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No hybridization and marked interspecific differences in individual growth rate in mixed cultures of Manila clam (*Ruditapes philippinarum*) and grooved carpet-shell clam (*R. decussatus*)

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ABSTRACT

The Manila clam (*Ruditapes philippinarum*) was introduced in Europe in the 1970's and in the following years it became naturalized. Interactions with the native species include hybridization with the grooved carpet-shell (GCS) clam (*R. decussatus*), which may have both useful and undesirable consequences. Here we report an attempt to produce hybrids in captivity by crossing 3 females and 4–5 males of each species in a two-step protocol that favored hybrid fertilizations. One-hundred animals were sampled at 15 months after fertilization, and scored for one morphological diagnostic trait (siphon fusion) and two diagnostic genetic DNA markers (ITS-2 and Fas-i1). No hybrids were detected, although the 0% hybridization rate has an associated 95% confidence interval of 3.3%. This result suggests that successful hybrid fertilization may be infrequent and/or the hybrid offspring may have very low survival rate. Abundant offspring of the two parental species were obtained and provided an unprecedented opportunity to study the innate differences in biological traits between the two species without the confounding influence of environmental variability. Individuals with ripe gonads were significantly less frequent in the Manila clam, suggesting an innate trend to earlier summer spawning in this species. Manila clam grew 20% faster than GCS clam and showed 80% heavier shells. However GCS clam showed almost twice as much variability in size as Manila clam, and some individuals of this species were as large as the largest Manila clams. The observed difference in growth variability may reflect a general loss of genetic variability in Manila clam during the introduction in Europe, although a random effect from using a small number of parents in the mixed cross cannot be discarded. Discrimination between these explanations, as well as determining more precisely the occurrence of hybridization in hatcheries by studying larger numbers of parents and offspring, may help improving clam aquaculture in Europe while preserving the genetic resources of the GCS clam.

1. Introduction

Clams have been part of shellfish fisheries in Europe since the XIX century. Until the 1970's, the main fished species was the native grooved carpet-shell clam, *Ruditapes decussatus* (GCS clam from now on). However, its long growing period and high disease susceptibility favored the introduction of the Manila clam (*R. philippinarum*) in Europe. This species has established permanent, self-recruiting populations in many localities of the European Atlantic and Mediterranean coasts (Chiesa et al., 2016; Cordero et al., 2017). A stable hatchery-based aquaculture industry has developed (da Costa et al., 2020), and the two species coexist in the market and in the wild. In spite of these developments, the biological bases of differences and similarities in production traits and culture procedures have been only shallowly studied. Moreover, while Manila clam populations have experienced a sustained expansion in Europe, natural populations of GCS clam have declined in recent years, in spite of restocking programs carried out in some regions (Aranguren et al., 2014; Juanes et al., 2012; Moura et al., 2017). In the last decade, the landings of the GCS clam in Spain, which is one of the main European producers, have decreased continuously from 1023 T in 2010 to

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218 T in 2018 (Supplementary Fig. S1). The causes of this decrease are still under research. It has been suggested that they could result from some sort of competition of Manila clam with the native clam species, or from a poorer adaptation of the GCS clam to a changing climate (Albentosa et al., 2007; Anacleto et al., 2014; Aranguren et al., 2014; Dias et al., 2019; Juanes et al., 2012; Lopes et al., 2018; Macho et al., 2016; Vélez et al., 2017). Nevertheless, further biological study of the two clam species seems necessary, especially in relation to two aspects.

The first relates to reports of hybridization between the Manila and the GCS clam. Hybrids have been detected in areas of intensive aquaculture in spite of the large genetic distance that separates the two species(p-distance = 0.25 at the mitochondrial COI gene in Habtemariam et al., 2015; see also Mikkelsen et al., 2006). With the use of genetic markers (the ribosomal RNA internal transcribed spacer ITS-2, and the whole repeat sequence of the minor ribosomal RNA genes, 5S rDNA) and in-situ hybridization, Hurtado et al. (2011) detected 9 hybrids among 328 clams of both species (2.7%) collected in the Ría de Vigo estuary (NW Spain). Habtemariam et al. (2015), using morphological markers and three genetic markers (ITS-2, 5S, and the mitochondrial gene cytochrome oxidase I), found 6 individuals of hybrid origin (post-F1) (3.9%) among 154 clams from two populations from the Bay of Biscay. They also found one post-F1 hybrid among 51 GCS clams (2%) of hatchery origin used for restocking. Their results suggest that hybrids of the F1 generation could be fertile. Habtemariam et al. (2015) also reported that hybrids are often difficult to distinguish from parental species based on morphology. Uncontrolled hybridization in natural habitats and in hatcheries may result in genetic introgression, with potential negative consequences for both species (Allendorf et al., 2001). This possibility has raised concerns in the clam fisheries and aquaculture communities where hybridization was detected (Habtemariam et al., 2015; Hurtado et al., 2011). On the other hand, controlled hybridization in captivity may help in aquaculture through the production of hybrid strains showing improved performance (Rahman et al., 2018). The production of hybrid clams in captivity is a fundamental step to approach the scientific study of both aspects, but so far, no attempt to obtain hybrids between the Manila clam and the GCS clam in captivity has been reported.

The second aspect in need of further research is related to the culture performance of the two species. In this respect, growth and reproduction are two traits of the highest interest. The species' fast growth and robustness were the reasons for the introduction of Manila clam for aquaculture in Europe (Breber, 1985). The GCS clam has usually been considered a slow-growing species compared to the Manila clam. However, comparative studies of growth of the two species in the same conditions are scarce, and have produced ambiguous and sometimes controversial results. Laing et al. (1987) compared the average growth of groups of juveniles (0.3–3 mm) of the two species obtained in captivity in an Atlantic (British) locality. Growth-rate coefficients were 30%–90% higher in the Manila clam when fed with *Isochrysis* T-iso at temperatures ranging from 20 °C to 30 °C. However, growth rate was very similar at 12 °C – 16 °C, a temperature range more typical of the main Atlantic culture areas. These results suggest a strong species-specific effect of temperature on growth, and suggest that growth rate differences could be realized differently in cool, Atlantic coastal environments as compared to Mediterranean environments, which are characterized by warmer waters during most of the year. Bodoy et al., 1980 studied average growth in groups of adult clams during a 3-month period (April to June) in ponds located in the NW Mediterranean coast, in which temperature varied between 14^0C and 26.5^0C. Growth was maximum for the two species in April, but Manila clam growth rates were twice as high as those shown by the GCS clam. Bidegain and Juanes (2013) studied the mortality and growth rates of adult clams of both species within a 1-year period in the Santander bay (Bay of Biscay, N Spain). Adult animals were collected in the estuary, individually marked in the laboratory, and then transplanted to culture plots in the same bay. The authors did not find significant differences in mortality rates between species, but the Manila clam average individual growth rate was nearly twice as high as that of the GCS clam in all plots (although with a wide overlap). The general view of a fast-growing Manila clam demonstrated by the previous studies -at least in the warmer environments- has been recently challenged by a study by Moura et al. (2017). These authors studied the Manila clam population growth rates in the Tagus estuary (Portugal) and compared it with published results on Manila and GCS clams in other locations worldwide. They found that the growth performance of GCS populations was comparable to that observed in European Manila clam populations. Interestingly, they also found that population growth rates of Manila clam were lower in Asia than in Europe.

As to reproduction, there have been multiple studies of the gonadal cycle of each species in several locations (e.g.: Beninger and Lucas, 1984; Ojea et al., 2005, 2004; Rodríguez-Moscoso et al., 1992; Rodríguez-Moscoso and Arnaiz, 1998; Xie and Burnell, 1994), but to our knowledge only the study by Laruelle et al. (1994) has focused on natural populations of both species in the same locality and time points. These authors studied reproduction in two localities in Brittany (France), and found a more extended reproductive period and a greater number of spawning events in the Manila clams. Delgado and Pérez-Camacho, 2007 carried out a study of the gonad development in both species in captivity at two temperatures, and found a greater rate of gonadal development in Manila clam at 18^0C than at 14^0C, as well as other differences common to both temperatures.

As most of the previous studies have used clams which have experienced different environmental influences in one or more stages of their lives, or have focused on clam populations living in highly divergent environments, it is difficult to tell to what extent the reported differences and similarities between the two species have been influenced by genetic and/or environmental differences between study groups. This is a most interesting question, as its alternative answers may drive clam aquaculture practices of choosing the best environments for culture or of selecting the best stocks. But, as of today, questions such as "what is the average gain in growth that can be expected from using one or the other species for aquaculture in a given location?" have not received a clear answer. A way to circumvent this uncertainty is to study samples of the two species that have been kept under the same environment during their lifetime. The observation of trait differences between the two species under these conditions would reveal innate (probably genetic) underlying factors, while an absence of differences would point to an important effect of environmental heterogeneity in explaining the differences observed in previous studies. As related specifically to growth, one aspect affecting the earlier studies summarized above is that, with only one exception (Bidegain and Juanes, 2013), growth has been usually measured in groups of animals, and therefore individual variation has not been reported. However, recent approaches to investigate the genetic and molecular bases of trait differences reach their highest strength when inter-individual differences are taken into account (Crawford and Oleksiak, 2007; Saavedra et al., 2017). The knowledge of the relative genetic and environmental influences on individual variability is also essential for breeding purposes (Houston et al., 2020).

Here we present the results of a study of a mixed culture of Manila clam and GCS clam performed in captivity. The simultaneous spawning of parental individuals of the two species provided the opportunity to test for the ease of interspecific hybridization. The finding of individuals of hybrid origin among the hatchery seed reported by Habtemariam et al. (2015), which clearly were unintentionally produced, suggest that hybrids could be obtained in captivity if adequate protocols are designed. We developed a two-step fertilization protocol intended to avoid intraspecific fertilizations and force interspecific fertilizations as a first step. As a result of the crosses, large amounts of juveniles of the parental species were produced, which were kept in the same containers since the moment that fertilization took place until the moment of the sampling. This circumstance provided an unprecedented opportunity to study the innate differences and similarities of the two clam species as to

the individual growth rate, shell morphometrics and gonad maturation in a common environment, without the confounding effect of environmental variability.

2. Materials and methods

2.1. Clam crosses and hatchery procedures

A group of ca. 90 adult animals of each species were captured in Cambados (Ría de Arousa estuary, NW Spain, Atlantic Ocean) in May 2018 and sent to the hatchery facilities at the Institut de Recerca i Tecnologia Agroalimetaries (IRTA) in Sant Carles de la Ràpita (delta of the Ebro river, NE Spain, Mediterranean Sea) for reproductive conditioning. The hatchery techniques were based on classical work for these species with some modifications addressed to improve the performance in our facilities (Arranz et al., 2020; Delgado and Perez Larruscain, 2011; Jones et al., 1993). Clams were placed in 240 L tanks with running seawater, which was filtered at 1 μm, passed through activated charcoal, and sterilized with UV light. The animals were fed *Isochrysis galbana* and *Chaetoceros gracilis* at ca. 6% of their dry weight. The temperature was gradually increased from 16^{0}C to 21^{0}C during the following 6 weeks. In addition, photoperiod was increased 30 min per week until 14:10 h. Four clams of each species were sacrificed weekly to check the ripeness of the gonads.

At the end of the conditioning period, groups of ca. 30 animals of each species were placed on black trays with aerated seawater to maintain oxygen saturation. Spawning was induced by alternate 30 min long thermal shocks at 16^{0}C and 25^{0}C. As spawners were detected, they were immediately separated, rinsed with filtered seawater to exclude any potential contamination with undetected gametes in the common tank, and placed in an individual container until they completed gamete emission. The GCS clam is usually referred to have separate sexes, although occasional hermaphrodites have been reported (Delgado and Pérez-Camacho, 2007). We did not observed any simultaneous emission of both sexes gametes from a single individual. Quality of eggs was checked under the microscope by looking at size and roundness. Eggs were counted in duplicate in a Sedgewick-Rafter plate under a binocular microscope. Sperm quality was checked by inspection under the microscope at 400-800×. Sperm samples with less than ca. 50% of motile cells were discarded.

Three Manila clam females, and four GCS clam females, responded to heat shocks (Fig. 1 A). The egg quality was good and therefore they were kept for subsequent fertilization steps, with the exception of one GCS female that released a very low number of eggs and was discarded. The sperm of 5 GCS clam males and 4 Manila clam males was used. Four additional Manila clam males spawned but the mobility of the spermatozoa was low and were discarded. Approximately 1 million eggs from each female, and 3 mL of sperm from each male, were used for fertilizations.

Fertilization was carried out by mixing gametes of the two species in two steps. The first step (Fig. 1 B) was intended to force interspecific fertilizations, by mixing eggs of GCS clam with sperm from Manila clam

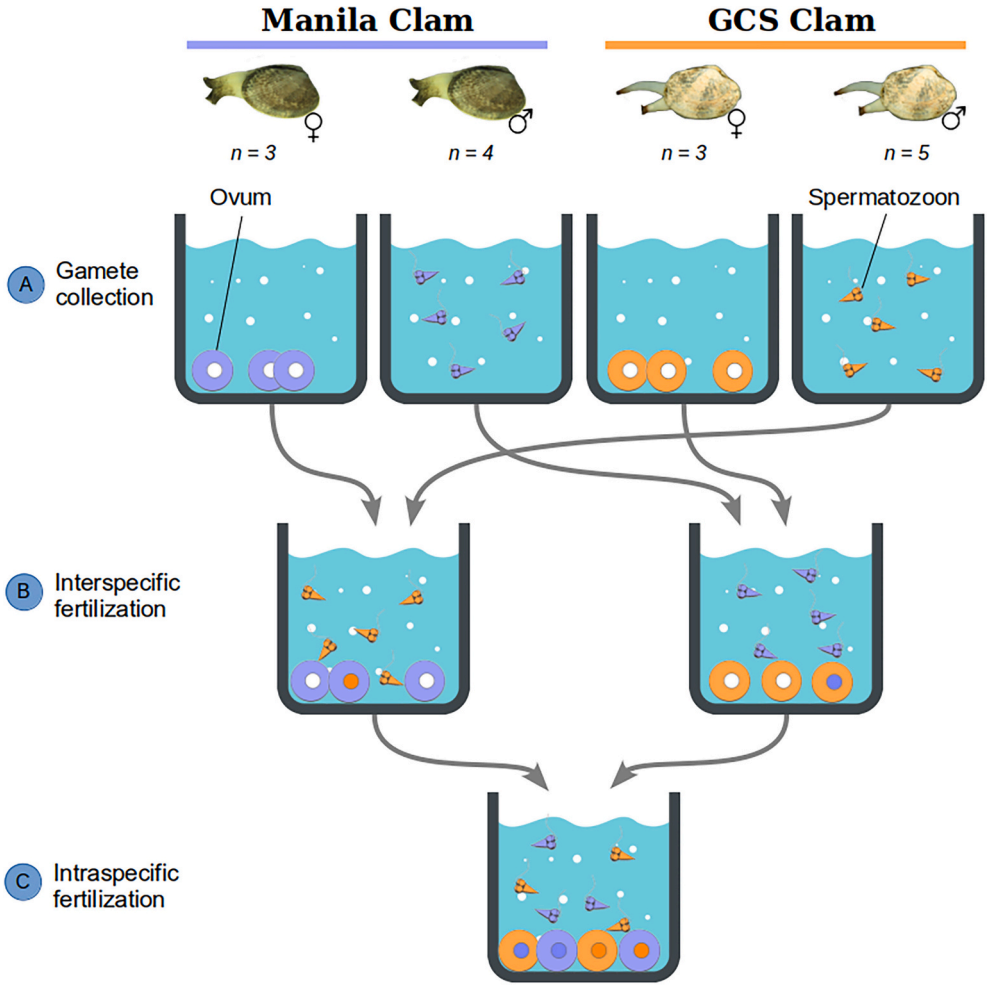

Fig. 1. The fertilization protocol employed to obtain the experimental mixed clam stock. Note in the clam pictures the partially fused siphons diagnostic for Manila clam and the completely separated siphons diagnostic for the GCS clam.

in a 10 L container, and the reciprocal in another container. In the second step (Fig. 1 C), which was performed 3 h later, the contents of the two containers of the previous step were pooled to allow for intraspecific fertilization. This second step had the double function of serving as a control of gamete quality (i.e.: the gamete's competence to produce embryos that undergo normal development) and to obtain clams of the parental species in the same environment for the comparative study.

One hour after the second fertilization step was completed, the embryos were transferred to a flat bottom tank with 200 L of filtered seawater. After 48 h larvae were counted and the volume of water was adjusted to allow 5 larvae / mL. Successive water changes took place every 48 h. Larvae were fed *Isochrysis galbana* at 50,000 cell/mL during first week, and later *Chaetoceros calcitrans* was incorporated gradually until a total concentration of 80,000 cells/ mL was reached at the end of the larval stage. Microalgae was added once every 48 h, coinciding with the water renewal. After 10 days, larvae started metamorphosis and settlement. Every 48 h the settled post-larvae were removed from the tank floor and transferred to mesh-bottom buckets (100 μm - 1 mm) provided with air-lifts and running seawater, where they were kept until 9.5 months post-fertilization. At this time point, 200 animals were collected and transferred to the Instituto de Acuicultura Torre de la Sal (IATS-CSIC), were they were placed in a mesh-bottom bucket, within 500 L tanks filled with running seawater. Clams were fed *I. galbana* and *Tetraselmis suecica* in a ration of ca. 4% of dry meat clam weight, which were delivered in fractions every hour with a peristaltic pump. Minimum and maximum seawater temperatures recorded during the study period were 10.5 °C and 28.3 °C, respectively, and the average was 19.4 °C (Supplementary Fig. S2).

2.2. Morphology, biometry and sex assignment

At 15 months post-fertilization (August 2019) a sample of 100 clams was collected. Clams were classified as Manila clam, GCS clam or intermediate (potential hybrids) according to the morphology of the siphons, as previous studies indicate that this is one of the most reliable traits to distinguish the two species (Habtemariam et al., 2015; Hurtado et al., 2011). Usually GCS clam siphons are completely separated from the base, while Manila clam siphons are fused at most of their length (Fig. 2 and Supplementary Fig. S3). Siphon morphology was first examined in live animals that were placed in a tray with aerated seawater for 30 min, in order to let them extend the siphons. The type of siphon was then recorded, and afterwards animals were sacrificed. The siphon morphology was again examined in the dead animals. Then a piece of mantle was dissected and preserved in 90% ethanol for DNA extraction. The gonad was punctured with a lancet and the extracted fluid was extended on a glass slide in order to determine the clam sex by searching for the presence of eggs or spermatozoa under the microscope. If no gametes were found, the animal was classified as "undetermined sex". The shell dimensions (length, height, width and weight; Supplementary Fig. S4) of each individual were measured to the nearest 0.01 mm with a digital Vernier caliper. The weight of the shell was measured with an electronic balance to the nearest 0.1 mg. The quantity of organic matter in the shell was measured as the weight loss after ignition of one of the valves in a muffle furnace at 450 °C for 6 h.

2.3. Statistical analysis

Averages, standard errors and coefficients of variation were computed for the analyzed shell traits. Differences between species and sexes were tested by the Student *t*-test on log-transformed data. Allometric differences for shell traits were examined using regression lines on log-transformed data and statistically tested by analysis of covariance (ANCOVA) (Zar, 1984). As a first step in the analysis, inter-specific differences in the slopes of the regressions were tested by the Student's t-test. When mass-exponents were not significantly different a common slope could be computed. Subsequently, the equality of elevations was tested, again by a Student's t-test. When elevations were not significantly different a common intercept could be computed. Sex-frequency differences were tested by chi-square tests. All tests were performed with the R statistical package.

2.4. Genetic markers and identification of species and hybrids

Although the two species have characteristics that allow them to be well recognized from their morphology (Supplementary Fig. S3), previous reports suggest that hybrids can be confused with both parental species (Habtemariam et al., 2015; Hurtado et al., 2011). For an unequivocal identification, the two PCR-based diagnostic genetic markers that were previously described by Hurtado et al. (2011) were initially used. One of them (*ITS-2*) produced PCR products of the expected size. The other (*5S*) presented numerous problems (lack of amplification and extra bands). To replace this marker, we developed a new one (*Fas-i1*) based on a length polymorphism of the 1st intron of the Fasciclin-like gene described by Cordero et al. (2014), for which we had observed between-species size differences (Cordero and Saavedra, unpublished results). As expected, PCR products of different sizes were obtained that allowed distinguishing species by running the PCR product in an agarose gel (Fig. 2). The performance of Fas-i1 was first tested in samples of the two species from their native ranges (Japan and Tunisia), where they do not coexist and therefore no introgression can interfere with the results.

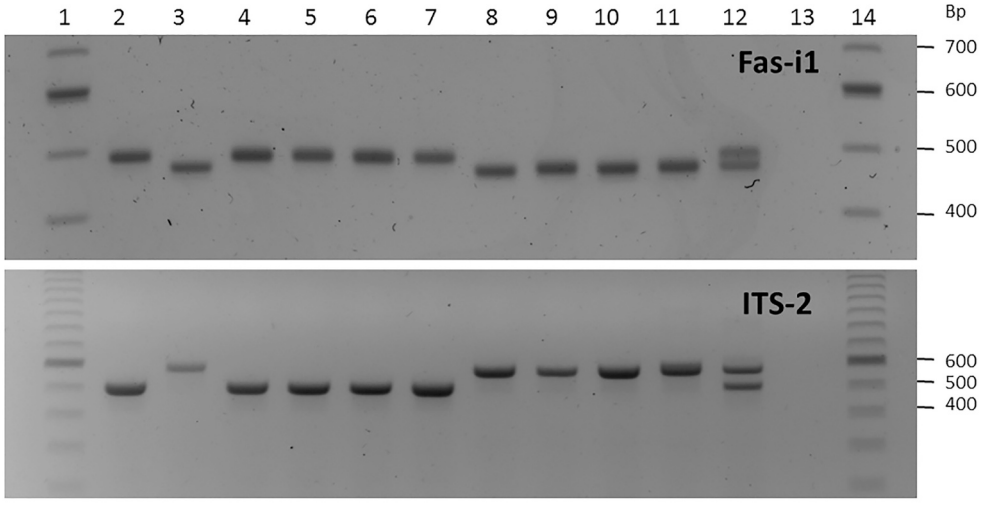

Fig. 2. DNA markers (*ITS2* and *Fas-i1*) used to discriminate species and potential hybrids with examples of their performance. A: Fas-i1. B: ITS-2. Individuals shown are the same for the two markers. Lanes 1 and 14: size markers. Lane2: one of the GCS clams parents. Lane 3: one of the Manila clam parents. Lanes 4–7: four randomly selected offspring with separated siphons (GCS clam siphon type). Lanes 8–11: four randomly selected offspring with fused siphons (Manila clam siphon type).Lane 12: fake hybrid made by mixing equal amounts of Manila clam and GCS clam DNA. Lane 13: PCR negative control.

Since the hybrids were all F1, they were expected to always show two bands of different size for the two markers in electrophoresis gels, and non-hybrids should always show single bands of their species' characteristic size.

Clam DNA was extracted from ethanol-preserved mantle tissues, by using an Invisorb DNA Tissue HTS 96 Kit (Isogen Life Science, Netherlands). PCR conditions for *ITS2* and *Fas-i1* were optimized to maximize amplification for both species, with 50 ng of DNA per reaction, 50 μM each dTNP and 0.8 μM of each primer pair. *ITS2* required 1.5 mM MgCl and 0.75 U of Taq polymerase (Invitrogen), while *Fas-i1* required 2 mM MgCl and 0.5 U of Taq polymerase. Thermocycling conditions for ITS2 included a denaturation step at 95 °C for 5 min, followed by 31 cycles at 95 °C for 30 s, 48 °C for 30 s, 72 °C for 35 s and a final extension at 72 °C for 7 min. For *Fas-i1* a denaturation step at 94 °C for 2 min was followed by 36 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 2 min. The forward and reverse primers used for the ITS-2 marker were ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) from Hurtado et al. (2011). In the case of Fas-i1, the sequence of the forward primer (CL13ibF) was 5'-GCTTTCAAAAAGCACAAGGA-3′, and the sequence of the reverse primer (CL13ibR) was 5'-TGTAGCCATGACGTCAGATG-3′. All reactions were performed in a Perkin-Elmer 9600 thermal cycler. PCR products were examined by electrophoresis on a 1% (*ITS2*) or 2% (*Fas-i1*) agarose gel.

3. Results

3.1. Screening for hybridization with morphology and genetic markers

After careful examination of the degree of siphon attachment, all individuals were assigned to one or the other species, with the exception of 2 animals (Table 1). One showed completely retracted siphons, and could not be diagnosed. The other showed siphons somewhat intermediate between the expected pattern of the two species. Out of the remaining 98 clams, 65 had separated siphons typical of GCS clams and 33 had fused siphons typical of Manila clams.

The new diagnostic marker *Fas-i1* produced bands of different size in each species: 470 base pairs (bp) in Manila clams and 495 bp in GCS clams (Fig. 2). In the case of *ITS-2* a single band of 565 bp corresponding to Manila clams, and another of 482 bp corresponding to GCS clams, were found, as described previously by Hurtado et al. (2011) and Habtemariam et al. (2015).

All 15 clams used as parents of the mixed cross were genotyped for the two markers and results were consistent with all being pure species (Supplementary Fig. S5). The frequencies of the genotypes for the two markers in the offspring are presented in Table 1. In total, 92 clams could be genotyped and diagnosed genetically, of which 76 were genotyped for both markers, and 16 were genotyped for only one marker (14 for *ITS-2*, and 2 for *Fas-i1*). Eight individuals produced no amplification products for both markers. The two individuals which had been classified as undetermined by siphon morphology could be genotyped for the two markers, which identified one as a Manila clam, and the other as a GCS clam (Table 1). After these tests, 64 animals were genetically diagnosed as GCS clam and 28 as Manila clam. No hybrids were detected with the genetic markers. The associate 95% upper limit of the confidence interval of the 0% proportion of hybrids in a sample size of $N = 92$ is 3.3%, as estimated by the "rule of 3" (Jovanovic et al., 1997).

Table 1
Results of species identification based on siphon morphology (separated siphons in GCS clam, and fused siphons in Manila clam) and genetic markers (ITS-2 and Fas-i1, as in Fig. 2), in the mixed offspring. The three numbers separated by a slash and in parentheses under the GCS and Manila columns indicate the number of individuals genotyped for both ITS-2 and Fas-i1, ITS-2 only, and Fas-i1 only, respectively. The None column records the number of individuals in which the PCR failed for both markers.

Siphon morphology		Genetic markers			
		GCS	Manila	Hybrid	None
Separated	65	63 (58/5/0)	0	0	2
Fused	33	0	27 (16/9/2)	0	6
Undetermined	2	1 (1/0/0)	1 (1/0/0)	0	0
Total	100	64(59/5/0)	28 (17/9/2)	0	8

3.2. Shell biometry

Descriptive statistics of the shell trait values at the moment of sampling are given in Table 2 and their distributions are shown in Fig. 3. The average shell length was larger in the Manila clam (19.6 ± 2.5 mm) than in the GCS clam (16.4 ± 3.4 mm) (Table 2), which indicates a 18% faster growth in the Manila clam. However, the distribution of the shell length was broader in the GCS clam (range = 9.8–23.7 mm) than in the Manila clam (range = 13.6–23.8 mm), resulting in coefficients of variation (C. V.) of 0.21 and 0.12, respectively (Fig. 3 A and Table 2). An interesting observation is that maximum lengths were similar in the two species. Similar results were obtained for the remaining measured traits (Fig. 3 B–D and Table 2). Average shell weight was double in Manila clam. As in the case of shell length, the distributions of shell height, width and weight were narrower in the Manila clam. Differences between sexes for shell traits were very small and were not statistically significant (Supplementary Table S1). There was more organic matter in the Manila clam shells (2.21 ± 0.12%; $n = 28$) than in the GCS clam shells (2.16 ± 0.17%; $n = 62$), as expected from their heavier weights, but the percentage was not significantly different between the two species (ANOVA, $F = 1.565$; $p = 0.214$) (Supplementary Fig. S6).

The regression of log-transformed shell height, shell width and shell weight on log-transformed shell length showed a clear dependence of all variables on size, as usual (Laing et al., 1987). ANCOVA rendered significant differences ($p < 0.05$) in elevations for the shell's height and weight, but no for width, indicating that the Manila clam has shorter and heavier shells than the GCS clam individuals of the same length (Fig. 4). Differences in slope were always non-significant ($p > 0.05$). Mass exponents were 1 (height) or nearly 1 (width), indicating isometric growth in shell dimensions (Fig. 4 A-B). However, the mass exponent for shell weight was positive allometric (2.97), indicating a fast weight increase with length (Fig. 4 C).

3.3. Gonad development and sex proportions

The sex frequencies in each species are given in Table 3. These numbers are based on the 92 clams that were unequivocally ascribed to one or another species with genetic markers. A total of 22 individuals could not be sexed or posed some uncertainty as to their sexual ascription, and were classified as undetermined. Differences in the distribution of the three categories between species were statistically significant (Chi-squared = 8.1, $p = 0.018$). The percent of undetermined was higher in the Manila clam (46%) than in the GCS clam (14%), and the difference was statistically significant (Chi-squared =6.92, $p = 0.009$). Females accounted for 55% of the GCS clam sample, and 36% of the Manila clam sample, but these differences were not statistically significant (Fisher exact test, $p = 0.23$). The sex-ratio fitting to the expected 1:1 proportions was tested by a binomial exact test, which was non-significant for the Manila clam ($p = 0.302$) and nearly significant for the GCS clam ($p = 0.058$). Pooling sexes of the two species resulted in a significant sex-ratio bias ($p = 0.022$).

4. Discussion

4.1. Hybridization

We found no hybrids among the 92 adult animals that were

Table 2
Shell biometry in the two clam species.

Trait	Species	Minimum	Maximum	Average	S.D.	C.V.	N
Length (mm)	GCS clam	9.8	23.7	16.6	3.53	0.21	64
	Manila clam	13.6	23.8	19.6	2.43	0.12	28
Height(mm)	GCS clam	6.6	15.9	11.1	2.37	0.21	64
	Manila clam	9.8	17.0	13.7	1.72	0.13	28
Width(mm)	GCS clam	3.7	10.1	6.6	1.55	0.24	64
	Manila clam	5.5	9.8	7.9	1.11	0.14	28
Shell weight (mg)	GCS clam	35	1091	408	255	0.62	64
	Manila clam	240	1209	735	255	0.35	28

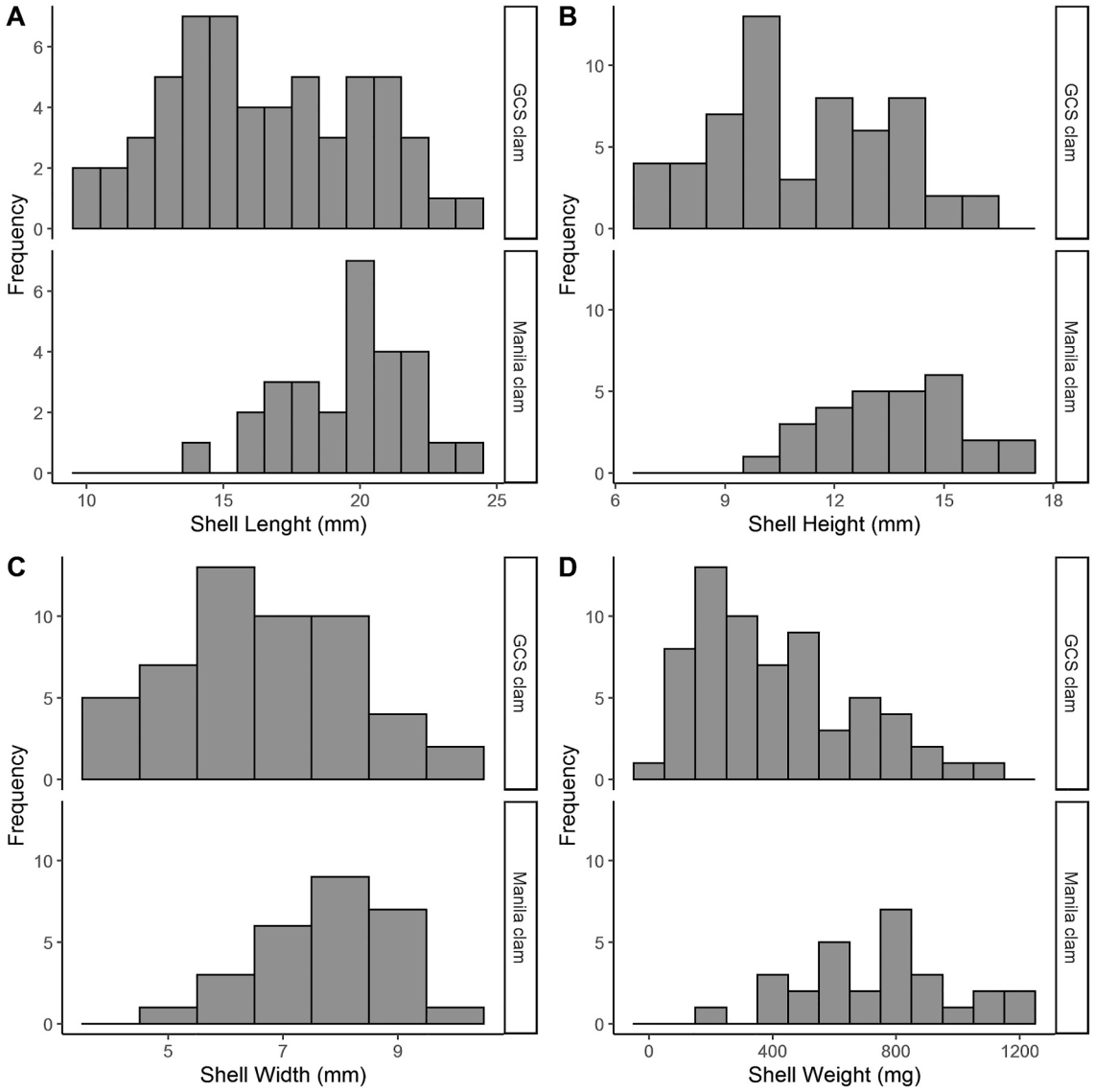

Fig. 3. Histograms showing the distribution of shell biometric traits in the two clam species. A: Shell length. B: Shell height. C: Shell width. D: Shell weight.

genotyped for 1 or 2 diagnostic markers, as all scored animals showed a single PCR product of the GCS or Manila type (were homozygotes). The genetic identity inferred from siphon morphology coincided with that inferred from genetic markers in the great majority of the scored clams, and the only two dubious individuals were unambiguously identified as non-hybrids by genetic markers. However, eight animals could not be genotyped for any marker, and therefore a genetic confirmation of their non-hybrid nature is lacking. This failure could be due to variability in the PCR primer regions, which is often reported in bivalves and associated to their high degree of nucleotide polymorphism and high numbers of repetitive regions in their genomes (Saavedra and Bachère, 2006; Suárez-Ulloa et al., 2013; Zhang et al., 2012), and prevent the annealing between the primer and the template DNA (Chiesa et al., 2016; Cordero et al., 2017, 2014). Nevertheless, the 8 individuals in which the PCR failed could be classified as GCS clam or Manila clam based on the siphon morphology. The good agreement between the results produced by siphon morphology and genetic markers in the rest of the clams, suggest that their identification based on siphons alone is

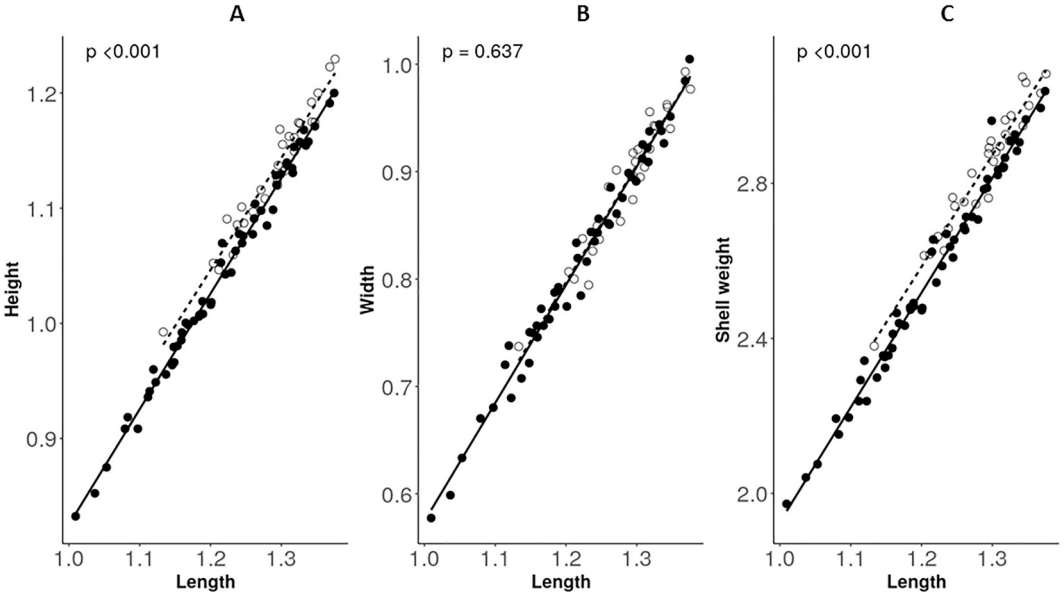

Fig. 4. Covariation between shell length and other shell size traits in the two clam species (values shown are log-transformed). A: Height. The regression equation for GCS clam was Height = −0.176 + 1.0 × Length, and for Manila clam was Height = −0.155 + 1.0 × Length. B: Width. The slope and intercept were non-significantly different, and the common regression equation was Width = −0.52 + 1.1 × Length. C: Weight. The regression equation for GCS clam was Weight = −1.04 + 2.97 × Length, and for Manila clam was Weight = −0.976 + 2.97 × Length. Open circles and broken lines correspond to Manila clam, and filled circles and black lines correspond to GCS clam. *P*-values in the figures are from the Student *t*-test for the intercept differences between species.

Table 3
Frequencies of sexes in the two clam species.

Species	Undetermined (%)	Female (%)	Male (%)	Total
GCS clam	9 (14)	35 (55)	20 (31)	64
Manila clam	13 (46)	10 (36)	5 (18)	28

correct and therefore these samples do not change the main conclusion of this study, i.e., the absence of hybrids in the studied sample of clams.

The finding of hybrid offspring in a hatchery sample by Habtemariam et al. (2015) suggest that hybrids can appear during hatchery operations. Our two-steps fertilization protocol, intended to favor hybrid fertilizations by excluding the other species' gametes in the first step, did not result in observable amounts of hybrids. While the detection of a single hybrid would have clearly indicated the success of this protocol, their absence does not necessarily imply that hybrids have not been produced in very low amounts, since our sample size has an associate error rate of hybrid identification of ca. 3%. A much more extensive survey of the population should have been carried out to detect their presence in very small amounts, but this would be costly.

There are two main explanations for our result. One is that only combinations of gametes from very few and special individuals result in a successful interspecific fertilization. Since we used only 7–8 parents per species, it is possible that such lucky combinations have not occurred. Another possibility is that, although hybrid fertilizations were successful, the viability of the hybrid offspring was so low that none, or a very low fraction of them (as many as the few percent suggested by the associated error of our sample size), have survived until the sampling time, and remained undetected. The implications of our results for future attempts to produce hybrids in captivity is that a much larger sample of parents should be used, the presence of hybrids should be scored since the embryo stage, and larger offspring numbers should be examined. Furthermore, a close scrutiny of the hybridization rates in wild and cultured populations should be pursued, in a framework of collaboration between fishermen, aquaculturists, management authorities and scientists, in order to favor the conservation of the genetic resources of indigenous GCS clam.

4.2. Comparative growth

Our experiment was designed to obtain growth data for the two species (and eventually hybrids) in a common controlled environment, allowing for a characterization of differences between species without the confounding effects of environmental variability. We gathered growth measurements on individual clams - as opposed to group measurements used in some comparative studies (e.g.: Bodoy et al., 1980; Laing et al., 1987)- which allowed us to study interindividual variation. Juanes et al. (2012) reported individual growth values for the two species in the common environment of a culture site, but the animals shared the same environment during the experimental period only, so previous environmental effects were not taken into account. Therefore, this study is the first to compare the growth performance of the two species for the whole life cycle in the same environment at the individual level.

The data presented here clearly show that the Manila clam grew faster on average than the GCS clam, which resulted in a 20% larger average size in the Manila clam at the moment of sampling. Since the culture sites involved in this study were located on the Mediterranean coast of the Iberian Peninsula, which is characterized by warmer temperatures than the native Atlantic environment of the parents during most of the year, this result agrees with the report of Laing et al. (1987) of faster growth of Manila clam at warmer temperatures. It will be interesting to see if such differences are still observed when GCS clams of Mediterranean origin are used as parents, as the genetic makeup of the species' Mediterranean populations is rather different from the Atlantic (Arias-Pérez et al., 2016; Cordero et al., 2014).

Our study has also showed a higher variability in individual growth rate in the GCS clam, and even some GCS clams reached the same size as the largest Manila clams. As the animals were kept in the same environment since fertilization, these observations suggest that the GCS clam has more genetic variability for growth rate than the Manila clam. This observation should be taken with caution, because due to the wide interfamily variability in offspring survival which is typical of bivalves (Plough et al., 2016, and references therein), and to the small number of parents used to perform the cross in our study, such difference in variability could be the unfortunate outcome of the Manila clam progeny

originating from a parental population of smaller effective size than the GCS clam. However, studies carried out with genetic markers indicate that the great majority of the parents that spawn in a hatchery contribute genetically to the adult progeny in clams (Smits et al., 2020). If such is the case here too, an alternative explanation for the interspecific difference in growth rate variability can be advanced. The lower growth variability of the Manila clam could reflect a general loss of genetic variability associated to the hatchery origin of the clams introduced in Europe in the 1970's, which are the source of the naturalized populations that live in this continent nowadays (Cordero et al., 2017). Moreover, unintentional selection for fast growing larvae and spat in the hatcheries make it possible that the imported North American clams from which the present day European clam populations derived were already genetically fast growers and carried low levels of genetic variability for growth-related genes. This would explain the observation of a higher growth performance of GCS clams with respect to native Asian Manila clams, but not with respect to introduced European Manila clams, reported by Moura et al. (2017). Additional studies using larger numbers of parents and varied grow-out environments are necessary to confirm these hypotheses.

The study of shell traits confirm the slight differences in shape between the two clam species described previously (Nerlović et al., 2016, and references therein), and suggest a small impact of environmental variability on them. The Manila clam has been reported to be usually slightly more rounded (more height for unit length) and the GCS clam more elongated, but a detailed morphometric study of their differences has yet to be carried out. The fact that the shape differences observed here under identical culture conditions fit the usual observations in natural settings indicates that shape is quite well controlled by the species' genetics. However, the influence of the culture environment in natural grow-out settings should be tested for a robust conclusion. It should be also noted that clams are infaunal animals, so the absence of sand in the culture tanks during this study might have influenced shape differences (or their absence).

Nerlović et al. (2016) found isometric and negative-allometric relationships between shell morphometric variables, while in this study all relationships were isometric. These differences could be due to the fact that we have studied a cohort while they studied a sample of wild animals, which probably was a mix of different cohorts. However, the shell weight to length relationship showed a clear positive allometry, indicating that the Manila clam usually has much heavier shells than the grooved carpet-shell clam at similar sizes. The Manila clam lives buried in the sand at more shallow depths than the GCS clam (Lee, 1996; Vilela, 1950), so they are probably more exposed to the attacks of predators such as crabs and drilling snails. Thicker shells therefore might provide the Manila clam with increased protection against predators. This trait could have contributed to the expansion of the species in European waters.

4.3. Gonad development and sex-ratio

This study was not intended to follow the reproductive cycle in detail. However, scoring the sex of the clams, addressed to test for growth differences between sexes, has evidenced some patterns that deserve some comments. In total 13 clams out of 92 could not be sexed. However, unsexed clams were 3 times more abundant in the Manila clam than in the GCS clam. This observation indicates that, at the moment of the sampling in mid-summer, many Manila clams had already undergone spawning, while the great majority of the GCS clams still were in functional reproductive state. These results agree generally with studies that reported a wide overlap in the reproductive period of the two species, but with an earlier onset of spawning events in the Manila clam in natural populations from NW Spain, were the broodstock for our study was collected (Ojea et al., 2002, 2005, 2004; Rodríguez-Moscoso et al., 1992; Rodríguez-Moscoso and Arnaiz, 1998). As the animals have been raised in a common environment since fertilization, these differences reflect an innate difference in the reproductive pattern. However, we note that, although the clams used in this study were of Atlantic origin, they were raised in a Mediterranean environment characterized by warmer water temperatures (up to 27.9 °C in summer; supplementary Fig. S2) than their parents' native Atlantic grounds. Therefore, our results suggest that under a warm environment the Manila clam tends innately to spawn earlier than the GCS clam. This conclusion agrees with reports by Delgado and Pérez-Camacho, 2007, who found that the Manila clam reached the ripe and post-spawning resting stages earlier than the GCS clam at 18 °C, but not at 14 °C. Detailed comparative studies of the gonadal cycle along the year in other environments, and involving the Mediterranean races of the GCS clam (Arias-Pérez et al., 2016; Cordero et al., 2014), are desirable in order to reach a full understanding of the differences in the reproductive cycle between the two species. Details of the gonad maturation cycle and the gamete release induction may also help to understand the occurrence of hybrids. It should be also noted that the same limitations due to the number of parents employed for the cross that were described above in relation to hybridization and growth apply to the previous considerations about reproduction.

Sex-ratios deviated markedly from the expected 1:1 ratio. Statistical significance disappeared if the unsexed animals were assumed to belong to the least frequent sex, suggesting that the observed deviations could be due to the high proportion of unsexed animals. However, it should be noted that within-family sex ratio in the Manila clam, as in other bivalves with separate sexes, is often biased in favor of one sex, and the sex-ratio is controlled by the female genotype (Ghiselli et al., 2011; Saavedra et al., 1997; Zouros et al., 1994). In this study only three females of each species were used to produce the experimental clam population, which would have resulted in an overall sex-ratio bias if the some females had produced a higher proportion of the same sex.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736824.

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