Effect of seasonal variation in trophic conditions and the gametogenic cycle on \( \delta^{13}C \) and \( \delta^{15}N \) levels of diploid and triploid Pacific oysters *Crassostrea gigas*

N. Malet\(^1,3,*\), P.-G. Sauriau\(^1\), N. Faury\(^2\), P. Soletchnik\(^2\), G. Guillou\(^1\)

\(^1\)CRELA (UMR 6217 CNRS-IFREMER-ULR), Place du Séminaire, BP 5, 17137 L’Houmeau, France

\(^2\)IFREMER, Laboratoire Environnement Ressources Poitou-Charente (LERPC), BP 133, 17390 La Tremblade, France

\(^3\)Present address: Great Lakes WATER Institute, University of Wisconsin-Milwaukee, 600 East Greenfield Avenue, Milwaukee, Wisconsin 53204, USA

ABSTRACT: Carbon and nitrogen stable isotopes were investigated in separate organs of diploid and sterile triploid Pacific oysters *Crassostrea gigas* for 13 mo, together with changes in chemical and isotope composition of suspended matter sampled from an intertidal mudflat within Marennes-Oléron Bay, France. Particulate organic matter (POM) was a mixture of pelagic and benthic material with a predominance of neritic phytoplankton in spring, and resuspended microphytobenthos in summer and autumn. A remarkable shift of +3‰ in \( \delta^{13}C \) was reflected in both diploids and triploids from spring to summer, and further temporal differences were observed amongst their tissues. Seasonal changes in POM \( \delta^{15}N \) were also reflected in oyster tissues, with digestive gland and muscle tissues showing the largest and the least variability, respectively. Use of \( \delta^{13}C \) and C:N ratio relationships in separate tissues allowed for an assessment of the influences of trophic condition, seasonal changes, and gametogenic cycle on tissue \( \delta^{13}C \). Diploid digestive gland \( \delta^{13}C \) matched those of gonads, and differences between diploids and triploids in digestive gland and mantle \( \delta^{13}C \) were less than –1‰ during gametogenesis. The reproductive and rest periods were easily distinguished in these tissues and were characterised by enriched \( \delta^{13}C \) values in summer–autumn compared with spring, which is consistent with POM \( \delta^{13}C \) seasonal changes. A similar trend was observed in muscle, with a preferential incorporation of \( ^{13}C \)-enriched carbon during the summer–autumn growing season. However, despite the similar roles of mantle and digestive gland in lipid synthesis in both diploids and triploids, the correlation of \( \delta^{13}C \) with the C:N ratio highlighted the transfer of lipids to gonads in diploids and their differential allocation to growing tissues in sterile triploids.

KEY WORDS: *Crassostrea gigas* · Stable isotopes · Reproduction · Ploidy · Lipids

INTRODUCTION

For decades, stable isotope ratios have been recognised as efficient tools for the identification of dietary sources incorporated by consumers (Fry & Sherr 1984), and consequently have been used as natural tracers of organic matter flow in aquatic food webs (Michener & Schell 1994). Routine reconstruction of diets from stable isotope ratios of whole animal bodies is commonly summarised by the maxim ‘you are what you eat’ or, more properly, ‘you are what you assimilate plus a few per mil’ (DeNiro & Epstein 1978, 1981, Fry & Sherr 1984). Indeed, stable isotopes in consumers provide time-integrated information, averaging the natural environment variability in dietary components. They also give us a clue on how animal tissues turn over in relation to growth and/or metabolic replacement (Tieszen et al. 1983). Comparative analysis of fast vs. slow turnover tissues (e.g. digestive gland vs. muscle) may reveal short- and long-term changes in food...
source composition, respectively (Tieszen et al. 1983, Fry & Sherr 1984). The δ^{13}C values of consumers reflect the δ^{13}C values of their diet with small changes (<1‰), whereas δ^{15}N values show a larger discrimination of 3 to 4‰ per trophic level (DeNiro & Epstein 1978, 1981, Vander Zanden & Rasmussen 2001). However, animal–diet differences (δ_{tissue} \sim \delta_{diet}) may vary among species (DeNiro & Epstein 1978, 1981, Peterson & Fry 1987), and within species among ontogenic stages and sizes (Gearing et al. 1984), physiological states (Hobson et al. 1993), body tissues (Tieszen et al. 1983, Piola et al. 2006) and biochemical compounds. For instance, in a given animal body, lipids are generally more depleted in 13C than are carbohydrates and proteins (DeNiro & Epstein 1978), the last being incorporated without significant isotopic changes from dietary protein. These variations, which reflect biological processes and physiology-driven kinetics, have not yet been widely determined (see e.g. Hobson et al. 1993) and, as argued by Gannes et al. (1997), still require experimental investigation.

In estuarine and coastal ecosystems, benthic bivalve molluscs have been successfully used to trace mixing processes between terrestrial, marine and autochthonous organic material for both spatial (Incze et al. 1982, Stephenson & Lyon 1982, Peterson et al. 1985, Riera & Richard 1996, Machás & Santos 1999) and temporal scales (Riera & Richard 1997, Piola et al. 2006). These organisms may reflect the degree of benthic–pelagic coupling in shallow waters through the utilisation of food sources of different origins (Incze et al. 1982, Gearing et al. 1984, Piola et al. 1985, Sauriau & Kang 2000). So far, metabolic processes that modify the diet-derived isotopic composition of bivalves have not been investigated in detail. The variable trophic enrichment of different bivalve tissues has already been reported (Stephenson & Lyon 1982, Machás & Santos 1999, Piola et al. 2006). Similarly, in view of the respective roles of different tissues in energy allocation among maintenance, growth and the reproductive cycle in bivalves (e.g. Gabbott 1983), Lorrain et al. (2002) suggested that seasonal changes in the magnitude of metabolic transfers between germinal and somatic tissues in the scallop *Pecten maximus* have significant consequences for their δ^{13}C and δ^{15}N compositions, irrespective of diet resources. The relative contributions of growth and metabolic replacement to isotopic turnover was also investigated by Datta-gupta et al. (2004) using transplanted methanotrophic mussels *Bathymodiolus childressi* between different hydrocarbon seep sites.

To investigate these issues in a bivalve species representative of estuarine areas, temporal changes in the δ^{13}C and δ^{15}N composition of separate tissues of the Pacific oyster *Crassostrea gigas* (Thunberg) were compared with changes in the isotopic composition of its food originating from a tidal mudflat of Marennes-Oléron Bay, France. Previous contributions to the comparative analysis of stable isotope ratios in food resources (Galois et al. 1996, Richard et al. 1997) and *C. gigas* in this bay (Riera & Richard 1996, 1997) revealed significant seasonal trends and spatial heterogeneity in trophic conditions. Riera & Richard (1996) suggested that *C. gigas* collected on bare mudflats at the mouth of the Charente Estuary (north of Marennes-Oléron Bay) are mainly nourished by benthic diatoms. However, depending on their geographic location within its salinity gradient, oysters may reflect short-term incorporation of continental material, as revealed by the more negative δ^{13}C values of their whole body following periods of high river discharge (Riera & Richard 1997). The aim of the present study was to investigate the isotopic composition of Pacific oysters *C. gigas* experimentally reared on sandy mudflats located in the southern part of Marennes-Oléron Bay, far from direct estuarine influence, and then to test the hypothesis that reared oysters from this culture site reflect the isotopic signature of the mudflat-based food web irrespective of estuarine influence.

The annual reproductive cycle of bivalve molluscs is closely linked to metabolic functions involved in the energy storage–utilisation cycle, with the biochemical carbohydrate–lipid conversion pathway (see review by Gabbott 1983). This cycle differs among species and depends on the type of tissues and cells involved in the storage and mobilization of energy (Mathieu & Lubet 1993). It is recognised that gonad development and accumulation of energy reserves in *Crassostrea gigas* may temporally overlap during spring and summer periods (Deslous-Paoli & Héral 1988, Kang et al. 2000, Matus de la Parra et al. 2005). The second hypothesis for our study was that biochemical modifications linked to gamete build-up would modify the isotopic signals in oyster tissues involved in energy transfers to the gonads. Thus, we analysed all oyster tissues separately, i.e. mantle, gills, gonads, and particular attention was paid to muscle with long turnover times and digestive gland with short turnover times in order to track temporal changes in diet assimilation (Tieszen et al. 1983, Fry & Sherr 1984). Moreover, to the best of our knowledge, this is the first time that in situ experiments simultaneously involved diploid and triploid oysters in stable isotope studies. In the latter, reproductive potential is reduced owing to disruption of meiosis, whereas growth of somatic organs is enhanced (Beaumont & Fairbrother 1991, Garnier-Géré et al. 2002). Consequently, triploids as sterile animals are expected to provide a useful tool with which to unmask metabolic processes linked to reproduction.
MATERIALS AND METHODS

Study area. Our study was deployed at Ronce-les-Bains, an intertidal oyster culture area (175 ha) located in the southern part of Marennes-Oléron Bay (French Atlantic coast, north of the Gironde Estuary) (Fig. 1). Details of hydrobiological features, shellfish activities, sedimentary conditions and benthic ecology of the study site were previously given by Soletchnik et al. (1998), Goulletquer & Héral (1997), Kang et al. (1999) and Sauriau & Kang (2000), respectively. Crassostrea gigas were cultured off-bottom using iron frames to which oyster bags were fastened (Fig. 1, see picture).

Sample collection. Adult diploid and triploid oysters originated from a commercial oyster farm at La Tremblade. Triploids were produced at IFREMER La Tremblade by mating tetraploids and diploids (Guo et al. 1996). Both diploid and triploid oysters were overwintered in saltmarsh-based earth ponds for conditioning, ca. 7 mo for diploids and 1 mo for triploids following the traditional rearing practice within Marennes-Oléron Bay.

At the start of the experiment, triploid oysters (58.8 ± 7.5 mm, n = 15) were significantly longer than diploids (40.7 ± 5.9 mm, n = 15) (1-tailed Student's t-test, p < 0.001); however, total dry weights were similar at 0.15 ± 0.05 g and 0.13 ± 0.04 g (n = 15, 2-tailed Student's t-test, p = 0.17), respectively. Every month from March 2002 to April 2003, samples of 35 diploid and triploid oysters were collected. During the summer reproductive period from May to August 2002, sampling intervals were shortened (see Table 1). Oysters were kept overnight in filtered seawater to remove gut contents. From each sample, 5 oysters were frozen and stored at –20°C for later dissection into mantle, gills, digestive gland, muscle and gonads, and subsequent isotope analyses. Labial palps were not separated from mantle tissues. To minimise seepage of tissue fluids, particularly from the gonads, dissection was performed on frozen oysters. The remaining 30 oysters were split into 3 groups, which were frozen, freeze dried, and analysed separately for lipid content according to the procedure of Deslous-Paoli & Héral (1988).

Hydrobiological parameters. Water samples were collected twice a month from March 2002 to May 2003 at Ronce-les-Bains within the first 2 h of the flood tide. About 5 l of water was collected and pre-filtered with a 63 µm screen to remove any zooplankton or algae debris. Total particulate matter (TPM) was determined after filtration through pre-combusted and pre-weighed Whatman GF/C filters and dried for 24 h at 60°C, and particulate inorganic matter (PIM) was determined after filters had been combusted for 4 h at 450°C. Chlorophyll a (chl a) was extracted from Whatman GF/F filters according to the method of Holm-Hansen & Riemann (1978), and its concentration was determined using a Turner fluorometer at 665 nm. All hydrobiological parameters were determined in triplicate. A final sample of water was filtered onto a single precombusted Whatman GF/C filter and frozen for subsequent C and N stable isotope analyses.

Stable isotope analysis. Five frozen oysters were carefully dissected in order to separate the adductor muscle, digestive gland and gonads from gills and mantle. To prevent contamination of tissues by shell fragments, oyster tissues (except the gonads) were
quickly rinsed in 10% v:v HCl, and briefly rinsed twice in de-ionised water. To confirm that the acidification did not modify the isotopic values, supplement analysis were performed on subdivided organs of 4 oysters either acidified with HCl and then rinsed twice in de-ionised water, or just rinsed twice in de-ionised water. The paired t-test did not show any difference between the mean of either treatment (n = 16; carbon, p = 0.54; nitrogen, p = 0.71). Digestive gland, mantle, gills and gonads were freeze-dried and ground thoroughly to a fine powder, whereas muscle tissue was cut into fine pieces with a scalpel. Water sample filters were acidified with 2 N HCl acid vapour in a glass desiccator for 4 h at room temperature in order to remove carbonates, and then kept frozen at −20°C until analysed. Particulate organic matter (POM) was scraped from the fibreglass filters.

Carbon and nitrogen isotope ratios of the oyster tissues (n = 3) and POM samples were measured by continuous flow isotope ratio mass spectrometry (CF-IRMS) analysis using an IsoPrime stable isotope mass spectrometer (Micromass) interfaced to an elemental analyser EuroEA3024-IRMS (Eurovector). The analytical precision of 10 consecutive measurements was <0.15‰ for both N and C isotope ratios. Data are expressed in the standard δ notation as parts per thousand (‰) relative to Peedee Belemnite Limestone (PDB) and atmospheric N₂ for carbon and nitrogen, respectively. The stable isotopic ratio is reported as δᵦX = [(R_sample/R_standard) – 1]10³ (‰), where A is the atomic mass of the heavy stable isotope of the element X, and R = ¹³C/¹²C for carbon and ¹⁵N/¹⁴N for nitrogen, respectively.

**Statistical analyses.** Basic statistics and ANOVA were performed using the Minitab Release 10.2 package. Homoscedasticity of data was tested prior to ANOVA, and non-parametric tests were used in case of rejection (Sokal & Rohlf 1981). Stable isotope ratio time series of oyster tissues were tested using a 2-way ANOVA with replication, and with ploidy type (diploid vs. triploid) and sampling date as fixed factors. The non-parametric test for association using Kendall’s coefficient of rank correlation (τ) was also used in case of non-linear relationship between 2 variables and/or data known not to be normally distributed (Sokal & Rohlf 1981, p. 601), as is the case for tidal range. Model II regressions were performed using a non-linear algorithm (SigmaPlot 1.02 curve fitter based on the Marquardt-Levenberg algorithm) in order to estimate the SD of all regression parameters. Biometrics (shell length, tissue dry weight) were presented as mean ± SD. Significant differences in biometrics and stable isotope ratios among oysters and/or tissues were tested using 1-tailed or 2-tailed tests at a significance level of 0.05.

---

**RESULTS**

**Environmental conditions and food quality**

Temperature and salinity exhibited similar seasonal cycles at Ronce-les-Bains, with maximum values from spring to early autumn and minimum values in winter (Fig. 2a). The temperature of the flooding tide reached 25.9°C in summer and 5.0°C in winter, with a slow
Malet et al.: $\delta^{13}C$ and $\delta^{15}N$ in Pacific oysters

decrease from August 2002 to January 2003. Higher salinity values of 32 were observed from April to October 2002, and lower salinity values of 22 in March of both years (Fig. 2a).

TPM concentrations were always higher than 100 mg l$^{-1}$ (Fig. 2b) and reached extremely high values of 560 and 721 mg l$^{-1}$ in May 2002 and January 2003, respectively. TPM concentrations between 200 and 400 mg l$^{-1}$ were more frequently recorded in winter and spring than in other seasons. A significant Kendall’s rank correlation was found between tidal range and both TPM and PIM concentrations ($\tau = 0.161$, $p = 0.030$, $n = 84$ and $\tau = 0.183$, $p = 0.014$, $n = 84$ for TPM and PIM, respectively). This suggests that a significant proportion of high and low TPM and PIM values were recorded during spring- and neap-tide periods, respectively. The POM fraction (POM to TPM ratio) averaged 16 ± 4%, with the lowest values recorded in spring 2002 (Fig. 2b).

Chl a concentrations presented no clear seasonal trend but reached maximum values (>15 µg l$^{-1}$) in spring, summer and winter, and minimal values (<5 µg l$^{-1}$) in autumn and early winter (Fig. 2c). Pheopigment concentrations did not follow the same pattern of variation; nevertheless, several maximum values of chl a and pheopigments matched (Fig. 2c). PIM and pheopigments were highly significantly correlated ($r = 0.67$, $p < 0.001$, $n = 42$) and a significant Kendall’s rank correlation was found between chloropigment concentrations and chl a ($r = 0.176$, $p = 0.020$, $n = 81$). POM and chl a did not show a significant correlation for the entire set of dates sampled in 2002 and 2003, but did show a highly significant correlation during spring and early summer 2002 ($r = 0.87$, $p < 0.001$, $n = 10$).

The combining of $\delta^{13}C$, the C:N ratio of POM, and the POC:chl a ratio revealed 3 distinct periods between which oyster food quality differed (Fig. 3):

1. Early spring (i.e. March to April 2002) was characterised by low POM concentrations closely associated with low chl a concentrations, high pheopigment and PIM concentrations, and with particulate organic carbon (POC):chl a values of <100 (Fig. 3b). Such values indicated that much of the POC derived from living algal sources. Accordingly, high C:N ratios (10.4 ± 0.4) associated with isotopic carbon-depleted values of $-23.2 ± 0.5$‰ (Fig. 3a) reflected the contribution of early spring phytoplankton blooms to bulk POM.

2. Spring, summer and autumn (i.e. May to November 2002) was defined by large pigment variability with several peaks in May, July and September. High chloropigment and PIM concentrations together with enriched $\delta^{13}C$ values of $-20.9 ± 0.4$‰ (Fig. 3a) indicated that benthic organic matter episodically contributed to the organic pool of the bay. Values of the POC:chl a ratio fluctuated between 26 and 254 in relation to the relative abundance of phytoplankton vs. re-suspended benthic algae.

3. Early winter to spring (i.e. December 2002 to April 2003) was characterised by high TPM concentrations but with chl a concentrations that were unrelated to pheopigment concentrations (Fig. 2c). Mean $\delta^{13}C$ values ($-21.8 ± 0.4$‰) were associated with C:N ratios ranging from 6.5 to 8.1 (Fig. 3). These low values reflected contributions by benthic sources to estuarine organic matter that were lower than those in summer and early autumn. Moreover, very high POC:chl a ratios suggested that detrital material was always a major constituent of POM (Fig. 3b), particularly in December 2002 and January 2003.

**Biometrics and proximate lipid contents**

Oyster total tissue dry weight increased approximately 10-fold between the start and the end of the experiment, i.e. from 0.13 ± 0.04 g to 1.33 ± 0.22 g for diploids, and from 0.15 ± 0.05 g to 1.75 ± 0.24 g for triploids (Table 1). Oyster growth occurred in spring and early summer for diploids (Fig. 4a), and until early autumn for triploid (Fig. 4b). The initial differ-
ence in shell size between diploids and triploids became insignificant after the 2 mo of cultivation, because diploids exhibited compensatory shell growth (Table 1). For both diploid and triploid oysters, the digestive gland and the gills accounted for 30 to 40% of total tissue dry weight, and muscle and mantle only for 10 to 20%. Major month-to-month changes in tissue dry weight occurred in the digestive gland and the gonads for diploids (Fig. 4a), and in the digestive gland and the gills for triploids (Fig. 4b).

Abrupt changes in the proportion (% of total weight) of gonads occurred in June, and one mass-spawning event occurred at the end of July (Fig. 4a, Table 1). During these respective periods, gonads represented between 27 and 54% of the total tissue dry weight in diploid oysters before gamete release (Table 1). As a consequence of biochemical replacement during reproduction, the lipid content of diploid oysters varied from 9.7 to 15.8% of total tissue dry weight during the spawning season (Table 1). Variation in the lipid content of triploid oysters was less abrupt than in the diploids, ranging from 9.4 to 12.2%. From June to mid-August 2002 and outside periods of gamete release (mid-June and August), lipid content of diploid oysters was significantly higher than that of triploid oysters (t-test, p < 0.001, n = 6, for 7 out of 9 sampling dates between 5 June and 21 August, Table 1).

Table 1. Crassostrea gigas. Total shell length (mm), total tissue dry weight (g), proportion of gonad, and total lipid content relative to dry weight (%) (mean ± SD, n = 5, 5, 5 and 3 pools of 10, respectively) for diploid and triploid oysters at Ronce-les-Bains. Dates given as mm/dd/year; nd = no data

![Figure 4](image_url)
Malet et al.: δ13C and δ15N in Pacific oysters

Before the start of the experiment at Ronce-les-Bains in March 2002, oysters were grown in intertidal areas for 1 yr and then stored in oyster ponds during the autumn and winter. Their stable isotope signatures were modified depending on the time spent in oyster ponds. Diploid oysters stored 7 mo in oyster ponds reached a δ13C value of –23.7 ± 0.3‰ for the digestive gland, –23.0 ± 0.4 ‰ for the mantle, and –22.6 ± 0.3 ‰ for muscle (Fig. 5a). However, δ13C values of triploid oysters, which spent only 1 mo in oyster ponds, remained more enriched, i.e. –21.7 ± 0.3‰, –19.8 ± 0.2‰, and –18.1 ± 0.4 ‰ for digestive gland, mantle and muscle, respectively (Fig. 5b). δ13C values of gills (not shown for clarity) were intermediate between those of muscle and mantle tissues whatever the ploidy group. At the end of the experiment in April 2003, there were no significant differences in δ13C values between diploid and triploid oysters for either digestive gland or mantle. However, for the majority of the time, the muscle tissues of triploids remained significantly more enriched in 13C (by approx. >1‰) than those of diploids (Fig. 5).

Over a 1 yr cycle in the intertidal area, both diploid and triploid oysters showed significant seasonal changes in digestive gland, mantle and muscle isotopic composition (2-way ANOVA, p < 0.001). Although a rather similar pattern was observed in month-to-month changes in the δ13C of their digestive glands (Fig. 5), highly significant differences occurred in the digestive gland within the first 3 mo and in autumn and early winter (p < 0.001 for the interaction between ploidy type and date). There was also a clear discrepancy between the 2 time series in muscle δ13C values of diploid and triploid oysters for the first 5 mo and in the autumn to early winter period (2-way ANOVA, p < 0.001 for date, ploidy type and interaction). Gill and mantle δ13C values were nearly always intermediate between digestive gland and muscle δ13C for both diploids and triploids, and significant differences between diploids and triploids were apparent within the first 4 mo and in early winter 2003 (Fig. 5).

δ15N in diploid and triploid tissues

Regardless of tissue type, δ15N values did not differ significantly between diploid and triploid oysters at the start of the experiment (Fig. 6). Mean values of δ15N ranged from 7.6 to 8.7 ‰ for diploid and triploid oysters, with similar digestive gland values in March 2002 (Fig. 6).

Mean δ15N values in the digestive gland of diploid vs. triploid oyster did not differ significantly (2-way ANOVA, p = 0.375 for ploidy type). However, month-to-month changes in digestive gland δ15N values were highly significant (p < 0.001), and a strong first-order interaction was indicative of varying overlap between

![Fig. 5. Crassostrea gigas. Temporal variation in δ13C values of digestive gland (■, □), mantle (●, ○), muscle (▲, △) and gonads (×) of (a) diploid (filled symbols) and (b) triploid (open symbols) oysters from March 2002 to April 2003. Gill data not shown for clarity. Grey areas represent spawning periods recorded for diploid oysters in 2002. Mean ± SD (n = 3)](image)

![Fig. 6. Crassostrea gigas. Temporal variation in δ15N values of digestive gland (■, □), mantle (●, ○), muscle (▲, △) and gonads (×) of (a) diploid (filled symbols) and (b) triploid (open symbols) oysters from March 2002 to April 2003. Gill data not shown for clarity. Grey areas represent spawning periods recorded for diploid oysters in 2002. Mean ± SD (n = 3)](image)
the 2 time series (p < 0.001, Fig. 6). Similar results were obtained when δ¹⁵N time series of mantle and gills (not shown for clarity) were compared, time series for the gills being intermediate between those of muscle and mantle tissues. Seasonal changes in muscle δ¹⁵N were also highly significant (2-way ANOVA, p < 0.001), but because the first-order interaction was not significant (p = 0.246), the highly significant effect of ploidy type (p < 0.001) suggested that diploid and triploid oysters had different values of muscle δ¹⁵N. One-tailed Student’s t-test thus revealed that mean values of muscle δ¹⁵N in triploid oysters were significantly higher than those of diploid oysters (p < 0.004), with a difference of +0.2‰ from April to July 2002 and +0.4‰ from August to April 2003.

Diploid vs. triploid δ¹³C and δ¹⁵N relationships

The relationship between digestive gland δ¹³C of diploid oysters and that of triploid oysters for the period May 2002 to April 2003 (excluding the acclimation period of March to April 2002) revealed 2 trends in relation to the reproductive season (Fig. 7a). From the end of May to August 2002, i.e. during the reproductive period, diploid oyster digestive glands were significantly more depleted in ¹³C than were triploid oyster digestive glands (1-tailed Student’s t-test, p < 0.001). In contrast, during the resting period from mid-August 2002 to April 2003, diploid oyster digestive glands were significantly more enriched in ¹³C than those of triploid oysters (1-tailed Student’s t-test, p < 0.017). Moreover, coupled changes in ¹³C values of diploids and triploids occurred during the rest period because of the significant correlation between δ¹³Ctriploids and δ¹³Cdiploids (r² = 0.49, n = 27, p < 0.001), with the y-intercept and the slope not differing significantly from 0 and 1 (1.01 ± 0.01), respectively.

Owing to the large differences in initial values of muscle δ¹³C between diploid and triploid oysters (Fig. 5), and because the turnover of muscle tissue is slow, changes in muscle δ¹³C of diploid and triploid oysters were not comparable. However, even after 7 mo on the same intertidal mudflats, diploid oyster muscle tissue was always more depleted in ¹³C than that of triploid oysters. Mantle tissues represented an intermediate situation between digestive gland and muscle tissues (scatter plot not shown), with δ¹³C values that were significantly different during the first 4 mo and then overlapped (Fig. 5).

The scatter plot of the relationship of digestive gland δ¹⁵N between diploid and triploid oysters did not show any significant differences related to the oysters’ reproductive activity (Fig. 7b). The linear regression for the whole data set was highly significant

δ¹³C vs. C:N ratio in diploid and triploid tissues

During the reproduction period, from April to August 2002, digestive gland δ¹³C values and C:N ratios were significantly and negatively correlated for both diploid and triploid oysters (δ¹³Cdiploids = -0.29±0.14, C:N = -20.7±7.1, r² = 0.14, n = 30, p < 0.05; δ¹³Ctriploids = -0.37±0.07, C:N = -19.6±0.4, r² = 0.46, n = 30, p < 0.001) (Fig. 8a). Gonads in diploid oysters also exhibited a similar negative correlation (δ¹³Cdiploids = -0.46±0.08, C:N = -19.9±0.4, r² = 0.61, n = 23, p < 0.001) (Fig. 8a). Slopes of these 3 linear regressions did not differ significantly. During the rest period, from August 2002 to April 2003, no significant correlations were observed in either
Malet et al.: $\delta^{13}$C and $\delta^{15}$N in Pacific oysters
diploid or triploid oysters, but $\delta^{13}$C values of the digestive gland during the reproductive period differed significantly from the 2 others of the reproductive period (Fig. 8a). The range of variation in mantle tissue C:N ratios was larger in triploids (4 to 8.5) than in diploids (4 to 6) (Fig. 8b).

Muscle $\delta^{13}$C values in both diploid and triploid oysters exhibited little scatter in C:N ratios, with no significant correlation (Fig. 8b). Muscle C:N ratios averaged $3.30 \pm 0.16$ (n = 104). $\delta^{13}$C values in both diploids and triploids significantly differed between the 2 periods, with $\delta^{13}$C_{rest period} $> \delta^{13}$C_{reproductive period}. A significant positive correlation was found between C:N ratios and muscle $\delta^{13}$C in diploids during the summertime rest period ($\delta^{13}$C_{diploids} = $-0.63(\pm0.20)$C:N $- 18.5(\pm0.9)$, $r^2 = 0.27$, n = 30, p < 0.01; $\delta^{13}$C_{triploids} = $-0.48(\pm0.10)$C:N $- 18.2(\pm0.6)$, $r^2 = 0.46$, n = 30, p < 0.001; and $\delta^{13}$C_{triploids} = $-0.60(\pm0.08)$C:N $- 16.4(\pm0.5)$, $r^2 = 0.72$, n = 27, p < 0.001, respectively (Fig. 8b). Owing to large confidence intervals, slopes of the diploid and triploid mantle linear regressions did not differ significantly during the reproductive period (Fig. 8b). However, slope of the linear regression observed for the triploid mantle during the rest period differed significantly from the 2 others of the reproductive period (Fig. 8b).

**DISCUSSION**

**Seasonal changes in sources of POM**

POM samples were collected from a bare sandy mudflat, where both Zostera noltii meadows and green macroalgae are rare (Kang et al. 1999, Sauriau & Kang 2000), the latter being confined to the vicinity of oyster culture structures (Fig. 1). At the same site, Kang et al. (1999) reported similar $\delta^{13}$C POM values (1995: $-24$ to $-20\%$). Previous hydrological analyses performed within Marennes-Oléron Bay (Galois et al. 1996, Riera & Richard 1996, Richard et al. 1997) suggested that a mixture of various POM sources was available to suspension-feeders. At that tidal site, water-column mixing is likely via: (1) current and wind-driven re-suspension that acts on sedimentary material (Kang et al. 1999); (2) tidal exchange through the nearest marine inlet, i.e. Pertuis de Maumusson (Soletchnik et al. 1998); and (3) north-to-south residual advection of water bodies that are characterised by high inorganic loads (Zurburg et al. 1994) and influenced by the Charente River and Gironde Estuary discharges (Soletchnik et al. 1998).

A significant contribution by benthic microalgae to the water column was expected because of large microphytobenthos mudflat biomass at our study site that was persistent throughout the year (Kang et al. 1999). $\delta^{13}$C values of microphytobenthos have been reported to range from $-15$ to $-17\%$ in Marennes-Oléron Bay (Riera & Richard 1996). It could be deduced from the temporal changes in $\delta^{13}$C POM values (Fig. 3a) that re-suspended microphytobenthos material contributed to suspended POM mainly from mid-spring to early winter. This is consistent with the occurrence of significant relationships among tidal ranges, PIM and pheopigments (Fig. 2c), most
Ported by ranges of an end-member in early spring is consistently sup-
during blooms in spring (diatoms) and summer and to –23‰, C:N ratios < 10, and POC:chl a (Middelburg & Nieuwenhuize 1998) — matches these

(Thornton & McManus 1994) and Schelde estuaries e.g. the Gironde (Fontugne & Jouanneau 1987), Tay estuaries, which includes discrete measurements made off Marennes-Oléron Bay (Fontugne & Jouanneau 1987, Richard et al. 1997). These authors indicated that, within the Pertuis d’Antioche (Fig. 1), POM was characterised by aged and refractory terrestrial material (1990: δ13C from –27 to –26‰ and C:N ratios > 20) during winter months with low Charente River discharge, and fresh estuarine phytoplankton (1991: δ13C from –24 to –23‰, C:N ratios < 10, and POC:chl a ratios < 100) during blooms in spring (diatoms) and summer and autumn (flagellates). That estuarine phytoplankton is an end-member in early spring is consistently supported by ranges of δ13C (–22.8 to –23.8‰), δ15N (+5 to +8), C:N ratios (10 to 11), and POC:chl a ratios (<100) (Fig. 3) recorded from March to April 2002 at Roncles-Bains. However, a contribution by sedimentary material cannot be excluded. The water column was thus characterised at that time (early spring) by high estuarine salinity (22 to 32) and very high turbidity (>100 to 600 mg l–1, Fig. 2a,b), which originated from local re-suspension and/or advection of estuarine waters through the Pertuis de Maumusson (Fig. 1). In fact, sedimentary POM from lower reaches of many estuaries — e.g. the Gironde (Fontugne & Jouanneau 1987), Tay (Thornton & McManus 1994) and Schelde estuaries (Middelburg & Nieuwenhuize 1998) — matches these δ13C values as the result of a progressive dilution of riverine (δ13C-depleted POC source) with marine organic matter (δ13C-enriched POC source). Within the Bay of Biscay and off Marennes-Oléron Bay, marine organic matter comprised of neritic species with δ13C values similar to those of marine plankton (Fontugne & Jouanneau 1987, Riera & Richard 1996, 1997), and is likely to nourish the studied mudflats every flood tide from mid-spring to early winter.

In winter 2003, freshwater discharges from both the Charente River and Gironde Estuary were much higher than in 2002, with flood conditions from mid-November 2002 to mid-March 2003. Consequently, salinities of <25 to 30, δ13C values of around –22‰, C:N ratio values of <9, and POC:chl a ratios with transient values higher than 300 were indicative of degraded phytoplankton and re-suspended sedimentary material contributions to the bulk of the estuarine organic matter.

### Stable isotope composition of tissues

**Initial vs. final stable isotope ratios of oyster tissues**

Before the start of the experiment, adult oysters originating from intertidal areas were transferred into oyster ponds or ‘claires’ that are traditionally used for oyster refining in late autumn and winter prior to market-ing (Goulletquer & Héral 1997). Oysters placed in such a shallow and rich environment during autumn and winter continue to grow, improving their body condition and biochemical composition (Deslous-Paoli et al. 1982). They consequently show significant increases in carbohydrate content compared with oysters reared on tidal flats (Deslous-Paoli & Héral 1988). In the present study, transplanted oysters presumably acquired new stable isotope signatures that reflected the incorporation of new dietary items, owing to both growth and metabolic tissue replacement (DeNiro & Epstein 1978, Datta Gupta et al. 2004). δ13C values of diploid oyster tissues that had spent 7 mo in oyster ponds before being transplanted back to tidal areas in March 2002 were depleted (~22.2 to ~24.1‰, Fig. 5). This suggests that either a significant quantity of C3-terrestrial organic matter or locally produced δ13C-depleted plankton lowered the initial stable isotope composition of those oyster tissues, even though salt-marsh-based oyster ponds are filled with Marennes-Oléron Bay waters every spring tide. Seasonal changes in stable isotope signatures of water-column POM in the oyster ponds clearly validated this hypothesis: δ13C and δ15N values ranged from ~29 to ~22.5‰ and from +4.5 to +12.5‰, respectively, over a 1 yr cycle (N. Malet unpubl. data). Similar values have been
reported worldwide from shallow, semi-enclosed salt marshes or near-shore systems free of C4-plants in which mixing of organic material from different origins may occur (Fry & Sherr 1984, Peterson & Fry 1987, Michener & Schell 1994).

In contrast, owing to practical supply difficulties, adult triploid oysters spent only 1 mo in oyster ponds. Triploid tissues retained the isotopic composition that they previously gained from their intertidal rearing location. Digestive gland in triploid oysters exhibited moderate changes in δ¹³C compared with the more depleted values recorded for diploid oysters. Mantle tissues presumably have a lower turnover rate than the digestive gland, and their δ¹³C values were intermediate between those of digestive gland and muscle. Moreover, muscle in triploid oysters maintained an isotope carbon ratio close to ~18‰. Differences in δ¹³C ratios among diploid and triploid muscle, mantle and digestive glands recorded before the start of our experiment are consistent with this time-integrated approach. Muscle tissue is recognised as a long-term integration of dietary sources owing to its slower turnover relative to more metabolically active tissues such as digestive gland, liver and mantle (Tieszen et al. 1983).

**Seasonal changes in stable isotope signature of tissues**

A large mid-summer seasonal shift of ca. +3.0‰ occurred in δ¹³C levels of digestive glands and mantle of triploid oysters, and in all diploid oyster tissues except gonads. This seasonal shift differs between ploidy groups and within groups among tissue types. This may be due to (1) a major seasonal change in the availability and/or incorporation by oyster tissues of pelagic vs. benthic food resources, (2) a differential ingestion and/or assimilation of specific compounds relative to the bulk POM, or (3) indirect consequences of the spring to summer reproductive activity in diploid *Crassostrea gigas*.

Our first hypothesis is based on the recognition that, in marine environments, the δ¹³C signature of benthic microalgae is more enriched than that of phytoplankton (Fry & Sherr 1984, Peterson & Fry 1987). Since the δ¹³C values of a consumer are closely related to that of its food (DeNiro & Epstein 1978), the assimilation of δ¹³C-enriched food sources (i.e. re-suspended benthic microalgae from intertidal mudflats) during summer and autumn could explain the enriched δ¹³C values recorded in oyster tissues for the 2 seasons (Fig. 5). The progressive incorporation of δ¹³C-enriched food led to more δ¹³C-depleted oyster tissues over high-growth-rate periods of diploid and particularly triploid tissues in summer and autumn 2002 (Figs. 4 & 5). However, temporal changes in δ¹³C values of oyster tissues did not closely match those of the POM pool in spring (Figs. 3 & 5), POM being more enriched in early May. This relatively short time lag (<2 mo) is nevertheless consistently linked to growth exhibited by somatic tissues (i.e. muscle and mantle) in both diploids and triploids, and digestive gland in triploids (Fig. 5). Specific tissue differences in δ¹³C time series suggested the influence of metabolic functions other than growth during that period of intense reproductive activity. Longer time lags are also expected during non-growing seasons, and lags of >3 mo can be deduced from the comparative analysis of POM and oyster-tissue δ¹³C time series in autumn and early winter, with respective decreases recorded in October 2002 and January 2003 (Figs. 3 & 5). Similar lags between POM and oyster-body δ¹³C time-series were also reported by Riera & Richard (1997) for oysters from the upper reaches of the Charente Estuary following a high flood period in winter 1992.

Similarly, summer-time changes in δ¹³C of muscle in both diploid and triploid oysters (Fig. 5) substantiated the diet-change hypothesis. As in most bivalve species, muscle tissues in *Crassostrea gigas* are mainly constituted by protein, ~60 to 85% of dry weight (Berthelin et al. 2000), and contain much lower proportions of lipids and glycogen than do other tissues (Whyte et al. 1990, Berthelin et al. 2000). As reported in this study, muscle tissues logically exhibited small C:N ratios in both ploidy groups. Consequently, most of their seasonal changes in δ¹³C values should have paralleled those of dietary protein components. Since δ¹³C in the muscle of diploids matched those of triploids (~18 to ~19‰) at the onset of winter (December 2002 to January 2003, Fig. 8), it could be concluded that diploids had been nourished by similar ¹³C-enriched dietary sources throughout the summer–autumn growing season. This conclusion is also in agreement with our results for tissue-δ¹⁵N time series, suggesting that both ploidy groups occupy similar trophic levels (Fig. 6). However, a systematic positive difference in muscle δ¹⁵N between triploids and diploids was found. This difference is approximately 10 times less than the accepted average value of 3 to 4‰ δ¹⁵N that represents the discrimination between each trophic level (DeNiro & Epstein 1981, Vander Zanden & Rasmussen 2001).

It was too small to allow us to discern any differences in trophic regime between triploid and diploid oysters. Moreover, it is recognised that higher growth performances in triploid *C. gigas* (Beaumont & Fairbrother 1991) are consistently linked to genetic and physiological differences, such as heterozygosity and higher metabolic process efficiencies (Garnier-Géré et al. 2002 and references therein). This may lead to subtle
changes in morphology, anabolism and/or biochemical features of adductor muscle in triploid C. gigas. This hypothesis would require further experimental investigation in C. gigas because differences found between ploidy groups of other bivalve families Pectinidae and Veneridae may not be applicable to Ostreidae (Beaumont & Fairbrother 1991).

Our second hypothesis is linked to the ingestion and/or assimilation of specific fractions and components among the ambient POM pool, owing to the ability of Crassostrea gigas to selectively ingest algae. (Cognie et al. 2003). Similarly, Bougrier et al. (1997) observed that C. gigas may preferentially filter and reject (as pseudofaeces) diatoms relative to flagellates depending on their shape and flexibility. As in most coastal systems within the Bay of Biscay (Gailhard et al. 2002) and within Marennes-Oléron Bay, flagellates dominate phytoplankton blooms only in the autumn (Galois et al. 1996 and references therein), partly due to lower turbulence conditions (Gailhard et al. 2002). Flagellates are also known to be more depleted in $^{13}$C than are pelagic diatoms (Cifuentes et al. 1988), and Gearing et al. (1984) showed that microflagellates may be 2‰ more negative than the diatom Skeletonema costatum, which is also a common neritic phytoplankton species in spring and autumn in coastal areas of the Bay of Biscay (Gailhard et al. 2002). However, time series of $\delta^{13}$C in oyster tissues (Fig. 5) did not exhibit any potential influence of $^{13}$C-depleted phytoplankton species on the food regime of oysters in autumn. The relative proportions of phytoplankton vs. microphytobenthos species and ratios of inorganic vs. organic material should considerably influence the food regime of opportunistic suspension-feeders such as C. gigas in Marennes-Oléron Bay (Bougrier et al. 1997, Riera & Richard 1997).

Our third hypothesis is connected with the time course of gametogenesis and associated biochemical changes in Crassostrea gigas. Gonad development is an energy-consuming process that mobilises nutrients from assimilated food and utilises reserves previously stored in somatic tissues. Although the time course and relative balance of processes between these 2 pools of energy required to sustain gametogenic demands are family- or species-specific, glyco-gen is regarded as the major source of energy in marine bivalves and is used for lipid synthesis (see Gabbott 1983 for a review). Increasing lipid contents with gonad build-up and ripe gamete production in C. gigas could explain the $^{13}$C depletion in oyster tissues involved in reproduction because lipids are much more $^{13}$C-depleted than are proteins and carbohydrates. This is ascribed to lipid synthesis, which discriminates against $^{13}$C in favour of the lighter isotope $^{12}$C (DeNiro & Epstein 1977).

In Marennes-Oléron Bay, the reproductive activity of Crassostrea gigas usually occurs from May to mid-August, with maximum lipid contents recorded just before the mid-summer mass spawning event (Deslous-Paoli & Héral 1988, Matus de la Parra et al. 2005). Our data agree with this pattern, which could not be generalised because the timing and duration of gametogenesis differ among C. gigas populations (Ruiz et al. 1992, Pazos et al. 1996, Kang et al. 2000, Li et al. 2000). As is usually found in animals, lipid classes differ in their metabolic roles, neutral lipids (triacylglycerols) being used as energy reserves and polar lipids (phospholipids) being the structural components of cells and membranes. Pazos et al. (1996), Li et al. (2000), and Matus de la Parra (2005) all indicated that fluctuations in lipid content in whole oyster bodies and/or separate organs are largely resultant from changes in triacylglycerols over the reproductive period. In C. gigas females, the triglyceride content of the ovaries faithfully reflects the course of sexual maturation, being greatest when oocytes have grown sufficiently and are ready to be spawned (Li et al. 2000). Our stable isotope and proximate lipid analysis results are in accordance with these biochemical findings. Time series of digestive gland $\delta^{13}$C in diplods differed from those of triploids until early August (Fig. 5), at which time mass spawning occurred in diploid oysters (Fig. 4). After that event, mid-summer to early-winter changes in the $\delta^{13}$C of digestive gland and mantle tissues of diplods paralleled those of triploids, suggesting that reproduction blurs $\delta^{12}$C signals in spring and early summer. During that period, $\delta^{13}$C values and ratios of $\delta^{13}$C to C:N of gonads were remarkably close to those of digestive gland in diplods, highlighting the major role of the digestive gland in controlling nutrient fluxes to gonads.

Small variations in $\delta^{13}$C time series of gonads and digestive glands were also observed but, as reported for other Crassostrea gigas populations, may be linked to differences between females and males or partial gamete releases prior to a mass spawning event. Significantly higher lipid content in females during the reproductive period has been reported (Deslous-Paoli & Héral 1988). This higher content is mainly linked to higher proportions of neutral lipids (triacylglycerols) in gonads (Li et al. 2000, Matus de la Parra et al. 2005) and digestive glands (Matus de la Parra et al. 2005) of female C. gigas. Partial gamete release before mass spawning has not been clearly established in C. gigas. Alternatively, a second autumnal peak in the reproductive cycle may occur, as reported in Spain (Pazos et al. 1996), or gametes may degenerate and be resorbed without release, as reported in Ireland (Steele & Mulcahy 1999). Similarly, the pattern of reproductive events may be greatly affected by unfavourable
thermic and food conditions, as reported in Marennes-Oléron Bay in 1981 (Deslous-Paoli & Héral 1988). This reinforces the view of a highly flexible potential in the reproductive cycle in C. gigas (Kang et al. 2000) and the importance of food availability to the production of ripe gametes (Ruiz et al. 1992). In this opportunistic species, the autumn–winter period constitutes a stage of sexual resting, and glycogen reserves stored over phytoplankton blooms from spring onwards are simultaneously used for both growth and lipid accumulation for gamete build-up (Deslous-Paoli & Héral 1988, Pazos et al. 1996, Kang et al. 2000, Matus de la Parra et al. 2005). Furthermore, differences in δ¹³C values between gonads and digestive glands, and between mantle and muscle, reflect the phenomenon of isotopic routing (Gannes et al. 1997), with differential allocation of dietary components to different tissues. After mass spawning had occurred, δ¹³C values of digestive glands of diploids closely followed those of triploids (Fig. 5). This may reflect the abrupt breakdown of lipid transfer from digestive glands to gonads for further summer–autumn gametogenic development.

Some gonad tissues may develop in triploids, both in females and males (Beaumont & Fairbrother 1991), because meiosis in triploid oysters is disrupted at first prophase. Thus, during periods favourable for gonad ripening in diploid oysters, gonadal tubules in triploids may contain pre-meiotic cells that subsequently abort. This explanation is consistent with our observation that all triploids used in our analyses were sterile, but may explain some unexpected parallel fluctuations in δ¹³C values of digestive gland and mantle tissues in both diploids and triploids over the beginning of the reproductive period prior to mass spawning (Fig. 5).

Somatic tissue reserves and gametogenic cycle

Our results are consistent with the view that the digestive gland of Crassostrea gigas might be an essential organ that controls nutrient fluxes not only for gametogenic development but also for other maintenance and growth functions (Berthelin et al. 2000, Matus de la Parra et al. 2005). Our results suggest that, in triploids, the digestive gland contributes to energy storage and transfer to other organs: lipids are used for gonad development in diploid oysters but are lost in gamete release, whereas unconverted glycogen is used for enhanced somatic growth in triploids (Beaumont & Fairbrother 1991). Gametogenesis in molluscs has been reported to be sustained by mobilisation of reserves from tissues other than the digestive gland (Gabbott 1983, Mathieu & Lubet 1993). For example, in Pecten maximus, the most important storage tissue is the adductor muscle, with the digestive gland only involved secondarily depending on the season (Lorrain et al. 2002). However, Berthelin et al. (2000) concluded that the muscle of C. gigas did not represent a storage compartment that could supply the energetic resources for reproduction. The dry weight of C. gigas muscle represents less than 15 to 20% of total oyster dry weight and its biochemical composition is largely dominated by proteins, with glycogen and lipid levels always being low (Berthelin et al. 2000). Most recently, Matus de la Parra et al. (2005) performed proximate biochemical and lipid class analyses on labial palps, gonads and digestive glands of C. gigas from Marennes-Oléron Bay. They concluded that labial palps are an organ with glycogen and triacylglycerol reserves, which are transferred to the gonads during the last stage of ripening. However, the weight-to-weight proportion of labial palps vs. soft parts revealed that labial palps represent less than 6% of the whole oyster soft body (Matus de la Parra et al. 2005). In mytilids, such as Mytilus edulis, the mantle tissue is the principal organ of glycogen reserve and the site of gonad development (Gabbott 1983 and references therein, Mathieu & Lubet 1993). However, from both the analyses of Berthelin et al. (2000) and our data set, it appears that mantle tissues comprise reserves with which to fuel the reproductive cycle in C. gigas. Similar correlations obtained in this study between δ¹³C and C:N ratios in gonads, digestive glands and mantle in diploid oysters during their reproductive period might confirm this hypothesis, but large scatter appeared in δ¹³C mantle values (Fig. 8). A more definitive answer to this hypothesis must await further biochemical comparisons between diploid and triploid oyster tissues.

Acknowledgements. We thank S. Bourgeois and J. Seugnet for assistance with sample processing and field work. We are indebted to the technical staff of LERPC at La Tremblade, particularly to F. Blouin, who died tragically during the course of this study. We also thank R. Galois, P. Goulletquer, J. Prou, E. Durieux, P. Richard, P. Riera, and I. Jenkinson for helpful comments. This project was funded by the Region Poitou-Charentes, Ifremer MOREST programme and CNRS. N.M. was supported by a grant from Conseil Général de la Charente-Maritime.

LITERATURE CITED


Editorial responsibility: Matthias Seaman (Assistant Editor-in-Chief), Oldendorf/Luhe, Germany

Whyte JNC, Englar JR, Carswell BL (1990) Biochemical composition and energy reserves in Crassostrea gigas exposed to different levels of nutrition. Aquaculture 90: 157–172

Submitted: October 13, 2006; Accepted: February 27, 2007
Proofs received from author(s): September 3, 2007