - S - P-)$; Table 3]. For *P. serratus* we did not observe any variation in oxygen consumption with changing salinity, either at low or high temperature. Thus, the two crustacean species, at the corresponding higher values of temperature (20 °C for *P. serratus*, 30 °C for *P. monodon*; Table 1) show a similar behaviour, whereas they significantly differ in the domain of lower temperatures, indicating that *P. serratus*, the species adapted to the temperate and more variable climate, can better tolerate thermal perturbation than can the tropical species *P. monodon*.

The continuous recording of $pO_2 = f(t)$ obtained from the respiratory chamber made it possible to plot the rate of oxygen consumption as a function of pO_2. This approach allows evaluation of the degree of respiratory independence from pO_2, namely the capability to maintain constant oxygen consumption within a given range of pO_2. Tang (1933), Bayne (1971) and Taylor and Brand (1975), who first introduced this approach, used an empirical hyperbolic function to fit the experimental data and to calculate a ratio of empirical constants

k_1/k_2; a small value of this ratio would indicate that the VO_2 of the organism is quite constant in a wide range of pO_2, hence the animal has a high oxyregulating capability. On the contrary, a high value of k_1/k_2 would indicate that VO_2 is directly related to pO_2 and the organism does not exhibit oxyregulating behaviour. The use of the k_1/k_2 ratio for comparative studies is, however, limited by the fact that this is not a normalised parameter and, therefore, does not have an upper limit to its value. Our representation using "reduced variables" has the advantage that the K_0 and K_1 parameters range between 0 and 1; the latter parameter gives a direct indication of the oxyregulation efficiency, the former allows estimation of the range of pO_2 in which the animal exhibits such regulatory capability. Changes in experimental parameters may affect either or both parameters, in the same direction or in opposite directions. This approach is, therefore, more informative than previous methods because it allows the detection of changes in regulatory capability by differentiating the levels of oxyregulation efficiency and the range of pO_2 where this capacity is manifested.

The observed loss of regulatory capacity observed in *T. trunculus* due to temperature (significant effect on K_1) is in agreement with literature data on other invertebrates (Wiens and Armitage 1961; Taylor et al. 1977; Bridges and Brand 1980). The opposite effect is, however, observed with salinity. Furthermore, a strong and significant synergistic $T \times S$ effect is observed. Many molluscs react to a decrease in salinity with an interruption of respiration, which resumes after some time (Cheung 1997; Richmond and Woodin 1999). The intensity of this effect is expected to be related to temperature, since a temperature increment increases metabolism but also reduces resistance to hypoxia (Kinne 1956). The significant interaction between temperature and salinity on *T. trunculus* (both on K_0 and K_1) could, therefore, reflect a situation in which the animals, after resuming oxygen consumption, were in a phase of accelerated respiration due to the temperature increase. The tendency to increase the rate of oxygen consumption under conditions of limited oxygen availability should determine the characteristic synergistic effect. Analogous two-variable interactions also result from the experiments on *N. mutabilis* (significant effects on K_0). Furthermore, this latter species shows highly significant effects of salinity, indicating a lower tolerance to salinity changes.

In the experiments with crustaceans, the values of K_0 and K_1 were rather constant in all conditions (effects were not significant), indicating that the degree of respiratory independence is not affected by temperature or salinity, under our experimental conditions, as expected for euryhaline, osmoregulating species. This result is in agreement with the behaviour observed by studying $(VO_2)_i$ (Fig. 1). The only exception evident is the highly significant positive effect of salinity on K_1 for *P. monodon*. Since this species is not intertidal, like *P. serratus*, it is conceivable that it is less adapted to salinity changes and, therefore, the osmotic work may affect the oxyregulating capability rather than the $(VO_2)_i$.

In conclusion, our results show the great advantage of applying a factorial design in experiments when the influence of changes in environmental variables on a given physiological function is studied. This approach allows consideration of the effects brought about by each single variable, as well as the effects resulting from the positive or negative synergistic interactions of two or more variables, which are allowed to change simultaneously.

As far as the biology and physiology of the considered species are concerned, in this work we have shown significant interactions between variables for the snail species, which appear to be more susceptible to environmental changes than are the two shrimp species. This may be correlated to the strategies adopted by many gastropods which react to environmental variations mainly by behavioural mechanisms. In fact, shell and operculum permit them to isolate themselves from the environment when the physical-chemical conditions become dangerous; they can then survive in a microenvironment by switching their metabolism to anaerobic. It is worth noting that some gastropods can survive for over 1 week in anoxia (Kushins and Mangum 1971). Conversely, the two crustaceans do not have mechanisms which permit them to isolate themselves from the environment, but they have developed physiological mechanisms to adapt to environmental variations.

The results obtained for crustaceans seem to contradict the postulate that biological systems are subjected to interactive variables. However, it is worth noting that the homeostatic physiological control of salinity and pO_2 variations cancel the effect of their interactions. In the case of the two molluscs, which lack efficient homeostatic systems and exhibit a mainly behavioural response to environmental changes, the effects of single variables and of all their interactions are evident, and the extent of these effects is dependent on species-specific characteristics.

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