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RESEARCH ARTICLE

Influence of the parasite worm *Polydora* sp. on the behaviour of the oyster *Crassostrea gigas*: a study of the respiratory impact and associated oxidative stress

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Abstract The aim of this study was to investigate how the worm *Polydora* sp., which induces oysters into creating mud blisters in response to an irritation within their shells, could interfere with the oyster *Crassostrea gigas* physiology and ethology. The impact was characterized by studying two groups of oysters (non-parasitized and parasitized) during a 30 days period: (1) the animal behaviour by analysing their valve activity (valvometry), and (2) the animal respiratory physiology by measuring in vivo the oxygen partial pressure and the specific oxygen consumption in selected tissues (heart, fast and slow adductor muscle). We also researched a putative impact on the expression of several oxidative stress genes at the heart level. Our results show that *Polydora* sp. is clearly an oyster's parasite as it induces a decrease in oyster growth according to the infestation intensity. Moreover, it modifies the behaviour and the respiratory physiology of the molluscs. Infested animals opened more frequently but for less time and their level of blood oxygenation was systematically higher than healthy molluscs. These high levels of oxygenation had no effect on the oxidative metabolism of the tissues studied but they induced oxidative stress. Indeed, the superoxide dismutase gene showed a threefold increase in expression in the heart of infested oysters. A putative scenario of the weakening mechanism is proposed.

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Introduction

Many aquatic animals have a strategy which enables them to regulate oxygen partial pressures (PO_2) in the arterial blood, independently of many oxygenation conditions of the environment, to low levels of between 1 and 3 kPa (Massabuau et al. 1991; Forgue et al. 1992; Legeay and Massabuau 1999), levels which are similar to PO_2 values in most mammalian tissues (Vanderkooi et al. 1991). As a reminder, PO_2 in water equilibrated with air is 21 kPa (for reference 1 kPa = 10^{-2} bar; in seawater when PO_2 = 1 kPa, it corresponds to an O_2 fraction of 1% and an O_2 concentration of 0.4 mg l^{-1} at 10°C). This ability to regulate respiration has been demonstrated in crustaceans, fish and molluscs (Massabuau 2001, 2003), and the strategy appears to be a very ancient one, as it has been found to be already present in ostracods, which existed 450–500 million years ago (Corbari et al. 2004).

These observations show how generally widespread this form of oxygen management is; however, it can have a variety of consequences at the level of the functioning of the cell. Although 90% of O_2 input is sufficient to meet mitochondrial requirements, oxygen is not used only during the production of ATP; oxygen and its derivatives can also have a messenger, modulator, or neuromodulator function (Massabuau and Meyrand 1996), all of which are directly dependent on intra- and extra-cellular PO_2 levels. It is also well known that if the oxygen concentration is too high, it becomes toxic for the cell through the production of reactive oxygen species (hydrogen peroxide, hydroxyl radical, superoxide anion), which causes very serious damage. Free radicals can also oxidize DNA, lipids, or proteins (Morel and Barouski 1998). Oxidative stress occurs when the production of free radicals exceeds the rate of detoxification (Lushchak 2001). Preventing the appearance of high PO_2

levels would therefore appear to be one of the most primitive, simple, and effective methods of limiting this production (Massabuau 2001). To date, however, it has never been demonstrated that arterial PO_2s chronically higher than 1–3 kPa could have a deleterious effect at the cell level in normoxic aquatic animals although the deleterious effect of PO_2 higher than 3 kPa has been clearly demonstrated in cultured cells (Busuttil et al. 2003). This study reports on such an example in the oyster *Crassostrea gigas* parasitized by the worm *Polydora sp*.

In an ecological context, it is interesting to note that parasitism in ecosystems with limited anthropogenic influence is often considered to be a means of regulating the dynamics of invertebrate benthic populations in coastal areas. It is therefore an important and often neglected ecosystem component (Poulin 1999). During the infestation process of the oyster by *Polydora* sp., the worm comes in contact with the mantle, stimulating the host, which then synthesizes a new layer of nacre on the inside of the valve in order to protect itself. This worm is often considered as a commensal, i.e. deriving benefit from this association by finding a host that provides it with support-habitat, whereas the oyster is indifferent, being neither advantaged nor disadvantaged (Handley and Bergquist 1997). However, other authors have reported that the presence of *Polydora* sp. has a negative impact on host growth (Kent 1979; Almeida et al. 1996; Handley 1998) and on shell quality (Kent 1981). In the conclusion of his paper about spionid polychaetes impact on oysters, Handley (1998) discussed the necessity of leading further studies to better understand the stress induced by these parasites.

The aim of this experiment was to study the impact of *Polydora* sp. on the oyster *C. gigas* by examining whether the worm could have an impact on the oyster's ventilatory physiology and ethology. To do this, we hypothesized that the presence of *Polydora* inhabiting the mollusc's shell could stimulate the animal and cause stress by modifying its valve and ventilatory activities. For example, the presence of H_2S in the burrow that the worm constructs within the oyster's shell is a clear feature of infestation by *Polydora* as it could trigger the host reaction. Lastly, we explored the possibility of finding evidence of problems associated with a pathological overoxygenation of the blood and an eventual increase in the expression level of genes implied in oxidative stress limitation.

Material and methods

Animals and experimental conditions

All experiments were performed at the Marine Biological Station of Arcachon, France, from February to May 2005. The average soft bodies weight of the animals was 5.94 ± 0.21 g fresh weight and 0.97 ± 0.05 g dry weight (oven-dried 3 days at 60°C). These oysters measured on average 8.80 ± 0.13 cm in length, 4.24 ± 0.07 cm in width, and 2.69 ± 0.05 cm in height (n = 161). All oysters, from the same age-group, were bought from the same supplier and came from the ''Grand Banc'', Arcachon Bay. Before the experimental manipulations the animals were kept in an external tank (V = 800 l), fed every 90 min with seawater directly from the Arcachon Bay so that the amount of phytoplankton in the tank would be identical to levels measured in the bay. During the experiments, the oysters were transferred to experimental tanks (PVC ''Gilac'' 40 cm × 60 cm × 16 cm; V = 24 l) inside the laboratory, and fed with the same water quality as outside, using a pump. The chlorophyll a level in this water was measured regularly and concentrations ranged, as in the Arcachon bay, from 0.150 to 0.258 μg l^{-1}. For each experiment, the water was renewed at the rate of 1 l min^{-1}, and PO_2 was maintained normoxic (water PO_2 = 21 kPa) by bubbling air. In order to reduce vibrations in the laboratory, and hence keep the stress experienced by the animals to a minimum, the experimental tanks were placed on anti-vibration benches, insulated from noise (Massabuau 2001).

The temperature in the experimental tanks was not regulated so that it would evolve naturally over time, in the same way as natural water, from 9 to 14°C.

Determining the degree of parasitism in the shells

Several authors have established classifications describing degrees of parasitism by *Polydora* (Handley and Bergquist 1997). The classification we applied was the Ifremer scheme, where the level of shell infestation is determined by a macroscopic examination of the most infested valve (Catherine et al. 1996). Five classes are thus defined: class 0: no worm present; class 1: presence of worm, burrows visible; class 2: fewer than two chambers and less than 10% of the shell surface infested; class 3: more than two chambers or 10–25% of surface infested; and class 4: >25% surface infested. *P. hoplura*, *P. ciliate*, and *Bocardia semibranciata* are the three species implied in oyster infestation in the Arcachon Bay.

Non-invasive valvometry

Using valvometry, it is possible to gather information on animal behaviour in normal environmental conditions. The technique we used was first described by Tran et al. (2003; see also http://www.domino.u-bordeaux.fr/molluscan_eye/). Light-weight electrodes are attached to the molluscs, which are able to move freely and are not constrained in any way. The electrodes are linked by flexible wires to an electronic

control unit some distance away which measures valve activity. The version we used in our study is based on the magnetic principle. Each electrode encloses a coil; one is a transmitter, the other a receiver. The strength of the electric field produced between the two coils decreases with distance, according to the law $1/D$, where D is the distance between the point of measurement and the centre of the transmitting coil. The smallest possible electrodes and wires were used so as not to disturb the oysters' valve movements (electrodes 3.2 mm × 2.5 mm × 2 mm, 57.3 mg; cable: ∅ = 0.91 mm, 2.14 g/m). These experiments were performed from 8 March to 18 April 2005. Fifteen animals were equipped and monitored simultaneously in the same experimental tank to avoid the possibility of any differences between tanks. At the end of the recordings, post-mortem analysis of the shells indicated the degree of parasitism in the animals (seven non-parasitized; eight parasitized).

Principle of the mathematical analysis

To model oyster behaviour, we used a nonparametric regression model based on the kernel estimator (Silverman 1986; Härdle 1992; Tran et al. 2003; Briollais et al. 2006). The regression model is:

$$Y_i = m(T_i) + \varepsilon_i, \text{ with } i = 1, \ldots, n,$$

where n, Y_i, t_i, and m denote, respectively, the sample size (total number of paired values), the distance between the two electrodes, the time of the measurement, and the unknown regression function to be estimated. The variation ε_i is a random variable with a mean value equal to 0 and the stochastic distribution f of these random variations enables us to characterize the variation of the random variable Y around:

$$m(t) = E(Y/T = t) = \frac{\int yf(y,t)\mathrm{d}y}{f(t)}.$$

The distribution f is typically unknown and is unlikely to follow any familiar distributions, such as the normal distribution; hence, no distribution is assumed (non parametric statistics).

This average function m (definition of the conditional expected value of Y given T) depends on the unknown joint distribution function of (Y,T), denoted by $f(y,t)$, and on the unknown distribution function T denoted by $f(t)$. The problem is thus to estimate the function m conditioned by the measurements with respect to the time of experiments. Based on kernel estimators of the two distribution functions, the estimator of m describing the behaviour of an animal is established (Härdle 1992; Briollais et al. 2006).

Thus, the daily activity of each animal was modelled; different parameters specifying openings and closures (duration, number, and degree of opening in mm) and also the number of partial closures could be studied for each day of recording by using the modelling approach. The computations were performed under the Linux system (Fedora 5) on a biprocessor xeon workstation DELL using R and C programming language.

Measuring oxygen partial pressure

Oxygen partial measurements were carried out on 100 oysters. Oysters were placed in two parallel experimental tanks (20 animals per tank). The experience was renewed thrice (only on 20 oysters the last time). As no difference was observed with time, all the results were pooled. PO_2 measurements were carried out directly in the animal using the oxygen optode technique (Presens, Needle-type housing fibre-optic oxygen micro-sensor NTH–PSt1). Only the oysters that were spontaneously open, and thus able to ventilate, were sampled. Each oyster was taken out of the experimental tank quickly but very carefully, without touching the other oysters, so as not to cause them stress, and then it was opened in the space of about 15 s by cutting through the adductor muscle. Next, the needle was quickly inserted into the target organ (heart, fast or slow fibres of the adductor muscle, see Fig. 1). Readings had to be taken in less than 1 min 30 s for the heart and 1 min for the muscle (t_0 being the moment when the animal was taken from the tank).

Oxygen consumption measurements occurred on another set of 36 oysters randomly distributed in two experimental tanks. To measure O_2 consumption in the heart, first the pericardial membrane was torn, and then the common efferent vein was clamped (with a laboratory-made microclamp) in order to block any input of blood

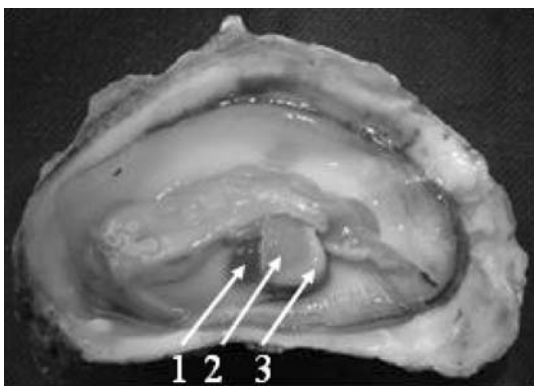

Fig. 1 Measuring oxygen partial pressure: localization of oyster organs measured (*1* represents heart, *2* represents slow fibres of the adductor muscle, *3* represents fast fibres of the adductor muscle)

from the gills. The optode was then inserted into the ventricle. For measurements in the muscle, the optode was inserted specifically into either the fast or slow muscle to carry out measurements in situ. Six oysters per condition (non-parasitized/parasitized; heart/slow muscle/fast muscle) were used.

Gene expression levels analyses

To study the impact of the variations in oxygenation on the functioning of the cell and look for the possible presence of oxidative stress, we studied the expression of genes coding for defence proteins against oxidative stress or demonstrating the existence of such stress. The experiment was carried out on five non-parasitized and five parasitized oysters (soft bodies fresh weight between 5.2 and 8.8 g) maintained in one experimental tank. The tissue selected was the heart, which, as it is located at the gill outlet, is working constantly and is in direct contact with the arterial blood and is therefore most exposed to variations in oxygenation. The hearts weighed between 20 and 40 mg and were taken from oysters within 1 min and placed immediately in ice, and then stored in the freezer at –80°C. The animals were in normoxia (water PO_2 ≈21 kPa). The degree of parasitism was determined post mortem.

Total RNA was extracted from the hearts using the ''Absolutely RNA RT-PCR miniprep'' kit (Stratagène). The quality of the RNAs obtained was assessed by electrophoresis on a 1% agarose–formaldehyde gel and concentration determined by spectrophotometry. Five micrograms of RNA were then retro-transcribed in complementary DNA (DNAc) using the ''Stratascript First-strand synthesis system'' (Stratagène). The DNAc obtained were then used as a matrix for quantitative polymerase chain reactions in real time (Lightcycler, Roche). The actin gene was chosen as reference gene. We studied the genes *nad5* (sub-unit 5 of N Acetyl dehydrogenase), *cox I* (sub-unit I of cytochrome c oxydase), *sod1* (superoxide dismutase Cu, Zn), *hsp70* (heat shock protein), and *mt2* (metallothionein 2). NADH 5 and COX I are respiratory chain proteins, enabling us to study the mitochondrial metabolism. SOD is located in the cytoplasm and outer mitochondrial space. This enzyme eliminates the superoxide radical anion, $O_2^{\bullet -}$, and produces hydrogen peroxide, H_2O_2 (Morel and Barouski 1998): it is thus involved in defence mechanisms against oxidative stress, like HSP 70, which is present in different compartments (nucleus, cytosol, mitochondria, and endoplasmic reticulum). Metallothioneins, whose main role is to sequester metals, can also be involved in the battle against oxidative stress (Viarengo et al. 1999). For each of these genes, specific primers were determined using the ''Lightcycler Probe design'' software (version 1.0, Roche). The primers and accession nos. of the five genes used in our study are given in Table 1.

Statistical analyses

All results from statistical analyses are given with a significance threshold of 5%. Comparisons between the distributions of the number of micro-closures and the duration of daily openings between the non-parasitized and parasitized animals were carried out using the goodness-of-fit Kolmogorov–Smirnov test. To compare two-sample means, parametric Student's *t*-tests *z* test (when $n > 30$) or Mann and Whitney *U* test were used. The computations were performed using R software (http://www.cran.r-project.org).

Results

Figure 2 shows the relationship between the dry weight of the soft bodies and the *Polydora* infestation class for a sample of 71 animals selected at random throughout the period of study. It is clear that the more infested the oysters are, the more their weight decreases. The average of the dry weights of individuals in classes 0–4 decreases steadily from 1.12 ± 0.13 to 0.71 ± 0.20 g dw; in other words, a decrease of almost 40%. This appears to be a linear

Table 1 Accession numbers and specific primer pairs for the six *Crassostrea gigas* genes used in the study

Gene name	Accession number	Primer (5′–3′)
nad5	AF177226	AGTGAGAGTACAGAATGGTGCT[a]
		TGATGGAGGAGACGCTCG[b]
cox I	NC001276	GTGGCTGGAATGGATATTGATACG[a]
		CTCTTGATAGAATAAGTCCTGTAAGACCC[b]
sod1	AJ496219	AAGGATTAACACCAGGACAGC[a]
		GTGATACTGATTTTGGCGACACC[b]
hsp70	AF144646	TTCCTCCCGCCCCTCG[a]
		TGTATGTCTCGGCCTCGT[b]
mt2	AJ297818	TCCGGATGTGGCTGCAAAGTCAAG[a]
		GGTCCTTTGTTACACGCACTCATTT[b]

nad5 NADH dehydrogenase subunit 5, *coxI* cytochrome C oxidase subunit 1, *sod1* superoxide dismutase Cu/Zn, *hsp* heat shock protein, *mt* metallothionein

[a] Upstream primer

[b] Forward primer

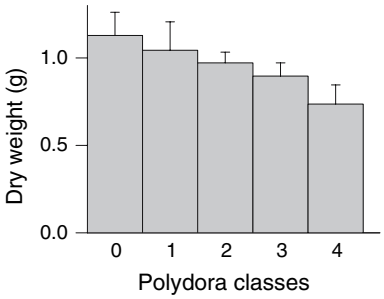

Fig. 2 Evolution of soft bodies dry weight (mean values ± SE; n = 100), in g dw, of oysters in *Polydora* classes 0 (not parasitized) to 4 (maximum infestation)

relationship, and the equation of the regression line modelling this decrease is: Average dry weight = –0.0932 *Polydora* class + 1.1411 with a coefficient of determination R^2 equal to 0.999. The average dry weight of the non-parasitized oysters (class 0) is significantly greater than that calculated for the parasitized animals in class 4 (P = 0.0346, t-test). This first result thus clearly shows that *Polydora* sp. can cause a decrease in the dry weight of infested oysters, proportional to the intensity of infestation, and should consequently be considered as a parasite. In follow-up experiments, we have studied its impact on the physiology of the host by analysing valve activity to look for the existence of any behavioural changes.

The ethology of parasitized and non-parasitized oysters

A crucial problem when studying animals in the laboratory is determining the time required for adaptation to the experimental conditions (Massabuau and Forgue 1996). Figure 3 shows two recordings of typical valve activity by non-parasitized animals, brought into the laboratory at time zero (J_0), during an acclimatization period in the laboratory. These recordings demonstrate a clear change over time with decreasing amounts of activity in the first days. Figure 3a shows that the number of daily periods of opening stabilized after 5 days. It peaked at 11.4 ± 1.2 openings.j^{-1} at J_1, then decreased gradually to stabilize at 2.6 ± 0.4 j^{-1} after J_5. The most frequent level of maximum closure, on the other hand, did not stabilize until after J_{11} (Fig. 3b). We were therefore able to define a period of adaptation of 11 days, and the study of behavioural differences between parasitized and non-parasitized animals was carried out from J_{12} to J_{33}. Note that during this period, temperature increased from 9 to 14°C in the Basin and in the experimental tanks, but with no corresponding increase in the number of openings and the level of maximum closure.

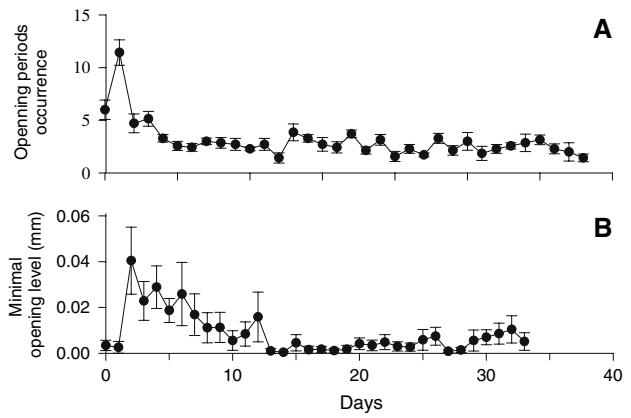

Fig. 3 Number of periods of opening per day (**a**; mean ± SE) and level of minimal daily opening in mm (**b**; mean ± SE), indicating a period of adaptation

The distribution of the number of daily openings is presented in Fig. 4a and b. Mean values in the non-parasitized animals were 2.57 ± 0.1 j^{-1} and in the parasitized animals 3.14 ± 0.12 j^{-1} (P = 0.0005, z test), which represents a 20% increase in activity in the parasitized animal. Figure 4c and d shows the distribution of the duration of this activity in the non-parasitized (7.7 ± 0.4 h) and the parasitized animals (8.1 ± 0.4 h). All the points shown represent 147 days of observation in the non-parasitized oysters and 168 days in the parasitized oysters. The distributions of durations do not differ significantly, although we note a shift between 4 and 6 h of daily opening in the non-parasitized animals, and around 2–4 h in the parasitized animals. Minimal and maximal values were also similar. Note that, overall, the oysters were most often open for 2–8 h (or only 8–33% of 24 h). To sum up, the parasitized animals appeared to open more often but for less time than the non-parasitized ones.

Tran et al. (2003; see their Fig. 3) reported that in the Asian clam *Corbicula fluminea*, the number of partial closures (small incomplete closures ≤ 3 mm) was linked with stress when the animal was placed in experimental conditions. We therefore looked to see whether the stress believed to be caused by the presence of *Polydora* would also result in a change in the number of partial closures. Figure 4e and f shows that these do indeed differ significantly between the two batches of oysters (Fig. 4e and f; P = 0.007, Kolmogorov–Smirnov) with numbers being greater in the parasitized animals.

Blood oxygenation status and tissue O_2-metabolism

Since results from the previous analysis showed differences in valve behaviour, and hence potentially also in respiratory behaviour between parasitized and non-parasitized oysters, we also examined whether these characteristics were associated with changes in oxygenation in the internal environment. Bearing in mind our initial hypothesis that the

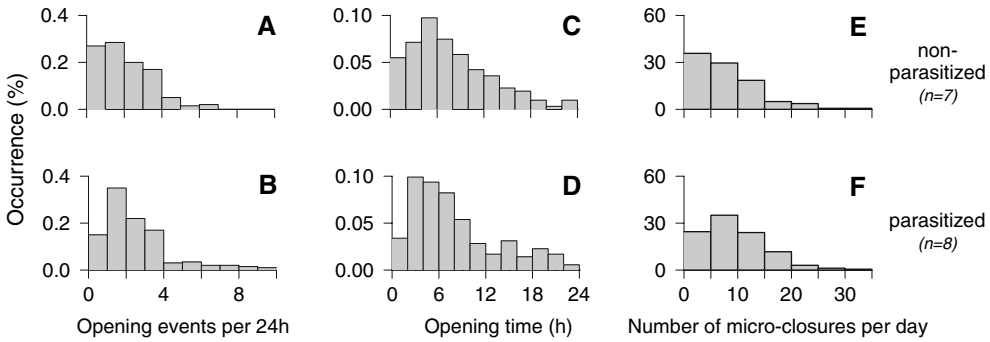

Fig. 4 Comparison between non-parasitized and parasitized animals (classes 1–4) of numbers of openings (**a** and **b**), duration of openings (**c** and **d**), number of micro-closures (**e** and **f**)

presence of *Polydora* in the shell could stimulate the animal and cause it stress by modifying valve activity and stimulating ventilatory activity, we wanted to test the hypothesis of a relationship between the degree of parasitism and blood oxygenation; therefore, we measured arterial PO_2 for each *Polydora* class. Figure 5 clearly shows a relationship between the two factors since in the most heavily infested oysters (class 4), arterial PO_2 was systematically high, within the range 10–18 kPa. In the non-parasitized oysters, on the other hand, arterial PO_2 varied considerably with values between 0 and 18 kPa. Note the regular appearance of the transition in the intermediate classes which clearly illustrates the relationship between degree of parasitism and level of blood oxygenation.

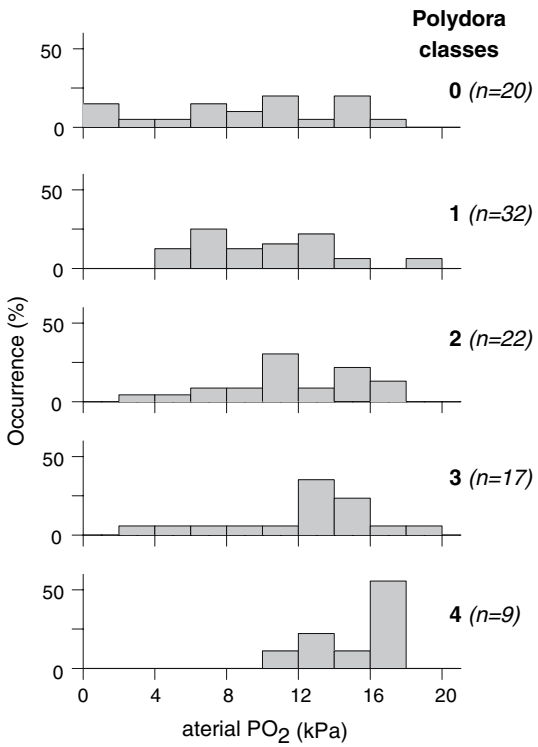

Fig. 5 Arterial PO_2 values (kPa) as a function of level of parasitism, represented by *Polydora* classes 0–4 (n = 100)

In order to understand the impact of this change in oxygenation on the animal's physiology, we next explored whether it affected the level of oxidative metabolism in the tissues. Results from analyses carried out directly on the two types of adductor muscle fibre (slow and fast fibres) and the heart are shown in Fig. 6 for PO_2 ranging from 2 to 15 kPa. Since no difference in O_2 consumption was observed between the different classes of parasitism, all the data were combined and averaged together. It is clear that the high arterial PO_2 values measured in the parasitized animals are not associated with any increase in O_2 consumption (slow muscle fibres, $P = 0.953$; fast fibres, $P = 0.566$; heart, $P = 0.632$; t-tests), and hence that the differences in arterial PO_2 between the parasitized and the non-parasitized animals do not influence the aerobic metabolism of these tissues. We then examined whether the minimal PO_2 tissue values below which oxygen consumption is limited could differ between the parasitized and the non-parasitized oysters. Figure 7 shows the relationship between oxygen consumption and tissue PO_2 for the heart and the fast muscle. Independently of the presence of the parasite, oxygen consumption can be kept constant when PO_2 in the tissues is 0.4–0.6 kPa. It is only below this value that oxygen consumption in the tissues is systematically limited.

Oxidative stress

Since the differences in oxygenation in the internal environment of parasitized and non-parasitized oysters do not modify oxygen consumption or PO_2 up to levels at which the heart and muscle tissue can maintain their O_2 consumption constant, we explored whether high oxygenation levels could be correlated with the existence of oxidative stress in the heart muscle, as this is a muscle that is working constantly and is exposed to the highest PO_2. Table 2 shows an absence of overexpression of NADH 5, Cox1, Hsp70, GST, and Mt2 and hence there is no profound effect on cell metabolism. However, *sod1* was overexpressed by a factor of 2.8 in the parasitized oysters ($P = 0.044$; t-test). This result therefore demonstrates the

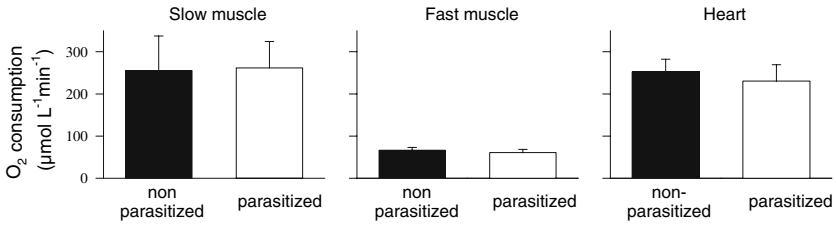

Fig. 6 Comparison of specific oxygen consumptions (mean ± SE; µmol l^{-1} min^{-1}), in non-parasitized and parasitized oysters (classes 1–4), in the slow adductor muscle fibre, the fast muscle fibre, and the heart (n = 6 for each plot)

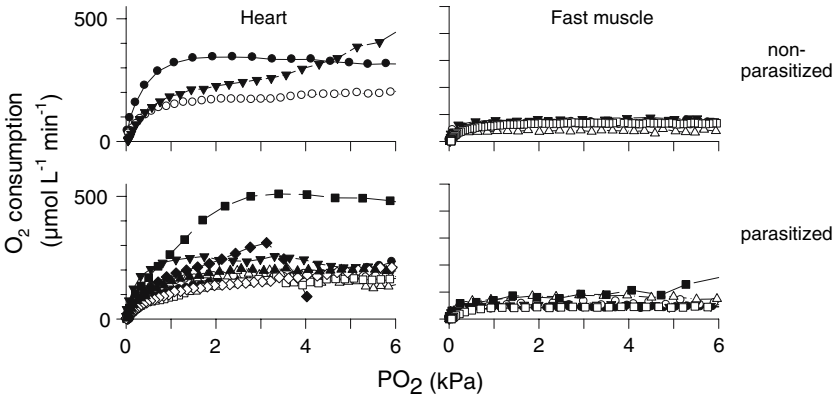

Fig. 7 Oxygen consumption (µmol l^{-1} min^{-1}) in the heart and the fast muscle fibre, as a function of PO_2, for non-parasitized and parasitized animals (classes 1–4)

Table 2 Expression of the different genes studied in relation to the expression of actin, and corresponding Mann and Whitney U test, to determine whether these differ between parasitized and non-parasitized oysters (classes 1–4)

	Parasitized (n = 5)		Non-parasitized (n = 5)		Mann and Whitney U test)
	Mean	SE	Mean	SE	
NAD5	1.29	0.14	1.60	0.19	0.2095
COX I	1.95	0.32	2.14	0.31	0.6842
SOD I	0.01	0.003	0.004	0.001	0.0440
GST	0.0002	0.0001	0.0003	0.0001	0.8036
HSP 70	0.0004	0.0003	0.0007	0.0002	0.4396
MT2	0.09	0.02	0.08	0.01	0.8075

existence of oxidative stress in the heart of parasitized oysters.

Discussion

We have shown that the worm *Polydora* sp. should indeed be considered as a parasite of the Pacific oyster *C. gigas*. Indeed, the scale of the parasitism directly affects the weight of the soft body in these animals. We have shown that valve activity is disturbed when *Polydora* is present: the parasitized oysters open more often, have a tendency to remain open for a shorter time, and sudden partial closure movements are more numerous. This overall increase in valve activity is accompanied by an increase in the general state of oxygenation in the internal environment which is not necessary to, and which does not lead to, an increase in aerobic metabolism (measured directly in three target tissues, the heart, the slow adductor muscle, and the fast adductor muscle). Note also that the minimal partial pressures below which this aerobic metabolism can be maintained are independent of the presence of the parasite. However, in the heart we demonstrated the existence of an oxidative stress associated with parasitism, and characterized by an increase in expression level of the *sod1*.

Experimental conditions and the variables measured

The animals we studied required a period of acclimation to laboratory conditions of over a week. This compares favourably with experiments described previously using the crab (Massabuau and Forgue 1996) and the bivalve *C. fluminea* (Tran et al. 2003). By demonstrating once again that this long period is necessary, we show not only how sensitive these aquatic animals are to environmental stress but also how experiments can be skewed if this period is not respected. The duration of the daily openings that we reported (in the laboratory, with no tide, but with continuously

renewed water that had been taken from the field site) varied a great deal, but the value that was most frequently observed, 7–8 h, should be compared with the 10–18 h observed in *C. gigas* (His 1976; Arcachon Bay) and 6–14 h in *C. angulata* (March 1971). The durations that we measured compare favourably with the results reported for *C. fluminea* in identical experimental conditions (Tran et al. 2003). In the case of *C. fluminea*, laboratory readings agreed satisfactorily with those obtained in situ in the Rhine by Ortmann and Grieshaber (2003).

During the course of our experiments, we did not carry out any direct measurement of ventilation rate even though it is essential to know what this rate is. The main reason for this is that in a filtering bivalve this reading can only be taken indirectly. The most efficient technique is clearing a volume of water of algae in a closed system. Several hypotheses must then be proposed: (1) the decrease in concentration of algae is due solely to the animal's filtration activity; (2) the ventilation rate is constant over time; (3) the algae are retained by the bivalve with an efficiency equal to or close to 100% (Tran et al. 2000); and (4) the algae are distributed uniformly through the medium. Note also that the most effective technique is to work with a single species of algae which meets the required criteria.

In the case of the oyster currently being studied in the waters of the Arcachon Basin, several problems have emerged. The amount of chlorophyll present in 2005 is in itself a problem as the level was particularly low and we were not able to take measurements for sufficiently long periods of time. Indeed, potentially, the more the concentration decreases, the more likely that the animal's ventilation will be stimulated (Tran et al. 2002). The same observation can perhaps be made in relation to the trophic quality of a water sample containing one or several species of plankton. For all these reasons, therefore, we chose not to measure the ventilation rate, but rather to focus our attention on measuring the oxygenation of the blood, as this is very closely linked with ventilation: low levels are the sign of a low ventilation rate, and high levels indicate a high rate.

Over a period of several years in our laboratory, considerable effort has been invested in developing a means of measuring valve activity. This technique is not invasive if light-weight electrodes are used mounted on flexible cables and it provides continuous recordings of groups of animals that can be studied in parallel (Tran et al. 2003). In the not too distant future, it will be essential to follow the same batch of oysters in their natural environment, and then in the laboratory, in order to observe any differences in behaviour. Finally, it should be noted that the developments in mathematical techniques that have been achieved have enabled us to carry out extremely detailed analyses, revealing differences which would have been impossible to detect without this type of tool.

Scenario of the weakening mechanism

Our results confirm that the worm *Polydora* sp. is a parasite that weakens its host. The animal bores into the shell, creates a burrow, and comes into contact with the mantle. The oyster then secretes a new protective layer of nacre (Ruellet 2004). We suggest the following scenario to account for, in part at least, the effects on the animal's metabolism: the activity of the worm overstimulates the oyster, since its valve behaviour is modified. As the oxygenation readings show, the parasitized animals must hyperventilate to account for the chronic overoxygenation of the blood, yet oxygen consumption does not increase at the tissue level. This excess of unused oxygen results in oxidative stress, observed in particular in the heart. The overexpression of SOD, a specialized enzyme which helps protect cells against the toxicity of free radicals, is a telltale sign. It participates in the defence mechanisms that tend to check these high arterial PO_2 levels. Note that since the parasitized animals exhibit a smaller soft body mass (Fig. 2), we can hypothesize that this defence mechanism is probably not totally efficient.

The results described above demonstrate the existence of stress, but clearly the damage caused by the worm must be multifactorial. For example, although the parasitized animal is open for less time than the non-parasitized animal, it hyperventilates, with the result that it must take in as much if not more food than the non-parasitized animal. It is interesting to note that although it should take in more food, transformation must be less efficient since the animal loses weight over time instead of gaining it. We know that oxidative stress acts by increasing lipid peroxidation, and creating DNA lesions and other macromolecules. The oxidative stress could therefore directly influence the metabolism of digestion, and/or of glycogen storage. The explanation for the weakening of the animal is not based on increased oxygen consumption, since the oxidative metabolism does not increase along with the increase in arterial and tissue PO_2 and the oxidative metabolism genes are not overexpressed. Among several other complementary scenarios, we should add the hypothesis put forward by various authors (Ruellet 2004) who suggest that when the oyster is parasitized and has to secrete a new shell layer, this extra work then contributes to the slowing down of growth, since some energy is spent on this repair. It could also participate to increase arterial PO_2 in parasitized animals.

Repositioning in terms of the low PO_2 strategy

As we recalled in the introduction, many aquatic animals have been observed to have an oxygen management strategy to regulate PO_2 in the arterial blood, independently of the many environmental conditions, to low and constant levels of between 1 and 3 kPa. This study shows that, at least in the conditions in which we were working, the oyster does not follow this strategy, especially when it is parasitized, and even when high oxygen levels are not absolutely necessary to maintain consumption in the different tissues studied. These observations contradict readings taken in 2001 when we did indeed measure low values (unpublished data) but the reasons for this apparent contradiction are yet to be studied. One working hypothesis is that since 2003, chlorophyll concentrations in the waters of the Basin have decreased. Measurements taken in 2001 ranged from 1 to 10 $\mu g\ l^{-1}$, whereas those taken this year were in the range 0.150–0.258 $\mu g\ l^{-1}$. This decrease in concentration may stimulate ventilatory activity to maintain a constant food intake, although this remains to be proved.

Conclusions

To conclude, by examining the case of parasitism by *Polydora sp* in the Pacific oyster *C. gigas*, we have shown here for the first time that maintaining permanently high levels of arterial PO_2 is associated with oxidative stress. Consequently, the low PO_2 strategy would certainly help provide protection for tissues faced with this type of stress. Our studies also show that the worm *Polydora* sp is indeed a parasite of the oyster *C. gigas* since it restricts the oyster's growth. We therefore suggest that in this case, the problem of oxygen and oxidative stress management should be taken into account for a better understanding of the weakening mechanism. In a broader ecological and ecotoxicological context, and a multiple stress context, it seems clear that parasitism by *Polydora*, via induced oxidative stress is an impairment factor which must inevitably decrease the oyster's resistance to other types of aggression.

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