ASSORTATIVE FERTILIZATION AND SELECTION AT LARVAL STAGE IN THE MUSSELS *MYTILUS EDULIS* AND *M. GALLOPROVINCIALIS*

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Abstract.—Assortative mating (prezygotic isolation) and reduced hybrid fitness (postzygotic isolation) are typically invoked to explain the stability of hybrid zones. In the tension zone model, these factors work in opposition to migration, which promotes genetic homogeneity. Many marine animals migrate over long distances through a planktonic larval stage. Therefore, strong reproductive isolation is needed to maintain stable marine hybrid zones. However, surprisingly little is known about mating preferences and hybrid fitness in marine organisms. Smooth-shelled mussels (*Mytilus* spp.) form a well-known species complex, with hybridization over extensive areas such as the contact zone of *M. edulis* and *M. galloprovincialis* around European Atlantic coasts. This paper reports direct experimental evidence of assortative fertilization, hybrid larval inviability, and early heterosis for growth rate in *M. edulis* and *M. galloprovincialis*. Four crosses between pure *M. edulis* and *M. galloprovincialis* were analyzed with a new polymerase-chain-reaction-based diagnostic marker. Gamete competition between taxa was allowed in two out of the four crosses. Genotype frequencies observed at an early stage (36 h after fertilization) unambiguously revealed assortative fertilization when gamete competition was allowed. A significant reduction in hybrid viability was subsequently observed during the larval stage. At the same stage an antagonistic effect, heterosis, was observed on growth rate. However, even if heterosis is observed in the F1, it is expected to vanish in subsequent hybrid generations. Although specialization for different habitats and asynchronous spawning have been mentioned as factors contributing to the maintenance of the blue mussel hybrid zone in Europe, we argue that assortative fertilization and reduced hybrid fitness are important factors that also contribute to the stabilization of this zone. These results emphasize that multiple factors may act concomitantly in a barrier to gene flow, especially in complex life cycles. Furthermore, they show that assortative mating through gamete preference, as already demonstrated for sea urchins, may play a role in speciation processes taking place in the sea.

Key words.—Gamete competition, heterosis, hybrid viability, *Mytilus edulis*, *Mytilus galloprovincialis*, preferential fertilization.

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Hybridization is not an uncommon phenomenon in marine organisms (Gardner 1997). However, empirical and theoretical studies on hybrid zones have focused mainly on terrestrial species (reviewed by Barton and Hewitt 1985, 1989; Harrison 1993; Arnold 1997). Exogenous and endogenous factors involved in preventing genomes of two species from merging depend on the life cycle. In most marine bivalves, this cycle is characterized by a sessile adult phase, high fecundity, external fertilization, and extensive larval dispersal, which provide extensive gene flow that should prevent geographic isolation and the potential for strong selection in an often unpredictable environment. Therefore, it is perhaps not surprising that one of the best-known marine hybrid zones, between the mussels *Mytilus edulis* and *M. galloprovincialis* in European Atlantic coasts, has revealed an unusual complexity. This zone shows a considerable extension, from the southwest of France to Scottish coasts (Skibinski et al. 1978, 1983; Coustau et al. 1991a) and has a mosaic structure, in which populations of pure genotypes alternate with hybrid populations (Skibinski et al. 1983; Daguen et al. 2001). Spatial distribution of alleles suggests the influence of environmental factors such as salinity (Skibinski 1983) and wave exposure or tidal height (Gosling and Wilkins 1981; Gardner and Skibinski 1988; Gosling and McGrath 1990; reviewed in Gardner 1994). Depending on location, hybrid populations display highly variable levels of introgression, from complete introgression (intermediate allele frequencies and Hardy-Weinberg plus linkage equilibrium, HWLE) to simple admixture (superposition of two parental populations; Skibinski et al. 1983; Daguen et al. 2001). Thus, despite extensive hybridization, there still remain two incompletely blended genetic entities, implying some barrier to gene flow. Habitat specialization and, although less studied, asynchronous spawning have been shown to be involved in this barrier (Gardner 1994). How these two mechanisms act exactly is not yet well understood, and it is not known whether they alone can explain the hybrid zone structure and stability observed. Other mechanisms such as gamete recognition and fertilization preference could be implicated (Arnold 1997).

Even though cellular mechanisms have been extensively examined between well-separated marine species (review in Palumbi 1992, 1994), experimental evidence from naturally occurring marine hybrid zones is lacking, although this is an important issue in the understanding of mechanisms that actually have a bearing on the speciation process. In the case of *M. edulis* and *M. galloprovincialis*, experimental crosses...
as well as genetic analyses of natural populations have demonstrated the existence of cross-fertilization (Gardner 1994), but we are not aware of any test of gamete preference. In any case, these mechanisms alone do not suffice to explain the long-term maintenance of separate gene pools once hybrids are produced in visible numbers. There has to be some sort of lower fitness of hybrids at the postzygotic stage (Barton and Hewitt 1989). This is why the evaluation of relative fitness parameters among parental and hybrid genotypes is a keystone in the study of hybrid zones (Barton and Hewitt 1989). The Mytilus hybrid zone in Europe has mainly been investigated using genotype-phenotype associations in adult natural populations (Coustau et al. 1991b; Gardner and Skibinski 1991; Gardner et al. 1993; Gardner 1994; Hilbish et al. 1994). These analyses concluded that M. galloprovincialis genotypes have an advantage over both hybrids and M. edulis genotypes for growth, fecundity, strength of attachment, and viability (Gardner and Skibinski 1990, 1991; Gardner 1994). However, such studies pose the problem of whether two allozyme loci are sufficient to represent the degree of mixing in the whole genome. Surprisingly, few studies of experimental crosses have been conducted. We were able to find only two such studies in the literature (Lubet et al. 1984; Beaumont et al. 1993). Lubet et al. (1984) did not find any differential viability or growth between parental and F1 categories at the adult stage, whereas results in Beaumont et al. (1993) suggested reduced hybrid viability during the early larval stage.

The aims of the present study were to investigate the effect of gamete competition on cross-fertilization success and the relative survival and growth rate of parental and F1 individuals from controlled crosses at different stages of the life cycle in the mussels M. edulis and M. galloprovincialis. We focused on early stages of the life cycle (larval stage, metamorphosis) during which most of the developmental processes and mortality take place and which remain largely unexplored. In this type of study, different genotypes are usually kept separate in different tanks (e.g., Beaumont et al. 1993). In our experiment, however, all progeny were reared in the same tank and genotypes were subsequently deduced from molecular marker analyses. This approach has several advantages: (1) environmental variation is erased; (2) interaction between genotypes, such as competition, is allowed; and (3) the effects of gamete competition can be investigated by comparing genotypic composition just after fertilization between crosses in which competition is allowed and crosses in which it is not (Huvet et al. 2001). We also present a new marker named EFbis, diagnostic between the M. edulis and M. galloprovincialis used in the crosses, which was obtained by the exon-primed intron-crossing polymerase chain reaction (EPIC-PCR) method (Palumbi 1995; Bierne et al. 2000) in the first intron of the elongation factor 1 α gene (Regier and Shultz 1998).

**Materials and Methods**

**Crosses**

*M. edulis* adults were collected from two North Sea populations, at Grand-Fort-Philippe (GFP, France) and in a population from Holland (HOL). *M. galloprovincialis* adults were collected from two populations at Sète (SET) on the French Mediterranean coast and at Vigo (VIG) in Galicia on the Atlantic coast of Spain. Temperature of maturation was controlled in the laboratory in closed-circuit tanks (around 24°C for *M. galloprovincialis* and around 18°C for *M. edulis*). Maturity was regularly checked by inducing spawning of a few individuals by a thermal shock (Lubet et al. 1984). Temperature and feeding were adjusted to synchronize maturation among species. *Mytilus galloprovincialis* reached full maturation slightly earlier than *M. edulis*. In December 1998, all the adults were individually induced to spawn. Four female *M. edulis* spawners, two from GFP and two from HOL, so we decided to equalize the crossing scheme by choosing four *M. edulis* males (two from GFP and two from HOL), four *M. galloprovincialis* females (two from SET and two from VIG), and four *M. galloprovincialis* males (two from SET and two from VIG). Oocytes were gently sieved, counted using Malassez slides, and adjusted to 10⁶ per female. Spermatozoa were treated in the same manner using Thoma slides and adjusted to 10⁶ per male to obtain a 1:1 sperm:egg ratio for the fertilization.

Two kinds of treatments were performed, by pooling either gametes (GP) or embryos (EP). For a GP cross, fertilization occurred within a mix of oocytes from all the females and a mix of spermatozoa from all the males. In this kind of cross, among-species gamete competition was allowed. For an EP cross, the following crosses were made in four different beakers: *M. edulis* oocytes x *M. edulis* sperm, *M. edulis* oocytes x *M. galloprovincialis* sperm, *M. galloprovincialis* oocytes x *M. edulis* sperm, and *M. galloprovincialis* oocytes x *M. galloprovincialis* sperm. Four hours after fertilization, embryos resulting from these four crosses were mixed together. Two replicates of the GP: GP1 and GP2, and of the EP: EP1 and EP2, were produced.

**Collection and Treatment of Larvae**

For each trial, larvae were transferred into a 30-L cylindrical larval growing pond (initial larval density = 10 larvae ml⁻¹). Sea water at 18°C, filtered at 0.2 μm, was changed every two days. Larvae were fed with Pavlova lutheri and Chaetoceros calcitrans every day. Thirty-five days after fertilization larvae were undergoing metamorphosis and were transferred in a 20-L closed-circuit tanks for five days. A coconut rope was placed in each tank to encourage fixation. The circuit was then opened to a continuous water flow, sieved at 200 μm to prevent contamination by local larvae (even though this was highly improbable during the season of the experiment), and kept at 18°C. Samples were taken 36 h after fertilization, at the end of the larval stage (day 35) before settlement, and at the juvenile stage (day 100). Unfortunately, all the individuals in EP2 died for an unknown reason between day 35 and day 100. Individualization of larvae as well as DNA extraction was performed as described in Bierne et al. (1998).

**Molecular Marker**

Intron-spanning primers were designed in the elongation factor 1 α cDNA sequences (Regier and Shultz 1998) as described in Bierne et al. (2000) and references therein. Prim-
er sequences are EFbis-F: 5'-ACAAGATGGAACAATAC CGAAACCACC-3' and EFbis-R: 5'-CTCAATCATGTTGT CTCCATGCC-3'. We used the PCR and electrophoresis procedures described in Daguin and Borsa (1999), except that annealing temperature was set to 54°C. The EFbis locus exhibited length polymorphism that appeared to be diagnostic between the M. edulis and M. galloprovincialis used in the present experiment (see Fig. 1).

**Statistical Analyses**

The four M. galloprovincialis alleles (G0, G1, G2, G3) were pooled as a single synthetic allele, G, for the analysis. Genotypes were: M. edulis (EE), F1 hybrid (EG), and M. galloprovincialis (GG). Random mating was tested for the 36-h samples using the Hardy-Weinberg exact test in the GENEPOP software (Raymond and Rousset 1995). Deviations from the expected heterozygote frequencies were estimated as $D = (H_{obs}/H_{exp}) - 1$, where $H_{obs}$ is the number of observed heterozygotes and $H_{exp}$, the number of expected heterozygotes under Hardy-Weinberg equilibrium (HWE). Homogeneity of genotypic frequencies was tested within crosses among time samples and between crosses within each sampling time using Fisher’s exact test in the GENEPOP software. Differences in growth rates among genotypes were tested using a one-way ANOVA with fixed effect.

Heterosis can be quantified as $h_p = Q/L$, where $L$ is the trait difference between the two parental lines and $Q$ is twice the deviation of the hybrid from the midparent value (Griffing 1990; Hedgecock et al. 1995). It is a useful synthetic coefficient that allows the hybrid to be easily compared with the parental phenotypic values: $h_p > 1$ means heterosis, $h_p < -1$ means hybrid depression and $-1 < h_p < 1$ means that hybrids are intermediate.

**RESULTS**

**Effect of Gamete Competition at 36 Hours**

No significant difference in genotype frequencies was observed between GP1 and GP2 or between EP1 and EP2 at 36 h. Data were thus pooled among GPs and EPs. Results are synthesized in Table 1. Estimated abortion rates, quantified by comparing the number of oocytes used and the number of larvae obtained at 36 h were 18% in GP and 12% in EP. Allele frequencies at 36 h departed significantly from equal species contribution (GP: $P = 0.017$; EP: $P < 0.001$) and genotype frequencies were significantly different between GP and EP ($P < 0.001$). This was partly due to a lower contribution of the M. edulis allele, E, especially when gametic competition was allowed (EP: E = 0.42; GP: E = 0.23). Another source of differences between GP and EP was that genotypic frequencies strongly departed from HWE in GP ($P < 0.001$), whereas no departure was observed in EP.

The aim of this experiment was first to test for random mating when gamete competition was allowed (GP). Clearly this was not the case, because a significant heterozygote deficiency was observed in this cross when compared to the expectation under HWE. The interpretation of this result is complicated because, despite our standardization, we do not know whether the effective contribution of each species was the same in eggs and sperm. Equal frequencies between sexes are a necessary condition for HWE. However, unequal frequencies generate an excess of heterozygotes under random mating. Using the observed frequencies in GP crosses (e.g., E = 0.23 and EE = 0.11), one can deduce that the effective frequency of the M. edulis oocytes in this cross, Ec, was 0.11 < Ef = 0.35. The predicted heterozygote excess therefore lies between 0.00 (sperm and egg frequencies both equal to 0.23) and 0.08 (frequency equal to 0.11 in one sex and 0.35 in the other). The observed value of $D = -0.34$ clearly means that there are less hybrids than expected under random mating.

**Differential Viability**

No significant difference in genotype frequencies was observed between GP1 and GP2 within the same sampling times.
(data not shown), and data were pooled. Genotypic frequencies are presented in Figure 2A. A significant difference in genotypic frequencies was observed between day 35 and day 100 ($P < 0.001$). EP1 and EP2 exhibited significant differences in genotypic frequencies at day 35 ($P = 0.04$) and data were not subsequently pooled. Figure 2B depicts the EP1 genotypic frequencies at day 35 and day 100. EP2 is not represented, because all individuals of this group were lost between day 35 and day 100. EP2 genotype frequencies at day 35 were $EE = 0.15$, $EG = 0.28$, and $GG = 0.57$ ($n = 92$). A significant difference in genotype frequencies was observed between 36 h and day 35 for EP1 ($P = 0.01$) and EP2 ($P = 0.014$) and between day 35 and day 100 for EP1 ($P < 0.001$). Table 2 summarizes the relative viability normalized by the fittest genotype and the corresponding heterosis coefficient, $h_p$. On the whole, $M. galloprovincialis$ exhibited the best viability, there was strong selection against $M. edulis$ genotypes, and hybrids were intermediate as shown by $-1 < h_p < 1$. Interestingly, in EP crosses, hybrid depression was detected during the larval stage (EP1: $h_p = -3$, EP2: $h_p = -1.6$), although a strong subsequent selection against $M. edulis$ during the settlement process did restore a slightly less negative $h_p = -0.8$.

**Differential Growth**

Table 3 presents the mean shell length and the 95% confidence interval for each genotype and heterosis coefficients in each trial at the pediveliger stage (day 35) and the spat stage (day 100). $F_1$ hybrids were significantly larger than parental genotypes in two out of the four trials (GP1: $F = 0.07$, df = 107, $P = 0.9$; GP2: $F = 2.2$, df = 99, $P = 0.11$; EP1: $F = 3.4$, df = 91, $P = 0.03$; EP2: $F = 6.7$, df = 91, $P = 0.002$) and overall ($P = 0.002$) using Fisher’s method to combine independent tests (Sokal and Rohlf 1996). An opposite trend was thus observed for larval growth rate to that for larval viability. After metamorphosis (day 100), $F_1$ hybrids became smaller than $M. galloprovincialis$ (Table 3, the comparison with $M. edulis$ was impossible because of strong mortality for this genotype). This was significant in one out of three trials (GP1: $F = 0.24$, df = 156, $P = 0.6$; GP2: $F = 0.4$, df = 135, $P = 0.5$; EP1: $F = 5.9$, df = 75, $P = 0.015$).

**Discussion**

Mechanisms known to be involved in gamete recognition among marine organisms have been examined (Palumbi 1994), and some molecules implicated in the egg-sperm interaction identified (Vacquier and Moy 1977; Ward et al. 1985; Vacquier et al. 1990). Interactions can take place at various stages from gamete release to fertilization (Garbers 1989), and it is not always possible to distinguish among specific spawning induction, gamete attraction, or fertilization processes. Analyses may be further complicated by early developmental failure. If certain mechanisms are now well known, it is also true that few studies have examined fertilization patterns between closely related marine species (Palumbi 1994). Furthermore, these patterns have been almost exclusively considered in the absence of gamete competition, although assortative fertilization has been recently demonstrated in the sea urchin *Echinometra mathaei* (Palumbi 1999). Before using our results to assess assortative fertilization and hybrid fitness in *Myltilus* species, we will discuss the unequal gamete contributions of the two species in our experiment.

### Table 2. Relative viability normalized by the fittest genotype and heterosis coefficient, $h_p$, during the larval stage (36 h to day 35) and globally (36 h to day 100). EE, *Myltilus edulis*; EG, $F_1$ hybrids; GG, *M. galloprovincialis*. Sample sizes as in Figure 2.

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Unequal Gametic Contributions of the Two Species

In such an experiment, it is nearly impossible to obtain equal parental contributions. Even if it were possible to adjust parents to a precise 1:1 ratio, there is no way to standardize gamete quality among parents. The realized effective gamete ratio is thus expected to be distorted and must be estimated a posteriori from the data. It is therefore not surprising to obtain a significant departure from equal species contribution. G alleles may be overrepresented either because errors in gamete counts have favored *M. galloprovincialis* by chance or because *M. galloprovincialis* gametes were more efficient or more viable. Differential early abortions may also be involved. The fact that the excess of G alleles is larger in GP than in EP crosses can be attributed either to a severe error in sperm counts (this should affect GP crosses but not EP crosses, in which the two sperm types are not in competition) or an enhanced effect of differences in sperm quality by gamete competition (Boudry et al. 2002). Because we suspect that *M. edulis* parents were less mature than *M. galloprovincialis* parents, we favor the hypothesis of better quality of *M. galloprovincialis* gametes.

Assortative Fertilization

The heterozygote deficiency obtained in the GP cross may be explained either by (1) assortative fertilization (gamete choice); or (2) differential fertilization (fertilization failure) or abortion. The lack of heterozygote deficiency in the EP cross favors the first hypothesis because differential fertilization or abortion should have occurred both in EP and GP crosses, although gamete choice was only possible in GP. Moreover, abortion percentages seem insufficient to generate the observed heterozygote deficiency in GP. Indeed, in the worst case, if we hypothesize that all the 18% aborted embryos in GP were hybrids, the heterozygote deficiency would reach the value of $D = -0.08$. Assortative fertilization must therefore have occurred in GP. In conclusion, when there is no other choice (EP cross), cross-fertilization occurs without much problem, whereas when choice is possible (GP) in-species fertilization is preferred.

Our experiment does not provide a direct demonstration of the asymmetry of the fertilization barrier (i.e., whether crosses between male *M. galloprovincialis* and female *M. edulis* are easier than the reverse). However, our results suggest asymmetry. As shown in the results section, the effective frequency of the *M. edulis* oocytes in GP crosses was $< E_f < 0.35$. We can use EP crosses to estimate the value of $E_f$. Both crosses are subject to equal counting biases (because the same gamete solutions were split among the two crosses). The $E$ frequency in the EP experiment (representative of the maximum count bias) was 0.42. Assuming, conservatively, that the 18% abortion in GP only affected embryos derived from E oocytes (initially in frequency 0.42), the minimal frequency of E in fertilized oocytes would be $E = 0.30$, which is in the upper range of possible values (0.11–0.35). The effective contribution of *M. edulis* males was therefore close to its minimum value (0.11). Thus, almost all *M. edulis* sperm might have been employed in conspecific fertilization, whereas hybrids might be virtually exclusively the product of *M. edulis* oocytes with *M. galloprovincialis* sperm. This observation calls for additional experiments with gamete ratio manipulation.

Hybrid Fitness

The overall viabilities from the embryo to the spat stage (day 100) seem to reflect directional selection favoring *M. galloprovincialis* genotypes. However, this is no longer true when the two different phases (the larval phase from embryo to pediveliger and metamorphosis from pediveliger to spat) are considered separately. During the larval phase, no significant viability differences were observed in GP crosses, whereas a significant depression for hybrid larval viability was observed in EP crosses (EP1: $h_x = -3$, $P = 0.008$; EP2: $h_x = -1.6$, $P = 0.015$). We confirm the results obtained by Beaumont et al. (1993) that hybrid depression for larval viability occurs between *M. edulis* and *M. galloprovincialis*, but we did not detect such depression in the GP crosses. However, the power to detect inviability was lower in GP crosses because of the imbalance among different types of embryos right from the beginning. Furthermore, in the EP crosses, both categories of hybrids (male G × female E or the reverse) are present in approximately equal frequencies, whereas most hybrids in the GP crosses are suspected to be of the male G × female E type. If both categories of hybrids have different viabilities, this may explain the differences between EP and GP. Results in Beaumont et al. (1993) also suggested that this might be the case as male G × female E hybrids had always exhibited a better viability than the reverse type in their experiment. Reduced $F_1$ viability is not a predictable outcome in interspecific hybrids, although it is not uncommon (Barton and Hewitt 1985). It is much more frequent in suc-

| Table 3. Mean shell length and 95% confidence interval at two sampling times (day 35 shell length [μm] and day 100 shell length [mm]) and heterosis coefficient, $h_x$, at day 35. EE, *Mytilus edulis*; EG, F, hybrids; GG, *M. galloprovincialis*. Sample sizes as in Figure 2. |
| --- | --- | --- | --- | --- | --- |
| | EE | EG | GG | EE | GG |
| GP1 | 281 ± 12 | 280 ± 21 | 271 ± 13 | 1.98 ± 0.17 | 2.02 ± 0.06 |
| GP2 | 256 ± 7 | 271 ± 11 | 262 ± 13 | 1.7 ± 0.15 | 1.75 ± 0.07 |
| EP1 | 257 ± 8 | 257 ± 8 | 261 ± 10 | 2.16 ± 0.2 | 2.45 ± 0.12 |
| EP2 | 208 ± 10 | 230 ± 12 | 190 ± 13 | — | — |
cessive backcross or F₂ generations because of gene coadaption breakup (Edmands 1999). Despite inferior viability, hybrid veligers exhibited an increased growth rate (mean \( h_p = 3.8, P = 0.002 \)), confirming the heterosis for larval growth rate suggested by Beaumont et al. (1993). Heterosis is usually expected for F₁ progeny because hybrid dysgenesis only appears in subsequent generations of hybridization. Indeed, it has long been recognized that interspecific hybrids, although often sterile, show increased vigor and growth (a phenomenon called “luxuriance” by Dobzhansky 1952).

Tendencies observed during the larval stage were reversed during metamorphosis and early spat stages. A strong selection against *M. edulis* genotypes restored an intermediate \( h_p \) for viability, and F₁ hybrids were smaller than *M. galloprovincialis* spat.

A general caveat on results such as ours is the influence of environment. Our rearing facilities are on the Mediterranean sea in a region where the environment should be favorable to *M. galloprovincialis*. Because genotype-by-environment interactions may be important, such experiments should be replicated in a gradient of environmental conditions. However, we are left with the fact that hybrids are less viable than either parent during the larval phase, which shows that, at least in some environmental conditions, selection is not simply directional.

**Implications for Stability of the Hybrid Zone**

Selection against hybrids is the most frequent explanation proposed for hybrid zone stability and is probably the most widespread (Barton and Hewitt 1989), although it may act in conjunction with other mechanisms (Arnold 1997). In the case of the *M. edulis* and *M. galloprovincialis* hybrid zone, habitat specialization or asynchronous spawning have been identified as barriers to gene flow (Gardner 1994), but alone they seemed to be insufficient to explain the intensity of the barrier, given the extensive hybridizations observed. Our results suggest two new isolation mechanisms that may have been overlooked previously because the larval phase was not studied. First, partial postzygotic isolation can proceed from a reduced hybrid fitness during the larval stage. Second, partial prezygotic isolation is observed when competition is possible (which is probably the case in natural mussel beds), providing another example of what may be an important contribution to speciation mechanisms in marine invertebrates.

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**Literature Cited**


