Effect of infection by the protistan parasite *Marteilia refringens* on the enzyme activity and energy reserves of oyster *Ostrea stentina* (Payraudeau, 1826) in Tunisia

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This is the first study of the metabolic enzyme activity and energy state of the dwarf oyster Ostrea stentina *(Payraudeau, 1826) in Tunisia and in the Mediterranean region. The main purpose of this study was to examine the modulation of the physiological status of the oyster* O. stentina *depending on the season and the presence of parasite* Marteilia refringens. *The prevalence of bonamiosis and marteiliosis were established by PCR. Bonamia exitiosa was detected only in 2.91% of oysters while the prevalence of* M. refringens *was 100% (30 Ind./30) in February and 93.93% (28 Ind./30) in March. A 42–87% mortality rate was reported during the study period. Biochemical analyses were carried out to evaluate the management of the energy resources in regard to the biochemical changes of lipids and carbohydrates. The enzyme activity of pyruvate kinase (PK), citrate synthase (CS) and cytochrome c oxidase (CCO) were measured and the mean level of enzyme activity was respectively 20.31 mU mg^{-1} protein; 12.06 mU mg^{-1} protein and 3.59 mU mg^{-1} protein. Carbohydrate contents of* O. stentina *were very low all year round with an average of 15.18% in dry weight, and lipid contents remain similar (11.77% in dry weight) compared with the values reported for most other temperate bivalves. Enzyme activity significantly decreased over time ($P < 0.001$). The energy resources of the oyster* O. stentina *wherein much of the energy was devoted to reproduction seem to affect the defence system.*

INTRODUCTION

Important mortality outbreaks have been reported in many bivalve species including the flat oyster *Ostrea edulis*, the mussels *Mytilus edulis, Mytilus galloprovincialis, Crassostrea gigas* (Grizel *et al.*, 1974; Villalba *et al.*, 1993b; Soletchnik *et al.*, 1996; Le Roux *et al.*, 2001; Lopez-Flores *et al.*, 2004; Novoa *et al.*, 2005). Some authors suggest that mortalities are the result of multiple factors, including elevated temperature, physiological stress, pathogens and pollutants (Goulletquer *et al.*, 1998; Samain *et al.*, 2007).

Ostrea stentina is an endemic oyster species in Tunisia, which is not exploited notably because of its small size. The size of oysters had a mean length of 37.2 mm (range of 32–44 mm) and the total weight varied between 16.51 and 9.57 g. Despite its intense reproductive cycle (Elgharsalli & Aloui, 2011) and the lack of fishery pressure, this species appears endangered because of unexplained mortalities occurring in Tunisia since 2007. Very little information is available

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concerning the health status of *O. stentina*. It has received no parasitological attention, even though it inhabits waters in which economically significant parasites of *O. edulis* and *M. galloprovincialis* including *Bonamia ostreae* (Pichot *et al.*, 1980), *Marteilia refringens* (Grizel *et al.*, 1974) and *Marteilia maurini* (Comps *et al.*, 1982) are enzootic (Tiscar *et al.*, 1991; Virvilis and Angelidis, 2006; Carrasco *et al.*, 2008).

A previous study reported the presence of a parasite *Bonamia exitiosa* in some oysters (Hill *et al.*, 2010) with a relatively low prevalence (10.6%). Other histological examinations and molecular analysis of *O. stentina* performed in 2009 and 2010 revealed the presence of *Marteilia refringens* DNA in 88% of the oysters (Elgharsalli *et al.*, 2013) while *B. exitiosa* DNA was detected in only 3% of the oysters (unpublished results) suggesting a possible involvement of *M. refringens* in the mortality of *O. stentina*. Infection by protozoan parasites causes severe alterations and could affect the reproductive cycle through energy depletion, particularly during the spawning period (Jeffs & Hickman, 2000). Indeed, infections cause a poor condition index with glycogen loss (emaciation), discolouration of the digestive gland, cessation of growth, tissue necrosis and mortalities. Severe infection may cause loss of condition as a consequence of reduced energy acquisition and the parasite may interfere with host feeding and

absorption simply by its physical pressure (OIE, 2011). To our knowledge, no published work is available on the biochemical study of the oyster *O. stentina*. The absence of a healthy population does not allow a comparative study between infected and uninfected oysters and monitoring of biochemical changes. Despite this, it is necessary to determine the effect of the parasite on the physiological state of infected oysters.

A study of the biochemical compositional changes of *O. stentina* is considered to be necessary so as to document the physiological status of the oyster in Tunisia depending on the presence of the parasite. (1) The biochemical compositional changes of *O. stentina* were investigated seasonally over a 1-year period, taking into account the effect of the reproductive cycle (sexual maturation) together with the detection of *M. refringens* through PCR. (2) Energy resources were estimated with respect to changes of lipids and carbohydrates. (3) The use of energy resources were measured by the activity of metabolic pathways, such as pyruvate kinase (PK), citrate synthase (CS) and cytochrome c oxidase (CCO). The result of this work will provide the basic parameters for a dynamic energy model to assess parasitic infection, which can also affect other bivalves, based on shellfish farming.

MATERIALS AND METHODS

Study area

The Stah Jeber port is a semi-enclosed artificial basin, located in the south-eastern Mediterranean Sea, in the Monastir Bay on the east coast of Tunisia ($35°45'29''N$ $10°50'13''E$, Figure 1). The mean depth is 3.5 m with a maximum of 5 m and a surface area of about 227.800 m². A study of physico-chemical parameters was carried out. The temperature (°C), dissolved oxygen (mg l^{-1}), pH and salinity in the study area were recorded monthly using a multi-parametric probe (multiline P4 WTW). Oysters, attached to the rocks in the Stah Jeber harbour, were seasonally and randomly collected over the dock at which boats are moored. The mortality rate of *O. stentina* was a measure of the number of dead oysters in an *O. stentina* population, scaled to 100 individuals of that population, per sampling date.

Sample processing

In 2009, specimens of *O. stentina* were seasonally and randomly collected on 11 February (30 oysters), 26 March (30 oysters), 9 June (30 oysters) and 13 October (13 oysters), designated hereafter as winter, spring, summer and autumn, in the Stah Jeber port from shallow areas at a depth of between 1.5 and 5 m. In the laboratory, oysters were cleaned and all fouling organisms were removed. Each individual oyster was measured including the total length (to the nearest 0.005 mm) using a vernier caliper and the total weight (nearest 0.001 g), wet meat weight (nearest 0.001 g) and dry shell weight (nearest 0.001 g) were determined. After the measurement of biometric parameters the whole oyster tissue was dissected and immediately frozen in liquid nitrogen. Frozen tissues at $-20°C$ from each individual, were suspended with buffer (PBS buffer + Tween 0.1% + EDTA 1 mM) and ground with a polytron homogenizer with a final concentration of 200 mg ml^{-1} of the homogenate. For analysis, 200 mg of the wet homogenate was suspended with 1 ml of buffer PBS and divided into seven aliquots (200 µl for carbohydrate and CCO analysis, 400 µl for PK, CS and total lipids analysis and 50 µl for DNA analysis).

Biochemical composition

LIPID AND CARBOHYDRATE CONTENT
Lipids were extracted with chloroform: methanol. After centrifugation (3246 *g*), the precipitate was extracted and the
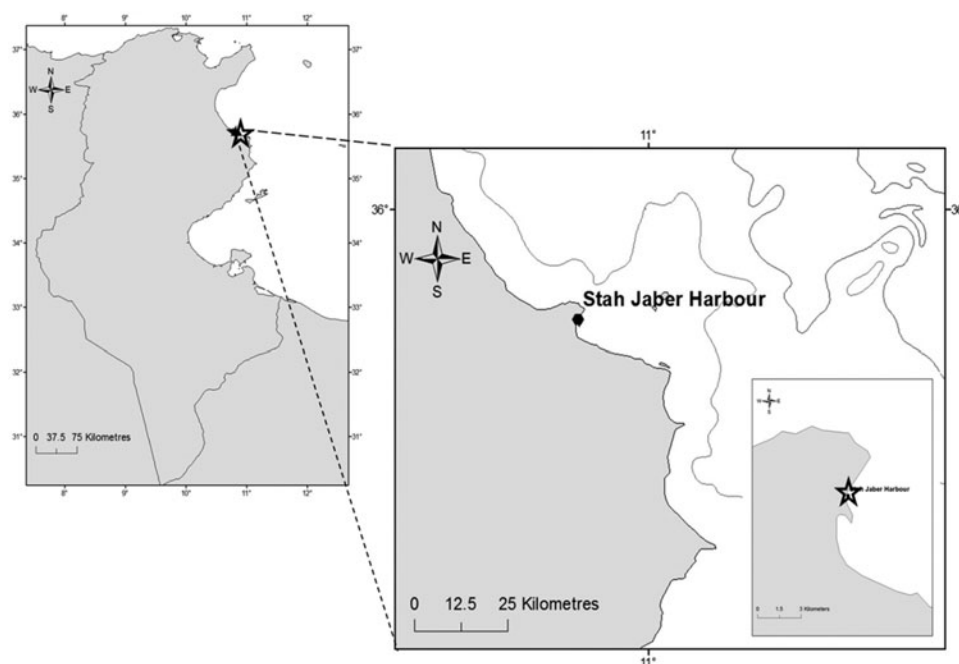

Fig. 1. Study site, Monastir port: Monastir Bay, the east coast of Tunisia.

supernatants were washed with chloroform: methanol: water as described by Bligh & Dyer (1959). Total lipids (percentage of total lipids per milligram of oyster dry weight) were determined gravimetrically by evaporation of the solvent in the purified extract on aluminium sheets at 60–80°C. Carbohydrates and protein contents were measured according to the method of Dubois *et al.* (1956) and Lowry *et al.* (1951), respectively. The results were calibrated against standard curves of glucose. Results were expressed as percentage of carbohydrate per milligram of oyster dry weight.

ENZYMATIC ACTIVITY

The homogenates were centrifuged for 10 min at 1,50,000 *g* at 4°C, the supernatant was used for enzymatic assays, citrate synthase (CS) and pyruvate kinase (PK). The results of enzymatic activities are expressed in mU mg^{-1} proteins. Protein concentration in the extracts were determined using the Lowry *et al.* (1951) method. The preparation for citrate synthase was as follows: 100-mM Tris HCL (pH 8), acetyl CoA 0.2 mM, DNTB 0.1 mM and oxaloacetate 500 µM. The reaction was initiated by mixing the supernatant 20 µl with the medium preparation 160 µl in a microplate. Changes in absorbance at 412 nm were monitored using a microplate reader (Bio-Tek Synergy HT, Bio Tek Instruments, USA).

The activity of PK in oyster tissues was determined as described by Greenway & Storey (1999). Frozen tissues were prepared at a 1:5 w/v ratio in a buffer composed of 50 mM imidazole-HCL buffer (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA and 15 mM 2-mercaptoethanol. The homogenates were centrifuged for 10 min at 15,000 *g* at 5°C. The preparation for pyruvate kinase was as follows: 100 mM imidazole –HCL buffer (pH 7.2), 50 mM KCL, 5 mM MgCl$_2$, 10 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH, 0.2% 2(v/v) retenone-saturated ethanol and 1.2 U ml^{-1} LDH. The assays were made in duplicate at room temperature and initiated by mixing the enzyme buffer with the 20 µl extract in 250 µl total microplate well volume. Changes in NADH absorbance at 340 nm were monitored using a microplate reader (Bio-Tek Synergy HT, Bio Tek Instrument, USA).

For the CCO activity the homogenates were centrifuged for 1000 *g*, the supernatant was re-centrifuged for 10 min at 9000 *g*, the pellets were removed and re-dissolved in the phosphate buffer pH 7.8 containing Tween 0.1% and EDTA 1 mM. The assays were made in duplicate at room temperature by mixing 66 mM reduced cytochrome c in phosphate buffer pH7.8, the reaction was initiated by adding 20 µl of mitochondrial extract. The absorbance was measured by using a microplate reader (Bio-Tek Synergy HT, Bio Tek Instruments, USA) at 550 nm during a period of 10 min.

DNA extraction and PCR-RFLP

DNA was extracted from the individuals of *O. stentina* collected from the Monastir Bay, by the method of phenol/chloroform extraction and precipitation by ethanol. Negative PCR controls consisting of water were included every 10 tested samples in order to check for any potential contamination. Positive PCR controls consisted of DNA extracted from *M. refringens* and *Bonamia* sp. purified cells provided by positive controls. *Bonamia* sp. was detected by PCR using Bo Boas's primers according to Cochennec *et al.* (2000). The parasite species was subsequently determined by restriction fragment length polymorphism (RFLP) analysis

(Hine *et al.*, 2001; Cochennec-Laureau *et al.*, 2003) carried out by separate digestions of 10 µl of PCR products with BglI and HaeII (Promega). The resulting fragment patterns were analysed electrophoretically on 2% agarose gel. *Marteilia refringens* was detected by PCR according to Le Roux *et al.* (2001). The parasite type was then determined using the PCR-RFLP approach developed by Le Roux *et al.* (2001). Analyses were carried out using the digestion method throughout the night at 37°C using 10 µl of PR4-PR5 PCR products with 0.25 units of HhaI (Promega). The resulting fragment patterns were analysed electrophoretically on 2% agarose gel.

Statistical analyses

Seasonal variation of the metabolic enzymes activity and energetic reserves, depending on the season were analysed by one-way ANOVA and Duncan's multiple range test to assess the significant differences ($P < 0.05$) between seasons (four levels). Differences were considered significant with a probability (P) value of 0.05 or less. All data are reported as mean $\pm$ standard deviation, except where indicated. Fisher's test was used as post-tests to compare the variation of biochemical analyses studied on the sampling dates. The metabolic enzymes activity, energetic reserves, biotic and abiotic parameters according to season were visualized and characterized by a Factorial Correspondence Analysis (FCA), which was conducted in order to establish the temporal clustering of the samples analysed. Pearson correlation coefficient (r) was applied to identify any significant correlation between parameters analysed. All statistical analyses were carried out using XLSTAT Software (version 2015.4.01 Addinsoft^{TM} SARL).

RESULTS

Physicochemical parameters

Seasonal values of the physicochemical parameters (temperature, salinity, dissolved oxygen and pH) measured over the study period are shown in Figure 2. The water temperature showed a typical seasonal variation, increasing from February (18.40°C) to June (30.02°C) and decreasing from June to October (17.10°C). The lowest value of dissolved oxygen was 3.05 mg l^{-1} observed in June and the highest values were measured in February 2009 (4.9 mg l^{-1}) and October (5.02 mg l^{-1}). The salinity was stable ranging from 36.20‰ in February 2009 to 37.80‰ in June 2009. The pH values were stable in February and March (respectively 8.10 and 8.06). The pH decreased between the March-June period, and during the June–October period, respectively from 7.80 to 7.13.

Biometric data

Individual oysters were collected randomly and their morphological characteristics were studied. The averages of these parameters are given in Table 1. Depending on the population density, availability of oysters to the collector and the sampling date, the length parameter of oysters varied between 32.41 $\pm$ 0.12 mm and 44.13 $\pm$ 1.20 mm and the total weight between 16.51 $\pm$ 1.28 g and 9.57 $\pm$ 0.10 g. Maximum values were
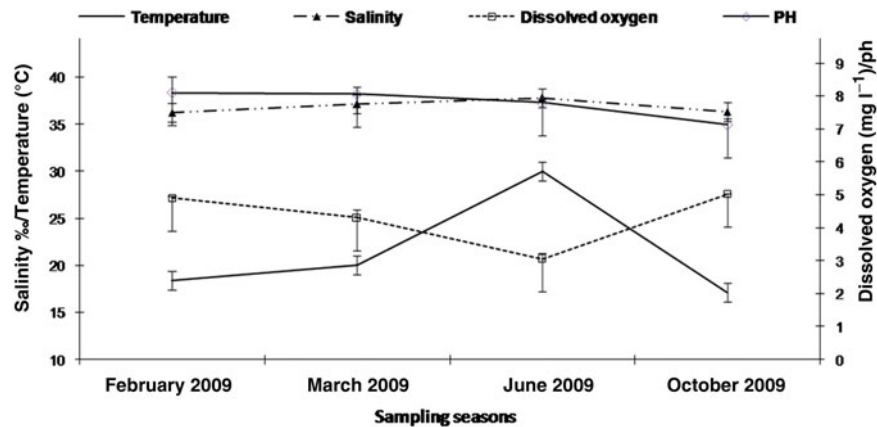
Fig. 2. Seasonal variation of physicochemical parameters (Salinity in ‰; Temperature in °C, Dissolved oxygen in mg l^{-1} and pH) from February 2009 to September 2009 in Monastir Bay, Tunisia (number of samples for each parameter = 3).

Table 1. Mean seasonal variation of length, total weight, shell weight and tissue weight of *Ostrea stentina* from February 2009 to October 2009 in the Monastir Bay, Tunisia.

Sampling date	24 February 2009 (N = 30)	26 March 2009 (N = 30)	21 June 2009 (N = 30)	28 October 2009 (N = 13)
Length (mm)	40.43 ± 1.06	44.13 ± 1.2	38.05 ± 1.50	32.41 ± 0.12
Total weight (g)	14.3 ± 0.91	16.51 ± 1.28	12.12 ± 0.99	9.57 ± 0.1
Shell weight (g)	10.73 ± 0.81	15.21 ± 1.21	8.45 ± 0.74	6.59 ± 0.08
Weight of the flesh (g)	1.47 ± 0.11	1.49 ± 0.15	0.93 ± 0.089	1.05 ± 0.014

observed in March and minimum values in October except the flesh weight.

PCR detection of *Bonamia exitiosa* and *M. refringens*

Following the detection of *M. refringens* by PCR analysis, we characterized the parasite using a histology examination in order to complete and to confirm our results. *Marteilia refringens* DNA was detected in 85 of the 103 tested oysters (80.95%) (Table 2). Detection frequency was lower in October (5 + /13 Ind.) and higher in February (30+/30 Ind.) and March (28+/30 Ind.) (Figure 5). All the PCR positive samples showed type O restriction profile after digestion of PCR products with *Hha*I. *Bonamia exitiosa* DNA was detected in three of the 103 tested oysters (2.91%) (Table 2). The PCR Bo-Boas positive samples showed *B. exitiosa* profile after digestion of PCR products with *Bgl*I and *Hae*II. During the study period, the mortality

rate ranged between 42 and 87% with the highest level recorded in autumn. The mortality rate increased from June (42%) to October (87%), and decreased to 73% in February (Figure 5).

Biochemical composition

The total lipid content did not show significant variations between the seasons (one-way ANOVA, $F = 1.51$, $P > 0.05$) and it varied little between seasons (Figure 3). Total lipids showed maximum values in the summer (13% lipids in dry weight) and lower values in the winter (10.65% lipids in dry weight). Carbohydrate content did not change significantly with the seasons (one-way ANOVA, $F = 1.52$, $P > 0.05$). Nevertheless, we observed a trend towards an increase of carbohydrate levels during the March–June period. A decrease in carbohydrates content was observed from 17.05% in dry weight in summer (9 June) to 11.60% in autumn (Figure 3).

Table 2. Identification of parasites from DNA extracts of samples of *Ostrea stentina* collected at different sampling dates in Monastir Bay, Tunisia. Detection of *Marteilia refringens* and *Bonamia* sp. by PCR using PR4-PR5, Bo-Boas primer pairs respectively, determination of the parasite species or type by RFLP after digestion by *BgI* and *HaeII*.

| Date of sampling	11.2.2009	26.3.2009	9.6.2009	13.10.2009
Identification of the parasite *Marteilia refringens*				
Number of tested oyster	30	30	30	13
Detection of *M. refringens*	30	29	21	5
Parasite typing (RFLP)	*M. refringens* type o	*M. refringens* type o	*M. refringens* type o	*M. refringens* type o
Identification of the parasite *Bonamia exitiosa*				
Detection of *B. exitiosa*	2	1	0	0
Parasite species (RFLP)	*B. exitiosa*	*B. exitiosa*		
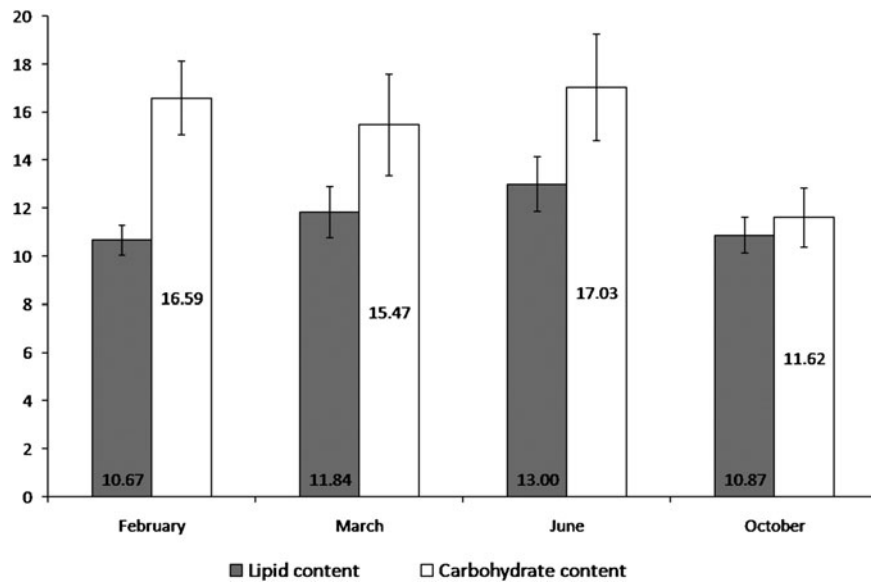
Fig. 3. Seasonal variation of lipids (% Lipids mg^{-1} dry weight) and carbohydrates content (% Carb. mg^{-1} dry weight) from February 2009 to September 2009 in Monastir Bay, Tunisia. Histograms present mean values. Number of individuals: February, N = 30; March, N = 30; June, N = 30; October, N = 13.

Enzymatic activity

The value for CS was significantly different over time (Table 3) (one-way ANOVA, $F = 31.22$, $P < 0.0001$). There was a decrease in the activity of CS from February (23.67 mU mg^{-1} prot) to October (3.36 mU mg^{-1} prot) (Figure 4). CCO activities continuously decreased significantly (one-way ANOVA, $F = 26.08$, $P < 0.0001$). The level of COO enzyme showed a seasonal variation with a maximum value recorded in winter, 11 February (8.26 mU mg^{-1} prot) and the lowest value was measured in autumn, 13 October (1.20 mU mg^{-1} prot) (Figure 4). The PK enzyme activity increased significantly (one-way ANOVA, $F = 10.96$, $P < 0.0001$) from February to June and decreased from June to October (Figure 4). The mean level of PK enzyme activity was higher in oysters during the summer period (9 June) with 31 mU mg^{-1} prot and lower in oysters during the autumn period (13 October) with 14.53 mU mg^{-1} prot.

Evaluation of the impact of *M. refringens* on *O. stentina*

The temporal variation of the metabolic enzyme activity and energy state of oysters is displayed in a FCA plot based on seasonal variation of several factors (biotic and abiotic parameters) acting simultaneously on the species *O. stentina*, in a two-dimensional graphical space (Figure 6). It reveals that the first axis contributes a value near 66%. The maturity of gonads and PK activity contribute significantly to the formation of the first factor, with respective values of 81.40% and 31%. Axis 1 (65.77%) separates *Marteilia* infection, maturity of gonads and PK activity from the other parameters. The CS activity and *Marteilia* infection have significantly higher contributions to the formation of the second factor, with respectively 59.80 and 57.70%. FCA analysis allowed separating maturity of gonads, mortality rate, CS and CCO activity and dissolved oxygen O_2 from *Marteilia* infection, energy reserves (lipids and carbohydrate content), growth rate and temperature seawater. The FCA showed that energy states (lipid and carbohydrate content) and PK activity were related to temperature since they correspond to highest registered values in summer. A high correlation of Pearson coefficient with proportional reaction was noted respectively ($r = 0.92$, $r = 0.60$ and $r = 0.99$). However, a high correlation of Pearson coefficient with inverse reaction was noted between energy reserves (lipids content, $r = -0.89$; carbohydrate content, $r = -0.72$) and maturity of gonads when the rise of energy reserves was followed by maturity of gonads during June–October period according to the reproductive cycle of *O. stentina*. The FCA showed that the growth rate of oysters was positively related to *Marteilia* infection since the parasite *Marteilia refringens* infected mature oysters. The Pearson correlation coefficient (between *M. refringens* infection and growth rate (length, total weight)) was $r > 0.9$. The FCA analysis demonstrated that contrary to maturity of gonads (Pearson coefficient $r = 0.90$), the energy reserves (Pearson coefficient $r = -0.74$) and PK activity (Pearson coefficient $r = -0.96$; $P < 0.001$) were negatively related with mortality rate.

Table 3. Results of one-way ANOVA and Student's t-test for biological data content according to the sampling date. Significance levels: ***($P < 0.0001$); ns, non significant; P, probability; F, Fisher test.

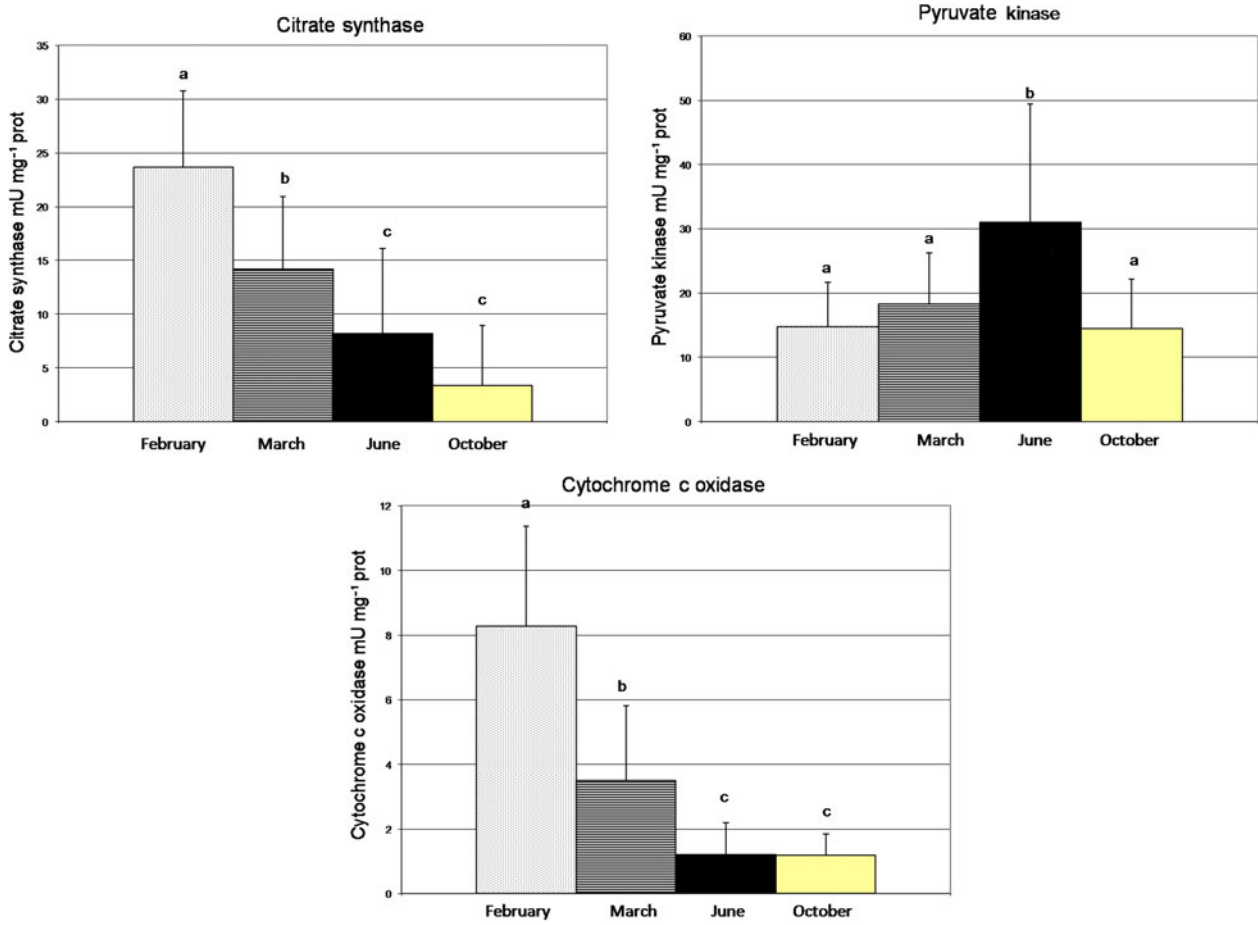| Biochemical analyses		Carbohydrates content		Lipids content		PK activity		CS activity		CCO activity	
	Df	F	P	F	P	F	P	F	P	F	P
Sampling date	3	0.84	ns	2.35	ns	10.96	***	31.22	***	26.08	***

Fig. 4. Seasonal variation of enzyme activity: PK, CS and CCO and temperature, from February 2009 to September 2009, in Monastir Bay, Tunisia. Number of individuals: February, N = 30; March, N = 30; June, N = 30; October, N = 13. Same letters are not significantly different.
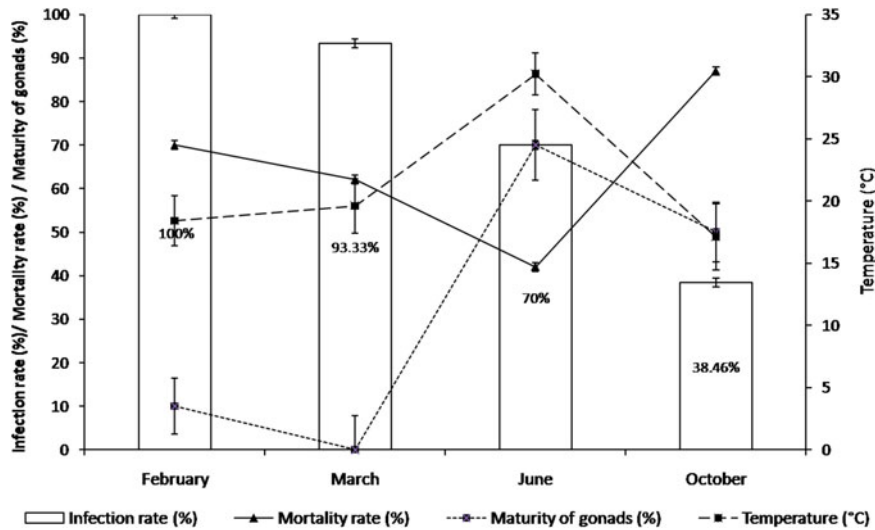

Fig. 5. Mortality rate (%) among oysters infected with *Marteilia refringens* (*Marteilia* infection %) during study period, with reference to water temperature (°C) and maturity of gonads (%).

DISCUSSION

Gonad maturity

The oyster *O. stentina* showed a successive hermaphroditism with a consecutive rhythmic sexuality on the basis of histological observation (Elgharsalli & Aloui Bejaoui, 2011). Reproduction occurs continuously throughout the year with two reproductive cycles for each sex. No resting period was observed. The sexual cycle clearly influences the morphological parameters of the oyster. The Pearson correlation coefficient (between maturity gonads and growth rate) was
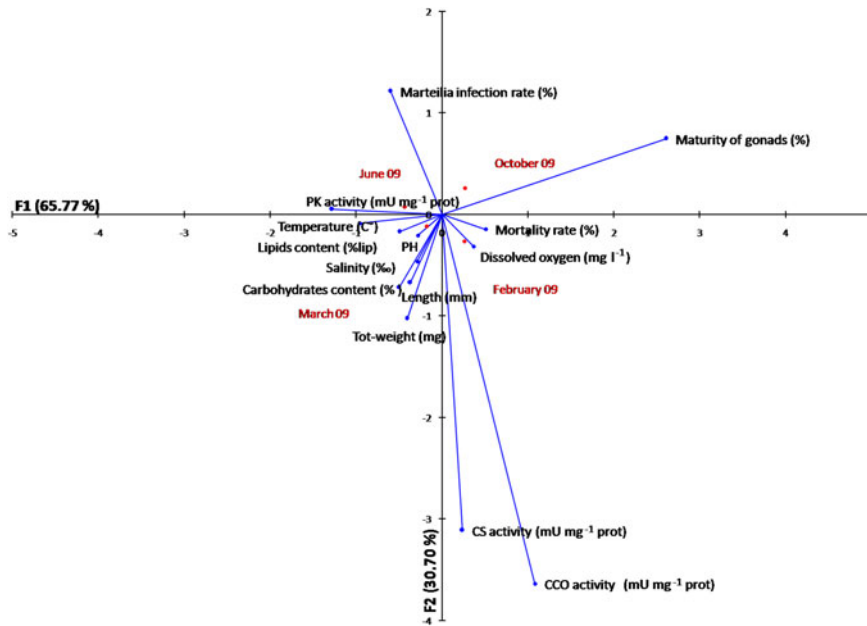
Fig. 6. Plot of the two first components (axes 1 and 2) of a factorial analysis (FCA) conducted based on the temporal metabolic enzymes activity, energy reserves, biotic and abiotic parameters according to season of the oyster *Ostrea stentina* infected with parasite *Marteilia refringens* from Monastir Bay, Tunisia.

$r > -0.71$ thus indicating negative relationships between the maturity gonads and growth rate. Wada *et al.* (1995) suggested that factors that affect the gametogenic cycle in bivalves might be the variation in the environment such as food availability, and particularly temperature, which may be the most important element. In the study area, the temperature fluctuated from $17.10°C$ in October to $30.20°C$ in June. In general, average temperatures above $17°C$ are common along the Tunisian coasts (Tlig-Zouari, 1993; Aloui-Bejaoui, 1998; Ben Kheder, 2001).

Biochemical analyses were carried out to evaluate the management of the energy resources in regard to the biochemical changes of lipids and carbohydrates. Lipid proportions in oyster tissues of *O. stentina* varied within the range $10.65-13\%$ in our study. Measuring the carbohydrate content revealed that it remained stable during the period studied and varied from February (16.60%) to October (11.60%). The metabolic pathways, such as pyruvate kinase (PK), will show the use of the energy resources. The correlation test between PK and lipids content showed a high Pearson coefficient with proportional reaction ($r = 0.95$, $P < 0.001$). The PK activity in *O. stentina* depended on the season. The major peak of PK activity was noted in summer with 33.60 mU mg^{-1} prot. The level of CCO activity for *O. stentina* ranged from 8.26 mU mg^{-1} prot in February to 1.20 mU mg^{-1} prot in October. The CS activity of *O. stentina* varied from February (23.67 mU mg^{-1} prot) to October (3.36 mU mg^{-1} prot) and remained low compared with the value reported for other oysters.

Biochemical composition

THE TOTAL LIPID CONTENT
Several patterns of temporal variability of lipids in bivalve molluscs, reported in previous studies (Pazos *et al.*, 1996; Li *et al.*, 2000; Dridi *et al.*, 2007) are the result of several environmental factors acting simultaneously, such as temperature, food availability, plankton composition and physiological

factors. The lipid contents of *O. stentina* remain similar compared with values reported for most other temperate bivalves (Pazos *et al.*, 1996; Almeida *et al.*, 1997; Kang *et al.*, 2000). Lipid proportions in oyster tissues of *O. stentina* varied between 10.65 and 13% in our study. Values for lipid contents of *M. edulis* by Zandee *et al.* (1980) were $10-11\%$; Wenne & Polak (1989) reported 18% for *Macoma baltica*. Values for lipid contents of *C. gigas* by Deslous Paoli & Héral (1988) were $6-12\%$, Costil *et al.* (2005) reported $8-10\%$ and Delaporte *et al.* (2006) reported 10%. There were peaks in lipid content in summer (June) which increased during gametogenesis and fell during spawning (July–December period). The total lipid reserves decrease in winter suggesting that they may have been mobilized to provide energy as the carbohydrate reserves become depleted (Bayne, 1976; Barber & Blake, 1991).

CARBOHYDRATES CONTENT
Carbohydrates are recognized as the major energy source in bivalves and the seasonal changes in glycogen levels correlated with seawater temperature, condition and tissue dry weight. Measuring the carbohydrate content revealed that it remained stable during the period studied and varied within the range $16.60-11.60\%$, from February to October. The carbohydrate content is low compared with the content of *C. gigas* in natural and controlled conditions (Berthelin *et al.*, 2000; Delaporte *et al.*, 2006) such as the carbohydrate content of *C. gigas* in natural conditions expressed in dry weight of $15-20\%$ (Le Moullac *et al.*, 2007). Accumulation of glycogen reserves in the spring–summer period followed by a fall synchronously with the rise of lipids, which reflects the maturity of the oysters, has been demonstrated by several authors (Maurer & Borel, 1986; Berthelin *et al.*, 2000). In fact, a high correlation of Pearson coefficient with inverse reaction was noted between energy reserves (lipids content, $r = -0.89$; carbohydrate content, $r = -0.72$) and maturity of gonads when the rise of energy reserves was followed by maturity of gonads during June – October period.

In general, when the gonad tubules are reduced, the mantle tissue contains numerous storage cells and there is maximal glycogen metabolism and storage capacity (Berthelin et al., 2000). Previously Gabbott & Walker (1971) have shown that glycogen may decrease during the spawning season when pre-stored glycogen is converted into lipid during vitellogenesis. In addition, the decrease of carbohydrate in the oyster tissues may correspond to the metabolization of glycogen during the vitellogenesis process (Berthelin et al., 2000) and muscle needs (Dunphy et al., 2006).

Other periods of limited glycogen content have often been associated with mortality events (Mori, 1979; Perdue et al., 1981). Mori (1979) considered that low carbohydrate content and high gonad development are combined factors increasing the mortality risk. As reported by Soletchnik et al. (2006), mortality was associated with a high reproductive activity (coefficient of Pearson, $r = 0.90$) when glycogen reserves (coefficient of Pearson, $r = -0.74$) decreased and reached a low point just before the mortality events. The Pearson correlation coefficient indicate a high correlation inversely proportional between mortality rate and carbohydrate content (Pearson coefficient $r = -0.74$), lipids content (Pearson coefficient $r = -0.94$), PK activity (Pearson coefficient $r = -0.96$, $P < 0.001$), and temperature (Pearson coefficient $r = -0.96$, $P < 0.001$). In fact, mortality increased from 42 to 87%, from June to October, when carbohydrate content declined.

Enzymatic activity

The use of the energy resources can be measured by activity of the metabolic pathways, such as the pyruvate kinase (PK). As in some other invertebrates, such as the oyster *Crassostrea virginica* (Greenway & Storey, 2000), the periwinkle *littorina littorea* (Greenway & Storey, 2001) and the horse mussel *Modiolus modiolus* (Greenway & Storey, 2001), the PK activity in *O. stentina* depended on the season. The temperature factor may have a seasonal effect on the PK activity by changing the kinetic properties (Le Moullac et al., 2007). In our study, changes in the water temperature were recorded with a maximum of 30.02°C in June 2009 and a minimum of 17.10°C in October 2009. The major peak of PK activity was noted in summer with 33.60 mU mgr^{-1} prot. As for the PK activity, the PK and temperature correlated significantly with a high Pearson coefficient reaction ($r = 0.99$, $P < 0.001$). The CS activity of *O. stentina* remained low compared with the value reported for *Crassostrea gigas*: 50 mU mg^{-1} prot (Gabbott & Walker, 1971) and 30 mU mg^{-1} prot (Dunphy et al., 2006). Accordingly, the reduction of CS activity observed in starved *C. gigas* (Sukhotin et al., 2006) and in starved mussels, *Mytilus californianus* (Dahlhoff et al., 2002), may be due to energy conservation. The level of CCO activity for *O. stentina* ranged from 8.26 mU mg^{-1} prot in February to 1.20 mU mg^{-1} prot in October. In general, decrease in enzyme activity may be due to environmental stress caused by a high temperature level or *M. refringens* infection. FCA analysis allowed separating CS and CCO activity from *Marteilia*. These enzyme activities seem to be opposite to *Marteilia* infection since they correspond to the lowest values during the high infection of parasite. In fact, the digestive gland, where *M. refringens* and other *Marteilia* species occur, is a site of intracellular food digestion and one of the main sites for the storage of metabolic reserves (Serve,

1969). Consequently, *Marteilia* infection causes a poor condition index with glycogen loss (emaciation) (Robert et al., 1991), discolouration of the digestive gland (Alderman, 1979), tissue necrosis (Comps et al., 1979), reproduction (Rebert et al., 1991) and mortalities (Berthe et al., 2004).

Based on our results, the products of glycolysis appear not to be used primarily for energy expenditure of the animal but rather oriented towards storage, gametogenesis or defence cells. Apparently, marteiliosis exacerbates the poor condition of the host. Oysters during reproduction may not have enough energy (lipids and carbohydrates) to defend themselves against *M. refringens* infection. Sexually mature oysters are therefore most affected by the infection. Furthermore, the drop of enzyme activity may be explained by the lack of nutritional resources due to necrosis of digestive tissues following infection by *M. refringens* (Comps et al., 1979) and depression of the energy state and the metabolic enzymes activities seen during our study.

CONCLUSION

Our results provide useful information on the energy state and enzyme activities in an attempt to describe the biochemical composition of *O. stentina* infected by *M. refringens* from Monastir Bay. The detection of parasites of the genus *Bonamia* and *M. refringens* in *O. stentina* from Monastir in 2009 with a higher prevalence of *M. refringens* (87.8%) suggested that *M. refringens* with other multiple factors could play an important role in the mass mortalities observed in Monastir Bay. The continuity of intense reproductive activity of *O. stentina* wherein much of the energy is devoted to reproduction led us to conclude that a weak defence system may be perturbed and cause a high parasite infection. Reproduction and the infection by the parasite *M. refringens* compounds could be involved in the depression of the energy state and the metabolic enzymes activities observed during our study.

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