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Separate effects of triploidy, parentage and genomic diversity upon feeding behaviour, metabolic efficiency and net energy balance in the Pacific oyster *Crassostrea gigas*

A. J. S. HAWKINS[1]*, A. MAGOULAS[3], M. HÉRAL[2], S. BOUGRIER[2], Y. NACIRI-GRAVEN[4], A. J. DAY[1] AND G. KOTOULAS[3]

[1] *Plymouth Marine Laboratory, Centre for Coastal Marine Sciences, Natural Environment Research Council, West Hoe, Plymouth PL1 3DH, UK*
[2] *Institut Français de Recherche pour l'Exploitation de la Mer, BP 133, Ronce les Bains, 17 390 La Tremblade, France*
[3] *Institute of Marine Biology of Crete, PO Box 2214, 710 03 Iraklion, Crete, Greece*
[4] *Conservatoire et Jardin botaniques, 1 chemin de l'Imperatrice, Case Poswtale 60, CH-1292 Chambesy, Geneva, Switzerland*

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Summary

Triploid oysters were induced using cytochalasin B upon retention of either the first (meiosis I triploids) or the second (meiosis II triploids) polar body in embryos from a single cohort derived from mixed parentage. Allozyme and microsatellite assays enabled the confirmation of both parentage and triploidy status in each oyster. Comparison of meiosis I triploids, meiosis II triploids and diploid siblings established that improved physiological performance in triploids was associated with increased allelic variation, rather than with the quantitative dosage effects of ploidy status. An unidentified maternal influence also interacted with genotype. Among full sibs, allelic variation measured as multi-locus enzyme heterozygosity accounted for up to 42% of the variance in physiological performance; significant positive influences were identified upon feeding rate, absorption efficiency, net energy balance and growth efficiency (= net energy balance ÷ energy absorbed). Whilst allelic variation was greater in both meiosis I and meiosis II triploids than in diploid siblings, both allelic variation and net energy balance were highest in triploids induced at meiosis I. This suggests that it may be preferable to induce triploidy by blocking meiosis I, rather than meiosis II as has traditionally been undertaken during commercial breeding programmes.

1. Introduction

Triploidy may be induced in shellfish by applying a thermal, pressure or chemical shock to ensure retention of either the first or second polar bodies during early divisions of the egg, with the result that each cell nucleus contains one additional set of unaltered chromosomes (Beaumont & Zouros, 1991). Different complementary hypotheses have been put forward to explain the faster growth that has frequently been observed in triploid versus diploid organisms. One explanation has been that triploids are sterile (e.g. Stanley *et al.*, 1981; Tabarini, 1984; Allen *et al.*, 1986, 1994), faster growth resulting because a relatively smaller proportion of metabolic flux is directed towards reproductive output (Allen & Downing, 1990; Allen *et al.*, 1994). Another explanation suggests an increased volume of polyploid cells that is not compensated by reduced cell number (Guo & Allen, 1994). It has also been suggested that faster growth may result from progressive haploidization, as a result of which the probability that chromosome loss will expose a recessive deleterious mutation, thereby depriving a cell completely of the activity of a gene, is much smaller in a triploid than a diploid cell (Zouros *et al.*, 1996). Of greater relevance to the present paper, it has been suggested that faster growth may result from increased allelic diversity known as heterozygosity (e.g. Mitton & Grant, 1984; Zouros & Foltz, 1987; Hedgecock *et al.*, 1996), which

* Corresponding author. Tel: +44 (0)1752 633100. Fax: +44 (0) 1752 633101. e-mail: ajsh@ccms.ac.uk

is expected to be higher in triploids as the probability of allelic variation increases in an individual possessing three rather than two copies of the same locus (Stanley *et al.*, 1984; Yamamoto *et al.*, 1988; Beaumont & Kelly, 1989). This, at least, has been confirmed among meiosis I triploids in the European oyster *Ostrea edulis*, for which both multi-locus enzyme heterozygosity and body growth were more than 49% greater than among either diploid siblings or meiosis II triploids (Hawkins *et al.*, 1994). Alternatively, rather than from any benefits of allelic diversity, it has been suggested that triploids may grow faster through having the same gene product in triple dose, compared with only a double dose in diploids (Zouros *et al.*, 1996).

Pacific oysters (*Crassostrea gigas*) are among the most widely cultured of all shellfish species. To help meet demand, advantages of increased sterility are currently being exploited during commercial growing of triploid *C. gigas* induced by the blocking of meiosis II (Allen *et al.*, 1989). The present paper describes work that was undertaken to help identify any further advantages that may result from inducing triploidy at meiosis I rather than meiosis II in *C. gigas*, and to better establish both the physiological mechanism and the genetic basis to anticipated increases in the rate of growth measured as net energy balance. We have applied several novel techniques. Firstly, epifluorescence analyses of early embryonic development allowed us to target meiosis I or meiosis II during the induction of triploidy (Gérard *et al.*, 1999). Secondly, image analysis of cells that had been biopsied from each individual oyster enabled the identification and selection of oysters within different ploidy classes before experimental comparisons of physiological performance (Gérard *et al.*, 1994). Thirdly, assays of polymorphism for both allozyme loci and microsatellite loci (Magoulas *et al.*, 1998) allowed the unambiguous determination of parentage for almost all experimental oysters, including the independent confirmation of ploidy status. This, in turn, allowed more accurate allozyme scoring of triploids than has been possible in many previous studies without microsatellite scoring. In associated papers, we compare genetic polymorphism and parentage in different ploidy classes (Magoulas *et al.*, 2000), and document effects of triploidy upon seasonal growth in contrasting natural environments (Naciri-Graven *et al.*, 2000). Here, we describe the influences of triploidy, parentage and genotype upon feeding behaviour, net energy balance and metabolic efficiency under standardized laboratory conditions. Our findings confirm that physiological benefits associated with greater genomic diversity result from inducing triploidy at meiosis I rather than meiosis II, whilst offering some fundamental insights into the genetic and physiological basis of those advantages.

2. Materials and methods

(i) *Overall programme*

Crassostrea gigas from a single mass-spawning were treated with cytochalasin B (CB) or 6-dimethylaminopurine (6-DMAP) to induce triploidy during April 1994 (Magoulas *et al.*, 2000; Naciri-Graven *et al.*, 2000). In the present paper, we compare ploidy classes among oysters that had been treated with CB alone. This is because survival was significantly lower among oysters treated with 6-DMAP (A. Gérard, unpublished data). In any case, 6-DMAP was only used to induce meiosis II triploids, whereas the main conclusions of the present paper result from the higher genetic polymorphism in meiosis I than in meiosis II triploids (see Section 3).

Once oysters treated to induce triploidy had grown to about 1 cm shell length, they were deployed for ongrowing within the natural environment at Bouin in the Bay of Marennes-Oléron. In August 1994, a random subsample of oysters were returned to the hatchery at La Tremblade, where they were maintained in natural seawater pumped from the Bay of Marennes-Oléron ($14{\cdot}0 \pm 1{\cdot}0$ °C; $33{\cdot}6 \pm 0{\cdot}2$‰), but which was supplemented every day with a different mixture that included three of the six algal species that were routinely cultured in the hatchery (*Isochrysis galbana* Parke and 'Tahiti clone', *Tetraselmis suecica* (Kylin Butcher), *Pavlova lutheri* (Droop) Green, *Skeletonema costatum* (Greville) Cleve and *Chaetoceros calcitrans* (Paulsen) Takano). Over the next 3 months, 1200 oysters were biopsied to provide more than 20 individuals within each of the four following classes:

1. diploids not treated with CB;
2. diploids refractory to treatment with CB upon extrusion of the second polar body;
3. triploids induced upon retention of the first polar body (meiosis I triploids); and
4. triploids induced upon retention of the second polar body (meiosis II triploids).

At least 14 days after biopsy, each experimental oyster was transferred to separate raceways containing natural seawater under unchanging conditions of temperature and salinity, and which was continuously supplemented with *Skeletonema costatum* (Greville) Cleve alone at an average (± 2 SE) concentration of 14555 ± 4806 cells ml^{-1}. Following 10–15 h acclimation within those raceways, we measured rates of oxygen uptake, net ammonia loss, feeding and absorption.

Oysters were later dissected on ice, determining the wet weight of gills, digestive gland and all remaining tissues. Subsamples of digestive gland and adductor muscle were air-freighted on dry ice to the IMBC laboratory in Heraklion for analyses of allozyme and

microsatellite variation, together with confirmation of ploidy status and the identification of families. Remaining tissues were weighed after drying to constant weight at 80 °C, and ratios of wet soft tissue weight to dry soft tissue weight used to compute the total soft tissue dry weight for each oyster, which averaged (± 2 SE, $n = 97$) $0{\cdot}42 \pm 0{\cdot}04$ g for all experimental animals. Physiological rates were weight-corrected for a standard oyster of 1·0 g soft dry tissue. Finally, weight-standardized physiological rates were used to compare physiological traits according to ploidy class, parentage and/or genomic diversity.

(ii) *Breeding, triploid induction and laboratory rearing*

Sperm and oocytes were stripped from the gonads of 3 male and 7 female *Crassostrea gigas* from the Bay of Marennes-Oléron. Following fertilization, embryos were treated with CB at 25 °C (Allen *et al*., 1986; Downing & Allen, 1987) to induce either meiosis I or meiosis II triploids according to the timing of chemical treatment as determined using epifluorescence analyses to monitor early development as described by Gérard *et al*. (1999).

Larvae were reared at a density of about 50 larvae ml^{-1} for 24 h in 30 or 150 l tanks at 24 °C. The D-shaped larvae were then collected, counted and diluted to 5 larvae ml^{-1} in filtered (2 μm) seawater. Seawater was changed every other day, and larvae fed a mixed diet of *Isochrysis galbana* Parke, Isochrysis 'Tahiti clone', *Pavlova lutheri* (Droop) Green and *Chaetoceros calcitrans* (*forma pumilum*) at 20 cells μl^{-1} per day for each algal species. Upon first larval metamorphosis, each treatment was sieved daily over up to 1 week, transferring metamorphosed larvae onto 150 μm sieves for ongrowing in a system of flowing natural unfiltered seawater. Although the great majority of living larvae metamorphosed within that week, some did not, such that any genotypes that resulted in especially slow development will have been lost. Oyster shell clutch was used for settlement of the larvae. During subsequent growth before deployment for ongrowing in the natural environment, seawater was enriched every day with a varying mixture of three algal species according to standard hatchery practice described above in our outline of the overall programme.

(iii) *Biopsy of ploidy status using image analysis*

Oysters were anaesthetized for biopsy using 50 g magnesium chloride l^{-1} filtered (2 μm) seawater. When shells gaped sufficiently, cells were scraped from the gill and spread on a glass slide. Slides were air-dried and fixed with a Bohm Sprenger solution for 10 min, and consecutively stained following the standard Feulgen-Rosalin method. Ploidy levels were determined using image analysis and the Samba 2005 computer program developed by Alcatel TITN ANSWARE. This program analyses the photometric intensity of stained nuclei and computes their individual integrated optical density which, when compared with a diploid control, gives their ploidy level according to Gérard *et al*. (1994).

(iv) *Allozyme and microsatellite analyses*

Oysters were genetically scored at eight polymorphic enzyme loci and three polymorphic microsatellite loci (*Cg44*, *Cg49* and *Cg108*) according to Magoulas *et al*. (1998, 2000). Enzyme loci included leucine aminopeptidase (*Lap*, EC 3.4.11.1, monomeric); phosphoglucomutase (*Pgm*, EC 2.7.5.1, monomeric); aspartate aminotransferase-1 and -2 (*Aat-1* and *Aat-2*, EC 2.6.1.1, both dimeric); phosphoglucose isomerase-1 and -2 (*Pgi-1* and *Pgi-2*, EC 5.3.1.9, dimeric and monomeric, respectively); isocitrate dehydrogenase-2 (*Idh*, EC 1.1.1.42, dimeric); and enolase (*Enol*, EC 4.2.1.11, dimeric).

(v) *Determinations of ploidy, parentage, heterozygosity and allelic diversity*

Magoulas *et al*. (2000) describe how we established different parental contributions within each ploidy class, and were able to use allozyme and microsatellite variations to confirm the ploidy status that we had determined from results of prior biopsy and image analysis in all but three of our experimental oysters. These three oysters were excluded from all data analyses.

Two different indices were used to characterize the genomic diversity of individual oysters. The first index was multi-locus heterozygosity, which represents the proportion of studied polymorphic loci for which that individual was heterozygous. We refer to the second index as total allelic diversity, which distinguished between triploids that possessed either two or three alleles at each locus. To calculate total allelic diversity for each individual oyster, each locus was assigned a score of 0, 1 or 2 if one, two or three alleles were present, respectively, and the scores of all loci summed.

(vi) *Physiological measures*

All physiological measures were undertaken whilst oysters were being fed under the same environmental conditions of laboratory acclimation as described above. To measure oxygen consumption (μmol h^{-1}) and ammonia loss (μmol h^{-1}), oysters were placed in

70 ml screw-top polycarbonate pots completely filled with unfiltered seawater. Duplicate water samples were collected at the beginning and end of 0·5 h incubations, before being analysed for oxygen and ammonium contents according to Winkler (1888) and Koroleff (1976), respectively. Changes were corrected using associated determinations within control pots without oysters. Following overnight recovery from measures of oxygen consumption and ammonia loss, processes of feeding and absorption were determined for oysters placed separately within raceways that were supplied with seawater at rates of 150 to 200 ml min^{-1}. These rates were sufficient to ensure that volumes of suspended particles determined using a Coulter Multisizer were not reduced by more than an average of 15% between the inflow and outflow from each oyster tray. After about 12 h, samples of suspended particles from the outflow of empty 'control' raceways, as well as all true faeces and all pseudofaeces from each individual oyster, were each collected separately. Samples of both seawater and faeces were filtered separately onto pre-weighed and ashed Whatman GFC filters. All filters were dried to constant weight at 60 °C before re-weighing and calculation of the total dry sample weight per filter. Each filter was then ashed at 450 °C for 4 h prior to final weighing, allowing further calculation of both the ash (inorganic) and ash-free (organic) masses of each filtered sample.

Food availability was measured as the total dry particulate mass of suspended particles (TPM; mg l^{-1}), and food quality as the organic content of that total dry particulate mass (OSC; fraction), assuming that concentrations measured in the outflow from the empty 'control' tray were representative of the available particulates. To account for particle retention efficiencies of less than 100% in *Crassostrea gigas* (Barillé *et al*., 1993), the organic content of filtered matter was calculated daily by calculating the organic mass within natural seston and cultured alga from average volumes that were measured daily for each using a Coulter Multisizer. Working with average (± 2 SE) measured organic fractions of $0{\cdot}29 \pm 0{\cdot}03$ and $0{\cdot}87 \pm 0{\cdot}05$ for natural seston and algae, respectively, it was assumed that (i) all particles larger than 6 μm diameter were cultured alga, (ii) densities were 2·6 mg mm^{-3} for silt and 1 mg mm^{-3} for algae, and (iii) particles smaller than 4 μm average particle diameter were retained with a reduced efficiency of 30% (L. Barillé, personal communication). The energy content of filtered organics could then be calculated assuming the energy content of algal organics to be 23 J mg^{-1} and the energy content of silt organics to be 7·25 J mg^{-1} as derived from the proximate biochemical composition of natural seston within the bay of Marennes-Oléron by Héral *et al*. (1983).

Processes of feeding and absorption were calculated according to Hawkins *et al*. (1998) as follows:

1. filtration rate (FR; total mg h^{-1}) = (mg inorganic matter egested both as true faeces and pseudofaeces h^{-1}) × (1 ÷ inorganic fraction in filtered matter);
2. rejection rate (RR; total mg h^{-1}) = mg total pseudofaeces egested h^{-1};
3. net organic selection efficiency (NOSE; fraction) = 1 − (organic fraction within pseudofaeces ÷ organic fraction within filtered matter);
4. net organic ingestion rate (NOIR; mg h^{-1}) = (FR × organic fraction within filtered matter) − (RR × organic fraction within pseudofaeces);
5. net organic absorption rate (NOAR; mg h^{-1}) = NOIR − (mg total true faeces egested h^{-1} × organic fraction within true faeces); and
6. net absorption efficiency from ingested organics (NAEIO; fraction) = OAR ÷ OIR.

Note that TPM was estimated as the integrated average over corresponding periods of faecal collection, assuming no significant delay in the deposition of filtered particles. This was justified given the stable feeding regime, together with previous measures establishing that gut transit occurs within 97 ± 12 min in *Crassostrea gigas* (Soletchnick *et al*., 1996). Note also that NOSE, NOIR, NOAR and NAEIO are net measures; NOSE and NOIR are influenced by mucous losses from the animal as pseudofaeces, whereas NOAR and NAEIO are influenced both by mucous losses as pseudofaeces and by metabolic faecal losses, which comprise endogenous materials lost from the animal following secretion, exocytosis and/or abrasion in the gut.

Net energy balance (NEB; J h^{-1}) was calculated according to Bayne *et al*. (1985), using energy contents that were calculated for the organic matter that was filtered each day as described above, and net production efficiency (%) computed as (NEB ÷ J absorbed h^{-1}) × 100.

All physiological rates were weight-corrected to those for an oyster of standard weight as follows: $Y_s = Y_e \times (W_s \div W_e)^b$, where Y_s is the standardized parameter, Y_e is the uncorrected parameter, W_s is the standard weight of 1 g dry soft tissue, W_e is the dry soft tissue weight of the experimental oyster, and b is the size exponent of 0·67 for feeding processes, 0·75 for oxygen consumption and 0·78 for ammonia loss (Hawkins & Bayne, 1992).

(vii) *Statistical analyses*

Analyses of variance, two-sample *t*-tests, least-squares regression analyses and Pearson product moment correlations were undertaken using SYSTAT for Windows, version 7 (SYSTAT, Evanston, IL). Homo-

scedasticity of the variance was confirmed for each dependent variable using F_{max} values. SYSTAT's General Linear Model was used to adjust for unbalanced factorial designs during analyses of variance when, to test each hypothesis, a series of hierarchical tests were undertaken, beginning with the interactions. If the highest-order interactions were insignificant, they were dropped, and the model recomputed with lower-order interactions. Only if the lower-order interactions were also insignificant was the model computed with main effects alone. In all pairwise comparisons between means, the Bonferroni adjustment was used as protection for multiple testing, dividing the usual probability by the number of tests to establish an adjusted significance level.

3. Results

(i) *Net energy balance*

(a) *Effects of treatment with cytochalasin B*

With net energy balance as the dependent variable, factorial analysis of variance indicated that there were no coincident effects of (i) treatment with CB (two levels: untreated diploids and refractory oysters that had remained diploid following treatment with CB at meiosis II), (ii) male parentage (three levels; each for a different male) or (iii) female parentage (seven levels; each for a different female). The largest *F*-ratio was that for treatment with CB, but this was also non-significant (*F*-ratio = 2·6; d.f. = 1, 18; $P = 0{\cdot}13$). This establishes that there were no long-term consequences of treatment with CB, other than were associated with the induction of triploidy (see below). Therefore, untreated and refractory diploids were pooled in tests to determine any effects of ploidy status.

(b) *Effects of ploidy status among the offspring from all families*

Factorial analysis of variance was performed to test the coincident effects of (i) ploidy status (three levels: diploids, meiosis I triploids and meiosis II triploids), (ii) male parentage (three levels; each for a different male) and (iii) female parentage (seven levels; each for a different female). This did not identify any influence of parentage ($P > 0{\cdot}05$), but established separate significant effects of ploidy status upon multi-locus enzyme heterozygosity (*F*-ratio = 13·7; d.f. = 2, 62; $P < 0{\cdot}001$), total allelic microsatellite diversity (*F*-ratio = 64·4; d.f. = 2, 62; $P < 0{\cdot}001$) and net energy balance (*F*-ratio = 5·0; d.f. = 2, 44; $P = 0{\cdot}01$).

Post-hoc Bonferroni-adjusted pairwise comparisons indicated that multi-locus enzyme heterozygosity and total allelic microsatellite diversity among the offspring from all families were each significantly higher

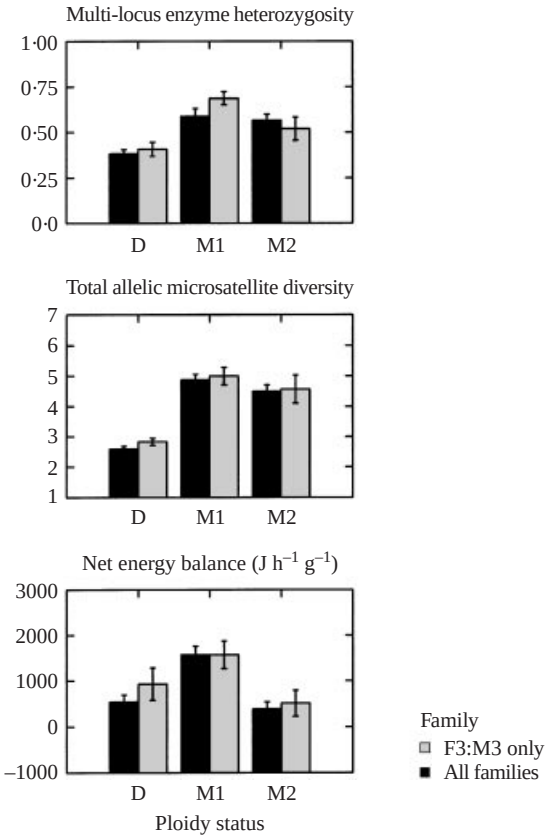

Fig. 1. Comparisons of multi-locus enzyme heterozygosity, total allelic microsatellite diversity and net energy balance (NEB; J d^{-1} g^{-1} dry soft tissue weight) between oysters from each ploidy class (D, diploids; M1, meiosis I triploids; M2, meiosis II triploids). Bars depict the average ± 1 SE, both for the combined offspring from all families (black columns) and for the F3:M3 family alone (grey columns).

in meiosis I triploids and meiosis II triploids than in diploids ($P < 0{\cdot}001$), with no significant difference between meiosis I triploids and meiosis II triploids ($P > 0{\cdot}05$) (Fig. 1, Table 1). In addition, net energy balance among the offspring from all families was higher in meiosis I triploids than in diploids ($P = 0{\cdot}02$) or meiosis II triploids ($P = 0{\cdot}03$), with no significant difference between diploids and meiosis II triploids ($P > 0{\cdot}05$) (Fig. 1, Table 1).

(c) *Single-locus effects among the offspring from all families*

Among the *Crassostrea gigas* used for experiments described here, the mean number of alleles per allozyme locus for all eight allozyme loci was 3·5 and per microsatellite locus for all three microsatellite loci was 13·3 (Magoulas *et al.*, 2000). Table 2 summarizes the results of factorial analyses of variance to establish influences of polymorphism at single enzyme or microsatellite loci on net energy balance as the

Table 1. *Genomic and physiological traits within each ploidy class, both among the combined offspring from all families, and for the F3 : M3 family alone*

	Combined offspring from all families (means ± 2 SE)			Offspring from F3 : M3 family alone (means ± 2 SE)		
Trait	D	M1	M2	D	M1	M2
Multi-locus enzyme heterozygosity	0·38 ± 0·05 $n = 36$	0·59 ± 0·07 $n = 18$ ***	0·57 ± 0·07 $n = 20$ ***	0·41 ± 0·06 $n = 14$	0·69 ± 0·09 $n = 8$ ***	0·52 ± 0·09 $n = 7$ NS
Total allelic microsatellite diversity	2·59 ± 0·96 $n = 32$	4·87 ± 1·43 $n = 16$ ***	4·50 ± 1·67 $n = 18$ ***	2·83 ± 0·43 $n = 12$	5·00 ± 0·52 $n = 8$ ***	4·57 ± 0·56 $n = 7$ ***
Filtration rate (mg h^{-1} g^{-1})	8·5 ± 1·9 $n = 33$	22·2 ± 2·6 $n = 18$ ***	12·3 ± 2·7 $n = 16$ *	9·4 ± 3·6 $n = 12$	22·4 ± 4·5 $n = 8$ ***	13·1 ± 5·2 $n = 6$ NS
Net organic selection efficiency (%)	23·8 ± 7·4 $n = 22$	36·9 ± 8·2 $n = 18$ NS	16·9 ± 12·3 $n = 8$ NS	37·7 ± 12·6 $n = 7$	35·0 ± 11·7 $n = 8$ NS	17·3 ± 19·2 $n = 3$ NS
Net absorption efficiency from ingested organics (%)	36·6 ± 6·8 $n = 30$	58·2 ± 8·9 $n = 18$ NS	36·8 ± 10·5 $n = 13$ NS	42·9 ± 11·6 $n = 10$	60·5 ± 13·0 $n = 8$ NS	44·6 ± 16·4 $n = 5$ NS
Net energy balance (J d^{-1} g^{-1})	537·7 ± 288·5 $n = 26$	1569·1 ± 346·7 $n = 18$ *	384·9 ± 424·7 $n = 12$ NS	928·3 ± 585·8 $n = 7$	1570·0 ± 547·9 $n = 8$ NS	508·3 ± 693 $n = 5$ NS
Net production efficiency (%)	22·7 ± 24·5 $n = 26$	72·0 ± 29·5 $n = 18$ NS	37·8 ± 36·0 $n = 12$ NS	33·6 ± 49·1 $n = 7$	77·7 ± 45·8 $n = 8$ NS	43·8 ± 58·1 $n = 5$ NS

Pairwise comparisons were each with diploids, for which probability values (P) are denoted as: NS, not significant for $P > 0{\cdot}05$; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; and *** $P < 0{\cdot}001$.
Among the combined offspring from all families, pairwise comparisons were undertaken following factorial analyses to assess the effects of ploidy class (three levels; D = diploids, M1 = meiosis I triploids and M2 = meiosis II triploids), male parentage (three levels, each for a different male) and female parentage (seven levels, each for a different female) upon each measured trait. Within the F3 : M3 family, similar pairwise comparisons were undertaken following one-way analyses of variance to test the effects of ploidy alone. All comparisons are Bonferonni-adjusted, and all data corrected for a standard-sized oyster of 1 g dry soft tissue weight.

dependent variable, testing the coincident effects of (i) heterozygosity (two levels: homozygosity and heterozygosity) or allelic diversity (three levels: one allele, two alleles and three alleles), (ii) male parentage (three levels; each for a different male) and (iii) female parentage (seven levels; each for a different female). Among the eight polymorphic enzyme loci that we studied here, significant effects of heterozygosity were evident only at the locus coding for *Aat-1*. Post-hoc Bonferroni-adjusted pairwise comparisons indicated that individual oysters heterozygous for *Aat-1* had a higher average (± 2 SE) net energy balance than individual oysters that were homozygous for *Aat-1* (1136 ± 286 versus 319 ± 146 J d^{-1} g^{-1}, respectively) ($P = 0{\cdot}03$). A similar effect was almost significant for *Aat-2* ($P = 0{\cdot}06$). There was no significant effect of heterozygosity at microsatellite locus *Cg44* (Table 1), which was the only one of our three microsatellite loci coding both for homozygous or heterozygous individuals (Magoulas *et al.*, 2000). No consequences of allelic diversity were evident either for any of the three microsatellite loci or the enzyme locus coding for *Pgm* ($P > 0{\cdot}05$), which was the only one of our enzyme loci for which we found individual oysters with more than two alleles (Magoulas *et al.*, 2000).

(d) *Multi-locus effects among the offspring from all families*

With net energy balance as the dependent variable, factorial analysis of variance indicated that there were no coincident influences of either (i) total allelic diversity calculated for all three microsatellite loci (five levels, designating scores between 2 and 6), (ii) male parentage (three levels, each for a different male) or (iii) female parentage (seven levels, each for a different female) ($P > 0{\cdot}05$). In addition, there were no significant effects of these same variables when total allelic diversity was summed over all microsatellite loci and the enzyme locus for *Pgm* ($P > 0{\cdot}05$). Alternatively, there was a strong interactive influence on net energy balance between (i) multi-locus heterozygosity calculated for all eight enzyme loci (12 levels, each for a different value of heterozygosity) and (ii) female parentage (seven levels, each for a different female) (F-ratio = 4·0; d.f. = 6, 48; $P = 0{\cdot}003$). This

Table 2. *Results of factorial analyses of variance to establish the effects of polymorphism at single enzyme or microsatellite loci on net energy balance as the dependent variable, testing the coincident effects of* (*i*) *heterozygosity* (*two levels: homozygosity and heterozygosity*), (*ii*) *male parentage* (*three levels, each for a different male*) *and* (*iii*) *female parentage* (*seven levels, each for a different female*)

	Independent variables	
Loci	Heterozygosity	Female parentage
Enzyme		
Lap	NS	$F = 3{\cdot}0$; d.f. = 6, 45; $P = 0{\cdot}04$
Pgm	NS	($F = 2{\cdot}3$; d.f. = 6, 44; $P = 0{\cdot}09$)
Aat-1	$F = 4{\cdot}7$; d.f. = 1, 45; $P = 0{\cdot}03$	NS
Aat-2	($F = 3{\cdot}7$; d.f. = 1, 45; $P = 0{\cdot}06$)	$F = 3{\cdot}2$; d.f. = 6, 45; $P = 0{\cdot}02$
Pgi-1	NS	$F = 2{\cdot}9$; d.f. = 6, 44; $P = 0{\cdot}05$
Pgi-2	NS	($F = 2{\cdot}5$; d.f. = 6, 44; $P = 0{\cdot}10$)
Idh-2	NS	$F = 2{\cdot}9$; d.f. = 6, 44; $P = 0{\cdot}04$
Enol	NS	NS
Microsatellite		
CG44	NS	($F = 2{\cdot}2$; d.f. = 6, 44; $P = 0{\cdot}10$)
	Allelic diversity	Female parentage
Enzyme		
Pgm	NS	NS
Microsatellite		
CG44	NS	NS
CG49	NS	NS
CG108	NS	$F = 2{\cdot}8$; d.f. = 6, 43; $P = 0{\cdot}04$

Each physiological trait was corrected for a standard-sized oyster of 1 g dry soft tissue weight.
F-ratios represent explained mean squares/unexplained mean squares. Probability values (*P*) are Bonferroni-corrected for the associated number of tests, where NS = not significant for $P > 0{\cdot}05$. For interest, results showing that effects were almost significant ($P < 0{\cdot}10$) are given in parentheses. There were no significant interactive effects between heterozygosity, female parentage and/or male parentage, and no significant effects of male parentage alone.

effect was not significantly improved if multi-locus heterozygosity was computed over all enzyme loci and the microsatellite locus *CG44* (*F*-ratio = 3·5; d.f. = 6, 48; $P = 0{\cdot}006$).

(e) *Effects of parentage*

In addition to the interactive influence of multi-locus enzyme heterozygosity and female parentage, many of our factorial analyses of the effects of heterozygosity or allelic diversity at individual loci indicated that net energy balance differed according to female parentage (Table 2). Following those factorial analyses, Bonferroni-adjusted pairwise comparisons consistently indicated that net energy balances in the offspring from parents F2 and/or F3 were greater than in the offspring from parent F5 ($P < 0{\cdot}05$).

The largest family used for physiological determinations described here was comprised of 20 sibling offspring from the F3 female and the M3 male parents (the F3 : M3 family). Within this F3 : M3 family, one-way analysis of variance (*F*-ratio = 11·6; d.f. = 2, 26; $P < 0{\cdot}001$) established that multi-locus enzyme heterozygosity was significantly greater among meiosis I triploids than among diploids ($P < 0{\cdot}001$), with no difference between either meiosis II triploids and diploids ($P > 0{\cdot}05$) or meiosis I and meiosis II triploids ($P > 0{\cdot}05$) (Table 1, Fig. 1). Alternatively, one-way analysis of variance (*F*-ratio = 23·7; d.f. = 2, 24; $P < 0{\cdot}001$) established that total allelic microsatellite diversities were similar between meiosis I and meiosis II triploids ($P > 0{\cdot}05$), and greater among both meiosis I triploids and meiosis II triploids than among diploids ($P < 0{\cdot}001$) (Table 1, Fig. 1).

Within the F3 : M3 family, with net energy balance as the dependent variable, factorial analysis of variance identified significant effects of multi-locus enzyme heterozygosity (12 levels, each for a different value of heterozygosity) (*F*-ratio = 5·0; d.f. = 1, 16; $P = 0{\cdot}04$), but no coincident effect of ploidy status (three levels : diploid, meiosis I triploids and meiosis II triploids) ($P > 0{\cdot}05$). Further, compared with the

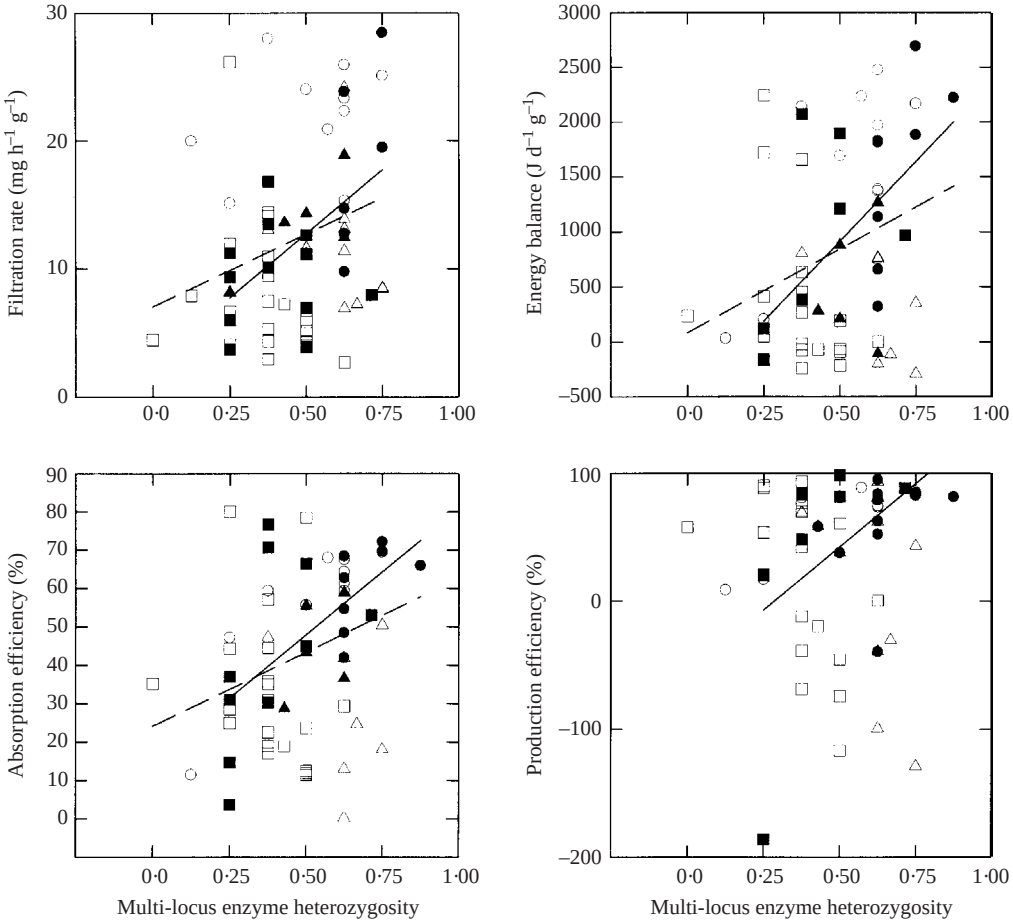

Fig. 2. Relations between filtration rate (mg h^{-1} g^{-1} dry soft tissue weight), absorption efficiency (%), net energy balance (J d^{-1} g^{-1} dry soft tissue weight), net production efficiency (%) and multi-locus enzyme heterozygosity. Filled symbols represent oysters from the M3:F3 family alone; open symbols represent the combined oysters from all remaining families. Both the M3:F3 family and all other oysters are illustrated according to ploidy status, where meiosis I triploids are designated by circles, meiosis II triploids by triangles and diploids by squares. Lines were fitted by least squares, each depicting a significant relation ($P < 0{\cdot}05$), dashed lines to data for all experimental oysters and continuous lines to data for the F3:M3 family alone (refer to Section 3 for correlation coefficients and significance levels).

combined offspring from all families, multi-locus enzyme heterozygosity within the F3:M3 family alone was statistically associated with more than 3 times the variance that was observed between oysters for net energy balance ($r^2 = 0{\cdot}10$ versus $r^2 = 0{\cdot}31$) (Fig. 2). Therefore, among full sibs, net energy balance varied according to multi-locus enzyme heterozygosity rather than ploidy class *per se*.

There was no relation between net energy balance and total allelic diversity for our three microsatellites within the F3:M3 family ($P > 0{\cdot}05$).

(ii) *Physiological traits*

Among the offspring from all families, there were positive correlations between multi-locus enzyme heterozygosity and physiological traits that included filtration rate ($r^2 = 0{\cdot}13$; $n = 67$; $p = 0{\cdot}002$), absorption efficiency from ingested organics ($r^2 = 0{\cdot}11$; $n = 61$; $p = 0{\cdot}01$) and net energy balance ($r^2 = 0{\cdot}10$; $n = 56$; $p = 0{\cdot}02$). As illustrated for net energy balance in Fig. 2, these same relations were clearer in the F3:M3 family alone. Compared with the combined offspring from all families, multi-locus enzyme heterozygosity in the F3:M3 family alone was statistically associated with more than 3 times the variances that were observed between oysters for filtration rate ($r^2 = 0{\cdot}42$, $n = 26$, $P < 0{\cdot}001$), absorption efficiency from ingested organics ($r^2 = 0{\cdot}37$, $n = 23$, $p = 0{\cdot}002$) and net energy balance ($r^2 = 0{\cdot}31$, $n = 20$, $P = 0{\cdot}01$, respectively). Within that F3:M3 family alone, there was an additional positive relation between multi-locus enzyme heterozygosity and net production efficiency ($r^2 = 0{\cdot}25$, $n = 20$, $P = 0{\cdot}02$) (Fig. 2). Therefore, the physiological basis of higher net energy balance in full-sib oysters with greater multi-locus enzyme heterozygosity included faster rates of nutrient acquisition, resulting both from faster feeding and more efficient absorption, together with enhanced metabolic efficiencies.

4. Discussion

To our knowledge, this is the first time in any animal that microsatellites have been used to check ploidy status or to establish parentage for the comparison of relative performance. In addition, by comparing microsatellite variation with differential allozyme staining, we have achieved very much more reliable scoring of allozyme variation than has previously been possible among triploids (Magoulas *et al*., 2000).

Our findings establish that a significant maternal influence interacts with genotype to affect physiological performance. Among the combined offspring from all families, different average net energy balances between ploidy classes resulted in part from varying parental contributions to the relative number of offspring within each class. When parentage was standardized by comparing full-sib offspring within our largest family (F3 : M3), there were no effects of ploidy *per se*. Rather, up to 42 % of the variance observed for each physiological trait among oysters was positively associated with multi-locus enzyme heterozygosity. These percentages were more than 3 times greater than for the same physiological traits among the combined offspring from all families, and higher than maximal values of about 25 % among the mixed offspring in all previous studies from other species (for reviews, see Mitton & Grant, 1984; Zouros & Foltz, 1987; Hedgecock *et al*., 1996). Such clarification of relations within the F3 : M3 family alone clearly illustrates the value of resolving parentage and other background genetic effects when studying fundamental genetic influences upon phenotype.

More importantly, by standardizing parentage, we have established that net energy balance and component physiological traits were affected by allelic variation, rather than by the quantitative effects of ploidy status which supplied cells with either double or triple doses of the same gene product. This significant finding is consistent with related work in the European flat oyster, *Ostrea edulis*, in which cumulative lifetime growth was compared directly between juvenile (not sexually mature) siblings of different ploidy status (Hawkins *et al*., 1994). Both in *O. edulis* and in the present *Crassostrea gigas*, multi-locus enzyme heterozygosity and net energy balance or growth were each greater among meiosis I triploids than in either meiosis II triploids or diploids (Hawkins *et al*., 1994) (Table 1). These observations are consistent with the finding that only moderate chiasma interference occurred in these same oysters (Magoulas *et al*., 2000). Therefore, associated recombination was not high enough to compromise the expectation that among meiosis I triploids, addition of heterozygosity from the female chromosome set with that from the haploid male chromosome set will have enhanced allelic variation above that both in diploids and in meiosis II triploids (Allen *et al*., 1982; Stanley *et al*., 1984; *Mason et al*., 1988). The above findings do not contradict evidence that faster triploid growth may also result from larger cell size or reduced reproductive output (see Section 1). They do, however, indicate that at least part of the higher net energy balance in meiosis I triploids results from metabolic and physiological consequences associated with an increase in allelic variation. Similar interrelations underlie the advantages of 'hybrid vigour' or 'heterosis' (Hawkins & Day, 1996, 1999).

The commercial induction of triploidy has traditionally been undertaken by blocking meiosis II (see Section 1), it being more difficult to block meiosis I (Gérard *et al*., 1999). Nevertheless, our collective findings indicate that greater benefits stem from blocking meiosis I. In *O. edulis* of mixed parentage, average multi-locus enzyme heterozygosity in meiosis I triploids was 49 % greater ($P < 0{\cdot}001$) than in diploid siblings, with no difference between diploids and meiosis II triploids ($P < 0{\cdot}05$) (Hawkins *et al*., 1994). In the present F3 : M3 family of *C. gigas*, average multi-locus enzyme heterozygosity was 68 % higher in meiosis I triploids than in diploid siblings ($P < 0{\cdot}05$), with a small but non-significant increment among meiosis II triploids (27 %; $P > 0{\cdot}05$) (Table 1). These findings may help to explain why faster growth of meiosis II triploids has generally only been observed among mature shellfish (e.g. Tabarini, 1984; as discussed by Beaumont & Zouros, 1991; but see Naciri-Graven *et al*., 2000), resulting primarily from lower reproductive output, rather than from increased heterozygosity.

Physiological consequences of greater multi-locus enzyme heterozygosity within our largest family included faster rates of nutrient acquisition, resulting both from faster feeding and more efficient absorption, together with higher net production efficiencies. Whilst findings based upon only one full-sib family should best be treated with caution, faster feeding has also been associated with greater multi-locus enzyme heterozygosity and growth in other species of filter-feeding shellfish (Garton, 1984; Garton *et al*., 1984; Holley & Foltz, 1987; Hawkins *et al*., 1989). It is not known whether the costs associated with faster feeding are afforded by increased energy availability that results from higher metabolic efficiency in more heterozygous individuals (see below), or whether faster feeding results from separate, as yet unidentified, effects of genomic diversity. Similarly, it is not known whether the additional positive relation observed here between multi-locus enzyme heterozygosity and the net absorption efficiency from ingested organics stemmed from any direct influence of heterozygosity on absorption efficiency or from functional interrelations whereby absorption efficiency may increase

with filtration rate and/or the organic content of ingested matter according to processes of particle selection (cf. Hawkins *et al.*, 1998). We are not aware of any previous study reporting an association between absorption efficiency and genomic diversity.

Alternatively, the metabolic basis of higher growth and production (= growth plus reproductive output) efficiencies among filter-feeding bivalve shellfish with greater multi-locus enzyme heterozygosity is known to include reduced energy requirements that result at least in part from slower intensities of protein renewal and replacement (= turnover) (for reviews, see Hawkins, 1996; Hawkins & Day, 1996, 1999). Reduced energy requirements in shellfish with greater multi-locus enzyme heterozygosity have most readily been observed indirectly as lower oxygen uptake under maintenance conditions of partial or complete starvation (e.g. Koehn & Shumway, 1982; Garton *et al.*, 1984; Diehl *et al.*, 1986; Hawkins *et al.*, 1986, 1989, 1994). When food is less limiting, those different energy requirements for maintenance alone become obscured by ancillary costs that are associated with faster rates of feeding, digestion and growth. Here, by measuring each physiological component of net energy balance, we have shown that this metabolic advantage extends to include higher net production efficiencies in *Crassostrea gigas* with greater multi-locus enzyme heterozygosity when reared under conditions of fast growth. These findings complement those in mussels (*Mytilus galloprovincialis*) for which, under conditions of growth, energy costs per unit of growth were lower in more heterozygous individuals (Bayne & Hawkins, 1997).

Several questions remain concerning the potential benefits of further genetic manipulation. We have established that net energy balance is increased following the indiscriminate amplification of allelic variation that results from inducing meiosis I triploids. Yet genetic polymorphism may only be beneficial at specific loci. In the present study, the only correlation between heterozygosity at a single locus and physiological performance within the F3:M3 family was evident for aspartate aminotransferase (*Aat*). This is consistent with previous findings in other species showing that enzymes involved in protein metabolism and energy flux make some of the greatest contributions to heterozygosity associations, including the established significance of protein turnover (for reviews, see Hawkins & Day, 1996, 1999). The evident functional basis to these findings suggests that phenotypic associations with multi-locus enzyme heterozygosity may result directly from studied loci, with different influences according to function, thereby implying that genetic manipulation might best be directed towards targeted loci. In this respect, it is of interest that strong viability selection in *Crassostrea gigas* is largely focused on two linkage groups, and that *Aat* is the only enzyme involved in protein metabolism within either of those two groups (McGoldrick & Hedgecock, 1997).

Of our studied enzyme loci, only that coding for phosphoglucomutase (*Pgm*) possessed more than two different alleles in any one oyster, with no significant phenotypic consequence. Similarly, Beaumont *et al.* (1995) reported that triallelic heterozygous triploid mussels (*Mytilus edulis*) did not grow any faster than diallelic heterozygous triploids. To date, therefore, there is no evidence establishing that the physiological performance of a triploid individual may be further enhanced upon possessing three rather than two different alleles.

Any relation between physiological traits and heterozygosity for microsatellites would have established that these associations may also exist for non-coding regions of the genome (cf. Pogson & Zouros, 1994), indicating a contribution of 'associative overdominance' to heterozygosity associations, in which studied loci may be neutral markers of other causative loci (Zouros & Pogson, 1994). Unfortunately, oysters were both homozygous and heterozygous at only one of our three microsatellite loci (*Cg44*) (Magoulas *et al.*, 2000), and although there was no relation between any physiological trait and heterozygosity at *Cg44*, one cannot draw any general conclusions from the present findings on the basis of that locus alone.

The present findings help to resolve the genetic and physiological basis of improved performance in juvenile triploids. To better establish the ecological benefits of the observed effects, we deployed a further cohort of mature *Crassostrea gigas* that included both meiosis II triploids and their diploid siblings for ongrowing at two different sites within the natural environment (Naciri-Graven *et al.*, 2000). As in the present study, multi-locus enzyme heterozygosity within that additional cohort was higher in meiosis II triploids than in diploid siblings. Meiosis II triploids also grew faster, apparently due to both higher allelic diversity and lower reproductive output (Naciri-Graven *et al.*, 2000). Collective findings therefore confirm that benefits of increased multi-locus enzyme heterozygosity resulting from the induction of triploidy may occur during both juvenile and adult life-stages in both the hatchery and natural environments.

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