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Comparative study of shell shape and muscle scar pigmentation in the closely related cupped oysters *Crassostrea angulata*, *C. gigas* and their reciprocal hybrids

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Abstract – The taxonomic status of the cupped oysters *Crassostrea angulata* and *C. gigas* has received considerable attention in the last decades. Based on larval shell morphology, experimental hybridization, allozymes and nuclear DNA studies several authors have considered these two taxa as being synonymous. However, mitochondrial data showed clear genetic differences between the two taxa. In addition, microsatellite-based studies and cytogenetic studies have also provided evidence that supports their differentiation. Considerable differences have also been observed at the phenotypic level in terms of growth rate and ecophysiological parameters. In the present study, *C. angulata* from Sado estuary (Portugal) and *C. gigas* from Seudre estuary (France) were collected and factorial crosses were performed. Juveniles of the different progenies were reared in Ria Formosa (Portugal) under common conditions to determine if they exhibited differences in shell shape and in pigmentation of the adductor muscle scar. Significant morphometric differences between *C. angulata* and *C. gigas* progenies were indicated by univariate and multivariate analyses. Univariate analysis of size-adjusted shell measurements revealed significant differences between the two taxa for shell depth, muscle scar height, and length of ligamental area. Both reciprocal hybrids showed intermediate morphometric characters between parental lines. In addition, significant differences were also observed between *C. angulata* and *C. gigas* progenies in terms of pigmentation of adductor muscle scar. *C. angulata* and both reciprocal hybrid progenies showed highly pigmented adductor muscle scars whereas in *C. gigas* progeny the pigmentation was lighter. The differences in shell shape and muscle scar pigmentation observed in the present study support the distinction of the two taxa.

Key words: Shell morphology / Shell pigmentation / Hybridization / *Crassostrea angulata* / *Crassostrea gigas*

Résumé – Étude comparative de la forme de la coquille et de la pigmentation de l'empreinte du muscle adducteur chez les huîtres *Crassostrea angulata*, *C. gigas* et leurs hybrides réciproques. Le statut taxonomique des huîtres creuses *Crassostrea angulata* et *C. gigas* a fait l'objet de nombreuses études ces dernières décennies. Se basant sur la morphologie des coquilles des larves, des croisements expérimentaux ou des marqueurs allozymiques ou des marqueurs ADN, plusieurs auteurs ont conclu que ces deux taxons appartenaient à la même espèce. Cependant, des données mitochondriales montrent de nettes différences entre ces deux taxons. De plus, des études basées sur des marqueurs microsatellites, et dans le domaine de la cytogénétique, montrent une différentiation significative. De plus, des différences phénotypiques ont également été observées en terme de croissance et de paramètres écophysiologiques. Dans notre étude, des huîtres *C. angulata* de l'estuaire du Sado (Portugal) et *C. gigas* de l'estuaire de la Seudre (France) ont été croisées selon un plan factoriel. Leurs descendants ont été élevés dans la « Ria Formosa » (Portugal) puis

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comparés pour la forme de leur coquille et la pigmentation de l'empreinte du muscle adducteur. Des différences morphologiques significatives entre descendants *C. angulata* et *C. gigas* sont mises en évidence par analyses uni- et multivariées. Après ajustement en ce qui concerne la taille des coquilles, l'analyse multivariée montre des différences entre les deux taxons au niveau de la profondeur de la valve inférieure, la hauteur de l'empreinte du muscle et la longueur du ligament de la charnière. Les deux hybrides réciproques présentent des valeurs intermédiaires à celles des lignées parentales pour ces caractères. De plus, des différences significatives entre descendants sont également observées pour la couleur de la coquille au niveau de l'empreinte du muscle adducteur ; les descendants *C. angulata* et les deux hybrides présentant des taches plus foncées que celles des descendants *C. gigas*. Les différences de forme de coquille et de pigmentation de l'empreinte du muscle adducteur, observées dans cette étude, contribuent à la distinction entre les deux taxons étudiés.

1 Introduction

The Portuguese oyster *Crassostrea angulata* (Lamarck 1819) and the Pacific oyster *C. gigas* (Thunberg 1793) are both important commercial species. According to FAO (2006), *C. gigas* is one of the most important aquatic animals produced in the world and is farmed in several countries. *C. angulata* sustained European oyster production for almost one century, with an annual production that reached 100 000 tons. Major mortalities between 1967 and 1973 almost wiped out *C. angulata* from Europe (Comps 1988). Nowadays, there is only a small production of *C. angulata* in Europe, namely in Portugal and Spain. However, *C. angulata* is presumed to be the main oyster species produced in Taiwan (Boudry et al. 1998) and possibly one of the main species produced in China (Lapègue et al. 2004; Guo et al. 2006).

The taxonomic status of *C. angulata* and *C. gigas* has received considerable attention in the last decades. Ranson (1960) based on the morphological similarity of late larval shells suggested that the two taxa should be classified as the same species. It was also observed that *C. angulata* and *C. gigas* interbreed readily in the laboratory (Imai and Sakai 1961) and F1 hybrids show normal meiosis and mitosis (Menzel 1974). In addition, Huvet et al. (2002) observed that the progeny of the hybrids is viable and fertile and it can be successfully backcrossed to either parental taxon. Studies using allozyme markers also showed that *C. angulata* and *C. gigas* are closely related, with genetic differences between populations at the species level (Mathers et al. 1974; Buroker et al. 1979; Biocca and Matta 1982; Mattiucci and Villani 1983). Further analysis based on centromeric satellite DNA confirmed the high genetic similarity between them (López-Flores et al. 2004). However, studies on the mitochondrial cytochrome oxidase subunit I (COI) gene demonstrated clear genetic differences between the two taxa (Boudry et al. 1998; O'Foighil et al. 1998; Boudry et al. 2003). An average of 2.3% differences in COI nucleotide sequence suggests that the two taxa may have diverged several hundred thousand years ago (Hedgecock et al. 2004). Huvet et al. (2000) observed low but significant differences between *C. angulata* and *C. gigas* populations using microsatellites markers. Cytogenetic studies have also provided evidence that supports their genetic differentiation (Leitão et al. 1999a; 1999b; 2004). More recently, Leitão et al. (2007) provided cytogenetic evidence of both parental genomes in the interspecific hybrids. At the phenotypic level, considerable differences have also been observed. A higher production yield has been reported for *C. gigas* when compared with *C. angulata*, due to the faster growth of *C. gigas* and higher mortality in *C. angulata* (His 1972; Bougrier et al. 1986; Soletchnik et al. 2002; Batista et al. 2007). Differences have also been revealed in terms of their ecophysiological characteristics, such as clearance rate (His 1972), oxygen consumption (Goulletquer et al. 1999) and feeding time activity (Haure et al. 2003).

Shell morphology is an extremely plastic trait in oysters (Stenzel 1971; Harry 1985; Lawrence 1995). Several factors influence the morphology of oyster shells such as the type of substrate on which they grow, degree of crowding and various physico-chemical parameters (Galtsoff 1964; Quayle 1988). However, several studies suggest that variation in shell shape and color in oysters is genetically controlled (Imai and Sakai 1961; Wada 1994; Brake et al. 2004; Ward et al. 2005).

It has been reported that adult shells of *C. angulata* and *C. gigas* are similar (Menzel 1974; Biocca and Matta 1982), although criteria used to compare them were not specified. The aim of this study was to re-examine differences in shell shape and pigmentation between *C. angulata* and *C. gigas* through the comparison of pure and hybrids progenies cultured under common conditions.

2 Material and methods

2.1 Biological material

Adults of *C. angulata* and *C. gigas* were collected from Sado River (Portugal) and Seudre Estuary (France), respectively. Oysters were identified according to their geographical origin (see Boudry et al. 1998; Huvet et al. 2000; Fabioux et al. 2002; Lapègue et al. 2004) and this was also confirmed by polymerase chain reactions with restriction fragment length polymorphism (PCR-RFLP) analysis of a fragment of the mitochondrial gene cytochrome oxidase C subunit I (Boudry et al. 1998). Gametes were collected by stripping the gonads from ripe adults of *C. angulata* (15 females and 5 males) and *C. gigas* (10 females and 8 males) as described by Stephano and Gould (1988). The gametes for each sex and taxon were pooled and crosses were performed in order to obtain the following groups: *C. angulata* progeny (AA); *C. gigas* progeny (GG); hybrids derived from *C. angulata* females and *C. gigas* males (AG); and hybrids derived from *C. gigas* females and *C. angulata* males (GA). Larvae from the four groups were reared in the IFREMER facilities in La Tremblade (France) as described by Taris et al. (2006). Single oyster spat was produced using cultch (finely crushed oyster shell chips,

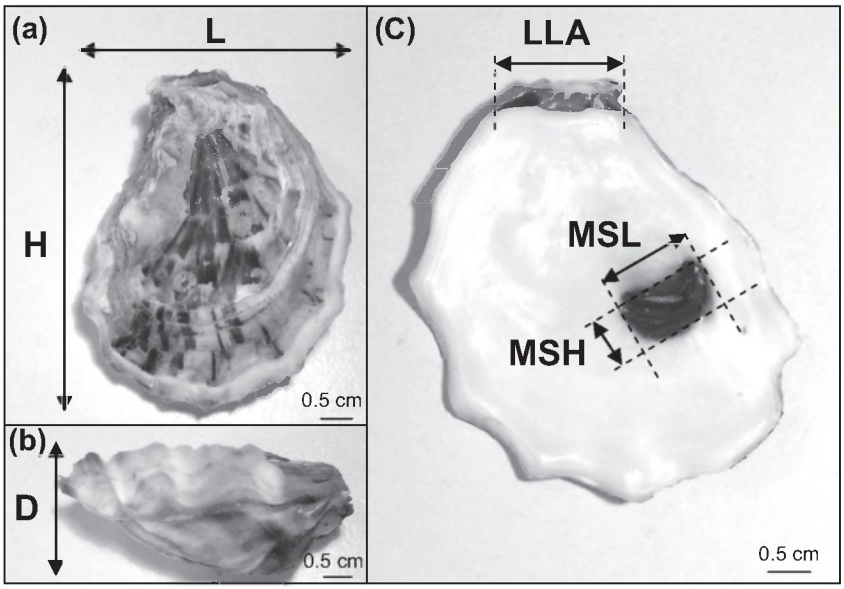

Fig. 1. Shell characters measured. (a) Shell height (H) and length (L) with both valves fitted together; (b) shell depth (D) with both valves fitted together; (c) muscle scar height (MSH), muscle scar length (MSL) and length of ligamental area (LLA).

<400 μm) as the substrate for settlement. The animals of the different groups were transferred to an indoor nursery system in the INIAP/IPIMAR facilities in Tavira (Portugal) and grown until 204 days after fertilization (DAF). The animals were finally transferred to the grow-out site in Ria Formosa (Portugal) and cultured off-bottom in oyster bags attached to iron frame tables until the age of 472 DAF. Four bags with 50 oysters each were prepared per group resulting in a low culture density (<100 individuals/m^2). The position of the bags on the tables was randomly changed every month. The sea floor of the study site was a mixture of sand and mud, and the emersion rate was approximately 50%.

2.2 Morphometric characters

At the end of the grow-out phase a total of 254 oyster shells (between 8 and 21 individuals per bag) were measured (Fig. 1). The following measurements were made from the oyster shells with both valves fitted together: (i) height (H); (ii) length (L); and (iii) depth (D). The measurements taken only on the right shell valve were muscle scar height (MSH), muscle scar length (MSL), and length of ligamental area (LLA). The terminology used for the shell measurements was adapted from Stenzel (1971) and the shells were measured using a digital caliper to a precision of 0.01 mm. The dry shell weight (DSW) of both valves was determined for each individual to a precision of 0.001 g.

2.3 Pigmentation of the adductor muscle scar

In order to characterize the pigmentation of the adductor muscle scar (MS) of the left and right valves two different methods were used: (i) the degree of pigmentation of the MS was estimated by a densitometric method using Quantity One Software (Bio-Rad, Hercules, CA), where the surface of the MS of the same oyster shell (showing no pigmentation) was used as background (quantitative method); (ii) a system with four MS pigmentation categories was used, based on the degree of darkness, as previously employed by Imai and Sakai (1961) and Brake et al. (2004) (semi-quantitative method). The categories were described as: (1) very light (whole surface having no or very little pigmentation); (2) light (a small parts having pigmentation); (3) dark (most parts having pigmentation); and (4) very dark (whole or almost whole surface having pigmentation). As described by Brake et al. (2004), a representative oyster shell for each category was selected based on shell pigmentation and chosen as a standard with which all shells were compared.

Table 1. Results of permutational multivariate analysis of variance from raw measurements and size-adjusted measurements (H, L, D, MSH, MSL and LLA) of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrid (AG and GA) progenies.

Source	*df*	*SS*	*F*-ratio	*p*-value
From raw measurements				
Groups	3	0.800	41.727	<0.001
Residual	12	0.077		
Total	15	0.877		
From size-adjusted measurements				
Groups	3	0.240	8.333	<0.001
Residual	12	0.115		
Total	15	0.356		

2.4 Statistical analysis

2.4.1 Raw data

All shell characters (DSW, H, L, D, MSH, MSL and LLA) were transformed using the natural logarithm. For each shell

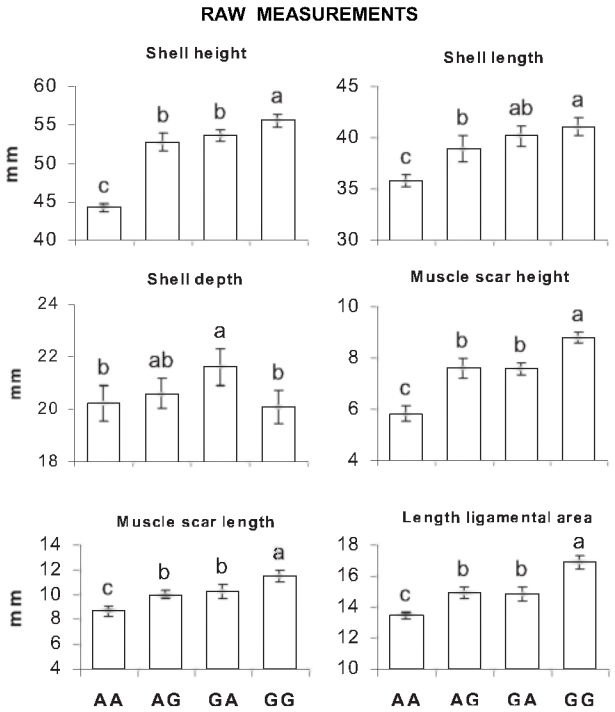

Fig. 2. Mean (± standard deviation) shell characters (n = 4) from raw measurements (H, L, D, MSH, MSL and LLA) of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrid (AG and GA) progenies. For each shell character, groups with different letters are significantly different ($p < 0.05$).

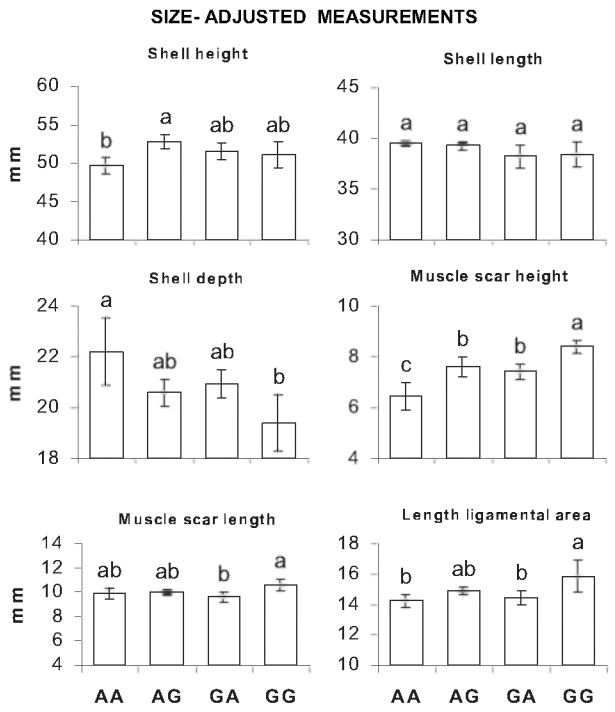

Fig. 3. Mean (± standard deviation) shell characters (n = 4) from size-adjusted measurements (H, L, D, MSH, MSL and LLA) of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrid (AG and GA) progenies. For each shell character, groups with different letters are significantly different ($p < 0.05$).

character, a mean value of all oysters measured in each bag was calculated and used for both univariate and multivariate analyses (n = 4). Data for H, L, D, MSH, MSL and LLA was analyzed using permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) based on Euclidean distances with 4999 permutations to identify differences between the groups. Univariate analyses were performed by analysis of variance (ANOVA) to determine if the groups differed from one another for the different shell measurements (H, L, D, MSH, MSL, and LLA). ANOVA was also used to determine if the groups differed in dry shell weight. Pairwise comparisons between groups were performed using Student-Newman-Keuls (SNK) tests when significant differences were revealed by ANOVA. The Kolmogorov-Smirnov test for goodness of fit was used to investigate departure from normality and the Cochran test was used to assess heteroscedasticity.

2.4.2 Size-adjusted data

Analysis of covariance (ANCOVA) has been used as a size correction method in morphometric studies to remove variation in size from variation in shape (Parsons et al. 2003; McCoy et al. 2006). Furthermore, the test of homogeneity of slopes in ANCOVA can also be used to infer if groups share common patterns of allometry (McCoy et al. 2006). Data was analysed using ANCOVA with dry shell weight (DSW) as the covariate. The slopes were not significantly different among the 16 bags for either shell character namely, shell height ($F_{15,222}$ = 1.22, p = 0.260), shell length ($F_{15,222}$ = 1.10, p = 0.359), shell depth ($F_{15,222}$ = 1.23, p = 0.249), muscle scar height ($F_{15,222}$ = 0.90, p = 0.563), muscle scar length ($F_{15,222}$ = 0.66, p = 0.825) and length of ligamental area ($F_{15,222}$ = 1.17, p = 0.296), which indicates that the four groups shared common patterns of allometry within the size range analyzed. The size-adjusted estimates for each shell character were then obtained by adding the residuals from the within-bag regression to the estimated marginal mean for the appropriate bag. The mean of each of the six size-adjusted shell measurements of all oysters in each bag was then used to determine if the groups differed from one another using univariate and multivariate analyses as described above for the raw data.

2.4.3 Pigmentation of the adductor muscle scar

The degree of pigmentation of the adductor muscle scar of the four groups, estimated with a densitometric method (quantitative method), was compared by ANOVA (40 individuals per group), and where significant, pairwise differences were analysed by Student-Newman-Keuls (SNK) tests. The Kolmogorov-Smirnov test for goodness of fit was used to investigate departure from normality and the Cochran test was used to assess heteroscedasticity. In order to compare the proportion of individuals assigned to the different pigmentation categories (semi-quantitative method) between

groups (the same 40 individuals per group used for the quantitative method), chi-square tests with Bonferroni corrections were used (i.e., 2 × 4 analyses). Data analysis was carried out using MATLAB (version 6.5; MathWorks Inc. 2002) and Statistica (version 6.1) software.

3 Results

3.1 Raw data

Multivariate analysis revealed significant differences among the four groups (Table 1) and multiple comparisons showed significant differences between all pairs ($p < 0.001$) with exception of the hybrids AG and GA ($p = 0.304$). Univariate analysis revealed significant differences in dry shell weight (DSW) among all groups ($F_{3,12} = 157.65$, $p < 0.001$). Pairwise comparisons showed that DSW was significantly higher ($p < 0.001$) in *C. gigas* progeny in comparison with *C. angulata* progeny, and both hybrids (AG and GA) showed intermediate values. Shell height (H), shell length (L), muscle scar height (MSH), muscle scar length (MSL) and length of ligamental area (LLA) were significantly greater in *C. gigas* progeny when compared with *C. angulata* progeny (Fig. 2). No significant differences were observed between the hybrids AG and GA for H, L, D, MSH, MSL, and LLA. Mean values of shell characters of both hybrid progenies were intermediate between, or similar to those of the pure progenies for all variables except for shell depth that was significantly higher for GA hybrids.

3.2 Size-adjusted data

There were significant multivariate differences among the four groups (Table 1) and multiple comparisons showed differences between all pairs ($p < 0.05$) with exception of the hybrids AG and GA ($p = 0.181$). Univariate analysis revealed that the *C. gigas* progeny had a significantly greater MSH and LLA than *C. angulata* progeny (Fig. 3). The MSH and LLA were on average 29.2 and 11.3%, respectively, greater in *C. gigas* than in *C. angulata*. However, shell depth was on average 14.4% greater in *C. angulata* than in *C. gigas* (Fig. 3). Shell characters of both hybrid progenies were intermediate between, or similar, to those of the parental lines. Pairwise comparisons between the reciprocal hybrids showed no significant differences for any shell characters analysed (Fig. 3).

3.3 Pigmentation of the adductor muscle scar

The pigmentation of the adductor muscle scar (MS) of the left (MSL) and right (MSR) valves was significantly correlated ($r = 0.61$, $p < 0.001$). ANOVA showed significant differences ($p < 0.001$) among the four groups for the degree of pigmentation of MSL and MSR (quantitative method). Pairwise comparisons revealed that *C. gigas* progeny had a significantly lower pigmentation of MSL and MSR than all the other groups (SNK test, $p < 0.001$). No significant differences were observed between *C. angulata*, AG and GA progenies for MSL and MSR pigmentation (SNK test, $p > 0.05$).

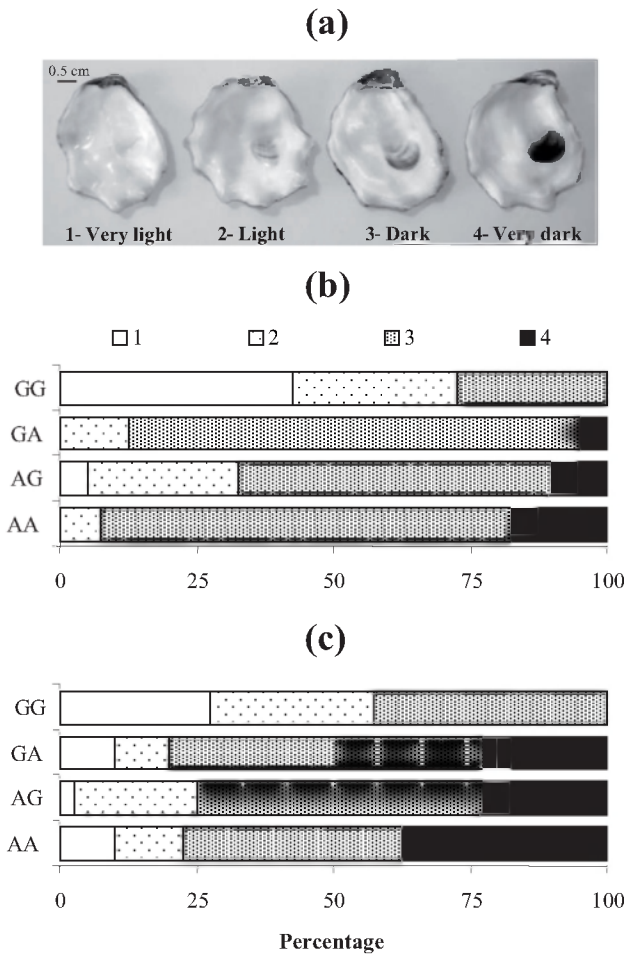

Fig. 4. Adductor muscle scar pigmentation. (a) Shells representative of the four categories used to classify the degree of pigmentation of the MS (1-very light, 2- light, 3- dark, 4-very dark). (b) Frequency of *C. angulata* (AA), *C. gigas* (GG) and hybrids (AG and GA) individuals for each category for the left valve. (c) Frequency of AA, GG, AG and GA individuals for each category for the right valve.

Similar results were obtained when individuals from the four groups were assigned to the different pigmentation categories (semi-quantitative method, Fig. 4). Chi-squared analysis revealed significant differences ($p < 0.01$) between *C. gigas* progeny and all the other groups, whereas no differences were observed between *C. angulata*, AG and GA progenies. The results of the chi-squared analyses were mainly due to *C. gigas* progeny being distributed in categories representative of lower levels of pigmentation.

4 Discussion

Univariate and multivariate analyses of the raw data showed that there were significant differences between *C. angulata* and *C. gigas* for all the studied shell characters (Table 1, Fig. 2). The differences observed in dry shell weight between the two taxa indicate that a large proportion of the variation observed was due to differences in size between *C. angulata* and *C. gigas* that resulted from differences in growth

rate. Indeed, previous studies showed that *C. gigas* grows faster than *C. angulata*, under culture conditions similar to the ones used in the present study (Bougrier et al. 1986; Soletchnik et al. 2002). Since much of the variation observed in shell characters among groups was apparently due to differences in size, it was necessary to remove this effect from the data in order to allow comparison of shell morphology. Univariate and multivariate analyses of size-adjusted measurements revealed significant morphological shell differences between *C. angulata* and *C. gigas* progenies (Table 1, Fig. 3). The same analyses also revealed that both reciprocal hybrids had intermediate shell characters between parental lines. The development of distinct morphologies in *C. angulata* and *C. gigas* progenies together with intermediate position of hybrids under the same environmental conditions indicates a genetic basis for this variation. *C. angulata* progeny had deeper shells with shorter adductor muscle scars (for MSH) as well as smaller ligamental areas compared with *C. gigas* progeny. A genetic basis for variation in shell flatness in *C. gigas* was first suggested by Imai and Sakai (1961) based on differences in relative shell depth observed among geographical races of this species. They reported that such differences persisted for several generations, which indicated its hereditary nature. The results obtained in the present study support the findings of Imai and Sakai (1961) of a genetic basis for shell depth relative to height. In addition, the differences observed between *C. angulata* and *C. gigas* progenies in the present study for MSH as well as for LLA suggest that these characters may also be genetically based. A genetic basis for hinge length, as well as for other shell characters, was also suggested in other bivalve mollusc species namely, in the scallops *Pecten maximus* (Wilding et al. 1998) and *Argopecten irradians* (Wilbur and Gaffney 1997).

Morphological divergence between *C. angulata* and *C. gigas* may have resulted from local adaptation by natural selection, genetic drift in absence of gene flow or a combination of these two evolutionary forces. The morphological differences observed may influence their fitness under certain environmental conditions. The size of the muscle scar reflects the size of the adductor muscle that controls the opening and closing of the valves and opposes the action of the hinge ligament (Quayle 1988). The ability of oysters to keep the shells closed avoids desiccation during the time of exposure in the tidal zone and allows them to remain protected against unfavourable conditions or attacks from predators (Galtsoff 1964). The relative smaller muscle scar height observed in *C. angulata* compared with *C. gigas* may result in a differential ability to keep the shells closed and hence might influence their fitness under some environmental conditions. Indeed, a positive correlation between MSH and muscle strength was observed in *C. gigas* juveniles (Poulet et al. 2003). Moreover, the previous authors also reported a link between muscle strength and summer mortality in *C. gigas*. Nevertheless, the adaptive significance of the other morphological differences observed between the two taxa remains unknown.

Other cases have been reported of closely related bivalve molluscs that interbreed and produce sexually viable offspring, but that are genetically distinct and show morphometric differences. One of the well documented cases is the *Mytilus* complex composed by the mussels *M. edulis*, *M. galloprovincialis* and *M. trossulus* for which morphometric differences were observed among the three taxa, although varying amounts of overlap between them have been reported (McDonald et al. 1991; Mallet and Carver 1995; Innes and Bate 1999; Gardner 2004). Another case is the clams *Mercenaria mercenaria* and *M. campechiensis* that also show significant morphometric differences, but overlap for some characters (Dillon and Manzi 1989).

For the oyster industry, shell morphology is considered an important trait since it can dramatically influence product value when animals are commercialised live or half shell (Mahon 1983; Ward et al. 2005). Brake et al. (2003) showed that length and depth indexes as well as the index of shell shape (which combines the information of the other two indexes) were useful to describe oyster shells with a desirable shape. The same authors observed that length index was not as important as depth index to describe a desirable shell shape. Although it is known that the relative depth of oyster shells is extremely influenced by environmental conditions and husbandry procedures (Galtsoff 1964; Quayle 1988), genetic variation has been observed for these traits (Wada 1994; Ward et al. 2005). However, Ward et al. (2005) observed that for *C. gigas* the genetic gains for depth indexes were low, and hence genetic selection was not a powerful tool to manipulate this trait. The results obtained in the present study, not only support the genetic basis for shell depth relative to height, but also suggest that cross-breeding between *C. angulata* and *C. gigas* can be seen as a possible strategy to manipulate shell depth to height with a limited decrease in growth performance.

The approximate normal distribution of MS pigmentation (estimated using the densitometric method) and the diversity of pigmentation colour/pattern observed suggests that pigmentation of MS in *C. angulata* and *C. gigas* can be considered a quantitative trait. This is in agreement with Brake et al. (2004), who hypothesised that the pigmentation of the shell surface in *C. gigas* is controlled by many genes. The results of the present study suggest that there are genetic differences between *C. angulata* and *C. gigas* for the degree of pigmentation of the MS. These results support earlier observations of Galtsoff (1964) that described the MS of *C. angulata* as being highly pigmented whereas in *C. gigas* pigmentation was either absent or very light. Furthermore, both AG and GA hybrids showed a pigmentation of the MS similar to *C. angulata*, which indicates that darker pigmentation is dominant over lighter.

The differences in shell shape and MS pigmentation observed in the present study support the distinctness of the two taxa as pointed out by other authors (Héral and Deslous-Paoli 1991; Boudry et al. 1998; O'Foighil et al. 1998; Leitão et al. 1999a; 1999b; 2004). However, identification of *C. angulata* and *C. gigas* based only on shell form may be prone to error due to the very high variability and plasticity in shell morphology. Furthermore, future studies should be performed using *C. angulata* and *C. gigas* progenitors from several populations to confirm that the differences observed are at the taxon level and not only at the population level.

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