Heritability of shell pigmentation in the Pacific oyster, *Crassostrea gigas* ☆

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**A B S T R A C T**

The Pacific oyster (*Crassostrea gigas*) is a species of considerable economic importance, with among the highest global production of any cultured aquatic animal species. In the interest of increasing the value of Pacific oysters sold as “singles” for the half-shell market, we explored the feasibility of modifying shell pigmentation through selective breeding by estimating both the broad- and narrow-sense heritability of total left-shell pigmentation in *C. gigas*. Twenty-six full-sib families derived from parents collected from a naturalized population in Dabob Bay, WA, were spawned in the hatchery and raised in an intertidal environment for 2 years. At harvest, we sampled shells from each family and quantified their total left-shell pigmentation using digital image analysis. We estimated broad-sense heritability based on full-sib intraindividual correlations and narrow-sense heritability based on midparent-offspring regression as 0.91±0.38 and 0.59±0.19, respectively. We further examined the distributions of pigmentation levels among individuals within full-sib families and found high within-family variation in total shell pigmentation that in the majority of families was normally and continuously distributed. However, offspring within two families segregated into phenotypically distinct “lighter” and “darker” shell groups in a 3:1 ratio (χ2, P=0.766) supporting the hypothesis that a single major gene is segregating in these families with the “light” allele being dominant over the “dark” allele. We conclude that selective breeding acting on this high additive genetic variance should be effective in altering total shell pigmentation and that further work is needed to confirm the existence and mode of inheritance of a putative major gene affecting total shell pigmentation in *C. gigas*.

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1. Introduction

Visual perception of food products is known to affect consumer preference and, therefore, product value (Kahn and Wansink, 2004). For example, the level of red pigmentation in salmon flesh is positively correlated with consumers’ enjoyment of the product (Sylvia et al., 1995) and their willingness to pay a premium price (Alfnes et al., 2006). Similarly, consumer preference for Pacific oysters (*Crassostrea gigas*) may also be influenced by visual cues including shell and meat color (R. Jacobsen, pers. comm.; Nell, 2001). In this paper, we explore the possibility of modifying an important component of visual perception, shell pigmentation, through selective breeding by examining this trait’s genetic basis.

Considerable evidence suggests that mollusk shell pigmentation is under at least partial genetic control, and therefore amenable to artificial selection. Furthermore, distinctive pigment colors or color patterns in several marine shellfish species have been shown to be controlled by genes segregating at only one or two loci. For example, variation in shell banding pattern in the hard clam *Mercenaria mercenaria* (Chanley, 1961) and the Chilean scallop *Argopecten purpuratus* (Winkler et al., 2001) are reported to be controlled by single genes. Some discrete shell color morphs also appear to have a simple genetic basis, being determined by either a single gene as in the case of the mussel *Mytilus edulis* (Innes and Halley, 1977; but see Newkirk, 1980), the bay scallop *Argopecten irradians* (Adamkewicz and Castagna, 1988), the pearl oyster *Pinctada fucata martensii* (Wada and Komaru, 1990) and the Japanese abalone *Haliotis rufescens* (Kobayashi et al., 2004) or by two interacting genes as in the Chilean scallop *Argopecten purpuratus* (Winkler et al., 2001). When a character is determined by a small number of genes with straightforward patterns of dominance, appropriate test-matings can reveal an individual’s exact genotype for that character and selection goals can be achieved in just a few generations.

In Pacific oysters, Nell (2001) reported true-breeding lines with golden shells and golden mantles were successfully developed over a small number of generations, implying that this discrete color variant is under the control of a limited number of genes. More typically,
However, Pacific oyster shell pigmentation spans a continuum from near-white, pigment-free shells to near-black, fully pigmented shells, and the absence of discrete phenotypic classes has led most researchers to view Pacific oyster shell pigmentation as a continuously distributed, quantitative trait under polygenic control (Imai and Sakai, 1961; Brake et al., 2004; Hedgcock and Grupe, 2006; Batista et al., 2008). Selection for such polygenic, quantitative traits differs from discretely-distributed, qualitative traits because the multi-locus genotype of any given individual cannot be determined with certainty. As a result, breeding strategies for quantitative traits rely upon population-level parameters that summarize the effects of all contributing loci such as how much of the total phenotypic variation in the trait ($\sigma^2_T$) can be attributed to additive genetic variance ($\sigma^2_A$) due to allele-specific contributions and to non-additive genetic variance ($\sigma^2_D$) due to interactions among alleles within or between loci (dominance and epistasis respectively). Polygenic traits strongly influenced by non-additive genetic variation require breeding strategies that identify optimal gene combinations such as cross-breeding, whereas traits strongly influenced by additive genetic variation (i.e. traits that are highly heritable) require breeding strategies that accumulate favorable genes in the selected population over time using selection (e.g. Falconer and Mackay, 1996). Although several studies have demonstrated that Pacific oyster shell pigmentation is under a high degree of genetic control (Imai and Sakai, 1961; Brake et al., 2004; Hedgcock et al., 2006), these studies used experimental designs that precluded partitioning total phenotypic variation into additive or non-additive genetic components. In this paper, we collected data from parents and their progeny to estimate both the broad-sense heritability, ($h^2=[\sigma^2_A+\sigma^2_D]/\sigma^2_T$) which includes both the additive and dominant genetic variation, and the narrow-sense heritability ($h^2=\sigma^2_A/\sigma^2_T$) which includes only additive genetic variation, of left (“cupped”) shell pigmentation in Pacific oysters collected from a naturalized population in the Pacific Northwest region of the United States. Variation among individuals within full-sib families was also examined to determine if offspring segregation patterns offer additional clues about the inheritance of left-shell pigmentation in C. gigas.

2. Materials and methods

2.1. Spawning and nursery protocol

We collected three hundred adult C. gigas from Dabob Bay, Washington, US (47°8' N, 122.87° W), and transported them to the Hatfield Marine Science Center (HMSC), Newport, Oregon, US (44°6.7' N, 124.1° W), in January 2002. Animals were held in flow-through tanks receiving 18 °C sand-filtered seawater and a mixture of Isochrysis galbana (Iso) and Cheatoceros calcitrans (Cc) at a concentration of approximately 50,000 to 80,000 cells ml$^{-1}$. In April 2002, we stripped spawned 52 individuals within a 48 h period as per Langdon et al. (2003) and paired them randomly to create 26 unrelated full-sib families. The left shells and tissue samples from all parental animals were retained for future analysis. Fertilized eggs were allowed to develop into veliger larvae for 24 h in family-specific 20-l containers filled with 0.2 μm filtered seawater held at 25 °C.

Veliger larvae from each family were then split into two 60-l larval culture tanks at a concentration of 10 larvae ml$^{-1}$. Each larval tank is referred to here as a culture and each single-pair mating is referred to as a family; therefore there were two cultures for each of 26 families resulting in 52 larval tanks. We fed each culture daily with a mixture of Iso and Cc and drained and re-filled them twice per week with 0.2 μm-filtered seawater at 25 °C. After 1 week we randomly thinned all cultures to 1 larva ml$^{-1}$. After 2 weeks, larval cultures were passed through stacked 243 μm and 80 μm sieves. Larvae retained on the 243 μm sieve were exposed to 2×10$^{-4}$ M epinephrine in order to induce metamorphosis (Coon et al., 1986). Larvae retained on the 80 μm sieve were returned to larval tanks and the setting process repeated for an additional week, by which time all viable larvae from each family had set.

Successfully metamorphosed spat were transferred to culture-specific upwellers held in semi-recirculating systems which received approximately 6 exchanges day$^{-1}$ of UV-irradiated, 1 μm filtered seawater and fed microalgae to a final concentration of approximately 80,000 cells ml$^{-1}$. Seawater temperature in the upweller system was gradually reduced from 25 °C to 18 °C. Spat grew in upwellers until they were retained on a 6.4 mm sieve, after which, they were transferred to culture-specific spat bags (2 mm mesh) held in storage tanks receiving ambient 1 μm filtered seawater (mean 12.4 °C) and batch-fed microalgae to a final concentration of approximately 80,000 to 100,000 cells ml$^{-1}$ twice per week (Langdon et al., 2003).

Prior to planting in the field, we collected tissue samples from a minimum of 16 spat from each family (mean count=25 spat) and stored them in 95% EtOH for subsequent microsatellite genotyping to test for cross-contamination among families during the larval and nursery stages of culture. Due to mortality in the sampled pools, families 10 and 34 provided only four and eight spat, respectively, for microsatellite genotyping.

2.2. Genotyping and parentage assignment

We extracted genomic DNA from both the parent and spat tissue samples and genotyped them at 4 microsatellite loci (umCg120, Huvet et al., 2000; imbCg049, Magoulas et al., 1998; ucdCg120 and ucdCg197, Li et al., 2003) following previously published protocols (Camara et al., 2008). Parental assignment was performed using PAPA v.1.1 (Package for the Analysis of Parental Allocation; Duchesne et al., 2002), and incorporated the pair-wise mating structure used in this study to increase assignment accuracy. PAPA employed likelihood-based parental allocation by computing the probability that each parental pair generated the observed offspring genotype and assigned parentage to the parental pair with the highest breeding likelihood. Failed allocations could also occur if all possible parental pairs of a given offspring showed zero breeding likelihood or if more than one parental pair shared the highest non-zero breeding likelihood. Two user defined parameters, the global level of transmission error and the distribution of transmission error over alleles, were set at 0.1 and 0.1, respectively, to minimize parental allocation error (Duchesne et al., 2002).

2.3. Field trials

Six replicate groups of 60 oysters from each culture were weighed and stocked into 0.3 m × 0.3 m small-mesh sleeves (2 mm mesh). Each small-mesh sleeve was then inserted into larger rectangular growout bags (0.53 m × 0.81 m; 6-mm mesh). Growout bags were planted intertidally on-bottom in Dabob Bay, Washington, in the Fall of 2002. The experimental plot was partitioned into three blocks, positioned at 0.0 m, +0.3 m, and +0.6 m MLW, respectively, to account for variation in intertidal aerial exposure on shell pigmentation. Due to variable survival in the nursery, six families were represented in the field by only one of the two cultures. In the Spring of 2003 (day 192 in the field), oysters were transferred from the small-mesh bags directly into the larger 6-mm mesh intertidal growout bags. Oysters were cleaned again in the Fall of 2003 and harvested in the Summer of 2004 after 664 days in the field.

2.4. Determination of total shell pigmentation

Immediately following harvest, we randomly sampled 10 animals from each culture-block combination and returned them to HMSC. These animals were shocked and their shells stored in plastic totes covered with reflective tarps to prevent shell damage and fading from
UV exposure. Shells were soaked in a 6% sodium hypochlorite solution for 2 h (Sturm et al., 2006), which preliminary tests found efficiently removed biotic and abiotic fouling without affecting shell pigmentation, and then rinsed thoroughly with freshwater and given a light coating of mineral oil. This protocol was also used to process left shells from all parental oysters.

We photographed all parental and progeny shells using a Sony digital camera (MVC-CD300) mounted on a light stand equipped with four 150 W bulbs to provide uniform and consistent illumination. In addition, all images included a gray-scale standard to ensure consistent exposure among photographs. Images were analyzed using Image-Pro Plus image analysis software (V. 4.5.1, 2003, Media Cybernetics Inc., Silver Springs, MD, USA) that allowed us to: 1 automatically outline oyster shells, which was faster and more accurate than outlining manually; and 2 determine the optical density of each shell (i.e. how much light it reflected independent of color). Total shell pigmentation was, therefore, defined as the overall lightness or darkness of the entire shell, due to variation in both pigmentation intensity and coverage, on a scale ranging from 0 (completely white) to 255 (completely black). We observed little variation in shell pigment color, with the primary pigment color in this group of oysters being purplish-black. We therefore did not separate shells by pigment color.

2.5. Data analysis

A random-effects, nested two-factor analysis of variance was performed using PROC GLM in SAS statistical software (SAS, V.8, 2002, SAS Institute, Cary, NC, USA) to test the significance of block, family, culture within family, block×family interaction, and block×culture within family interaction on total left-shell pigmentation in Pacific oysters raised intertidally in Dabob Bay, WA.

We then estimated the relative influence of dominance ($\sigma^2_D/\sigma^2_T$) by first computing the intraclass correlation among full sibs ($r_{FS}$, Falconer and Mackay, 1996), which represents the fraction of total phenotypic variation explained by among-family variation ($\sigma^2_T$):

$$t_{FS} = \frac{\sigma^2_F}{\sigma^2_T} = \frac{1}{2} + \frac{\sigma^2_A}{\sigma^2_T} = \frac{\sigma^2_D}{\sigma^2_T}.$$  

Because $t_{FS}$ includes both additive and dominance variance and $h^2$ includes only additive genetic variance, we were able to determine the proportion of the total phenotypic variance attributable to dominance ($\sigma^2_D/\sigma^2_T$) and thus the broad-sense heritability:

$$h^2 = \frac{\sigma^2_A}{\sigma^2_T}.$$  

Variance around $h^2$ was estimated using jackknife procedures outlined by Sokal and Rohlf (1995), based on the deletion of entire families rather than individuals observations.

To further examine the distribution of total left-shell pigmentation among individuals within families, we constructed histograms of the frequency distributions of shell pigmentation within each family. A total of 1312 oysters were used to generate these histograms, with the number of individuals used per family ranging from 22 to 60. Kolmogorov–Smirnov analysis was performed on a per family basis to determine if variation among siblings was normally distributed (Sokal and Rohlf, 1995). When these distributions showed discrete phenotypic classes, chi-square analysis was used to determine if observed segregation ratios in offspring total left-shell pigmentation differed from ratios expected from simple 1-locus Mendelian segregation (i.e. 1:1, 3:1, etc), on a per family basis (Sokal and Rohlf, 1995).

3. Results

Parentage analysis correctly assigned all 647 tested spat to their presumptive parents, with no failed assignments, indicating that cross-contamination among families in the hatchery and nursery was minimal or non-existent and that all cultures were properly labeled. We only genotyped four spat from family 10 and eight spat from family 34, and although all spat were correctly assigned to the expected parents, the small sample sizes from these families prevent drawing conclusions regarding cross-contamination with a high degree of certainty. Overall, however, our failure to detect cross-contaminants in all families suggests that such cross-contamination, if present at all, was not a systemic problem and had at most negligible effects on the results of this study.
Analysis of variance (Table 1) indicated that total left-shell pigmentation was significantly affected by family (P=0.001), but not by block (P=0.105), culture within family (P=0.070), block×family (P=0.334), or block×culture within family interaction (P=0.061). The partitioned variance components confirmed that family clearly had the largest effect on total left-shell pigmentation, accounting for 37.5% of the total phenotypic variation. Most of the remaining variation (57.3%) was accounted for by differences among individuals within each family (i.e. the error term in Table 1). The narrow-sense heritability of total left-shell pigmentation derived from the slope of the midparent-offspring regression was 0.59±0.19 (±1 S.E.; Fig. 1). Incorporating results from the midparent-offspring regression and intraclass correlation, we estimated the broad-sense heritability of total left-shell pigmentation as 0.91±0.38 (±1 S.E.).

Fig. 2 shows histograms of within family left-shell pigmentation for each of the 26 full-sib families. We found total left-shell pigmentation to be continuously and normally distributed within most of the families. However, eight families had significantly skewed distributions. The 3:1 segregation ratio within each of the 26 full-sib families. We found total left-shell pigmentation to be under a high degree of genetic control, with a broad-sense heritability of 0.91. It is worth noting that the covariance among full sibs can also be affected by siblings sharing a common, family-specific environment, resulting in an overestimate of h² and, consequently, an overestimate of H². In the present study, we raised siblings from each family in duplicate to determine the effect of common environment on average family total shell pigmentation. This culture within family effect was not statistically significant (Table 1), suggesting that common environmental effects were minimal. Indeed, the non-genetic effects of block, block×family interaction and block×culture within family interaction were also relatively minor, indicating that random-effects associated with variation among culture units in the nursery and tidal height in the field had little influence on shell pigmentation.

Broad-sense heritability, however, is a measure of total genetic determination, and includes both additive and non-additive genetic effects. The narrow-sense heritability refers specifically to the fraction of total phenotypic variation that can be explained by additive genetic effects. As a result, it is the narrow-sense heritability that is used by breeders to adopt appropriate selection strategies and to predict rate of phenotypic change (Falconer and Mackay, 1996). The high narrow-sense heritability in this study (h²=0.59) suggests that total left-shell pigmentation in C. gigas is strongly influenced by additive genetic variation and therefore amenable to selection. The difference between broad-sense heritability and narrow-sense heritability provided an estimate of the relative influence of dominance effects and, although most of the genetic variation was additive, approximately 32% of the total phenotypic variation was attributable to dominance.

As with previous authors, we treated Pacific oyster shell pigmentation as a continuously distributed, quantitatively inherited trait. Imai and Sakai (1961) assigned shells to one of five color morphs, ranging from a value of 1 ("whole surface is gray white with no dark coloration") to a value of 5 ("all [parts] dark colored"). Brake et al. (2004) divided shells into one of four groups based on overall shell pigmentation from 1 ("white, with little or no pigment present") to 4 ("having very dark pigmentation, mostly black or dark purple"). Batista et al. (2008) used a similar method to evaluate C. gigas and C. angulata muscle scar pigmentation. Recently, Hedgecock et al. (2006) used digital image analysis to quantify pigment saturation of the left-shell, allowing for a truly continuous measurement. With a few notable exceptions, variation among individuals within family in the present study was continuous and normally distributed. This within family variation, coupled with the high degree of genetic determination (H²=0.91), is consistent with a polygenic trait, controlled by the segregation of many genes, each having a small effect on shell pigmentation (e.g. Allard, 1999).

While the distribution of individuals within family was primarily normal and continuous, there were exceptions that may provide further insight into the inheritance of shell pigmentation in oysters. Positively skewed distributions were found in four families (1, 5, 33, and 34) and platykurtotic distributions were found in two families (17 and 22). Non-normal distribution among offspring has been considered evidence of a dominant major gene segregating in the offspring population, however, Lynch and Walsh (1998) advise extreme caution when inferring the presence or absence of a major gene based on subtle variations in phenotypic distribution. Perhaps more convincing are two families (10 and 21) that presented discontinuous bimodal distributions. The 3:1 segregation ratio within each of these families (i.e. three lighter individuals for every one darker individual) is consistent with the behavior of two alleles.
segregating at a single locus, with a dominant allele coding for the lighter phenotype and a recessive allele coding for the darker phenotype. The potential role of dominance in the expression of total left-shell pigmentation is therefore demonstrated by both comparing the difference between $H^2$ and $h^2$ as well as by examining phenotypic segregation patterns within families.

Previous studies have also suggested that $C$. gigas shell pigmentation and shell color may be influenced, in some cases, by only a small number of genes. Hedgcock et al. (2006) identified a single additively-inherited QTL that explained 32% of the phenotypic variation in total left-shell pigmentation within an experimental F2 population originally derived from two partially inbred lines. More recently, Nell (2001) reported that a hatchery in Tasmania had developed Pacific oysters displaying gold-colored mantles and shells. Strains were capable of breeding true after only a few generations of selection, implying a small number of genes control this trait. Taken together, our data and previous studies suggest that selective breeding to alter shell pigmentation in Pacific oysters could be highly effective, and that an initially rapid response to selection could result from the fixation of major genes followed by a more gradual and sustained response on polygenes.

In nature, shell pigmentation has been reported to serve a variety of adaptive roles, including crypsis/predator avoidance (Giesel, 1970; Allen, 1988; Owen and Whiteley, 1988; Cain, 1988), metabolic waste disposal (Comfort, 1951), thermoregulation (Mitton, 1977; Etter, 1988), developmental stability (Baucau, 2001), and shell strength (Cain, 1988). However, generally high phenotypic variation has led other researchers to conclude that shell pigmentation is under weak selective pressure and may have little or no adaptive function in many marine bivalve species (Seilacher, 1972; Ermentrout et al., 1986).

The large amount of additive genetic variation observed in this study further suggests that left-shell pigmentation is poorly correlated with overall fitness (Mousseau and Roff, 1987). However, genetic variance can be maintained by other mechanisms such as strong genotype-environment interaction, heterozygote advantage, or antagonistic pleiotropy, even in the presence of selective pressure (Falcoen and Mackay, 1996; Hartl and Clark, 1997). Further, inflated additive variance can also be a consequence of the offspring population experiencing a novel growing environment in mesh bags compared to the “ancestral” growing environment on a naturalized reef (Hoffman and Merila, 1999).

5. Conclusion

The large amount of additive genetic variation, as indicated by the high narrow-sense heritability, indicates that selective breeding should be effective in changing oyster shell pigmentation. Variation due to dominance may also play an important role in the expression of total left-shell pigmentation and the presence and mode of inheritance of the putative major gene reported here deserve further attention and verification through appropriate test crosses and/or mapping. Incorporating breeding strategies to fix the putative major gene observed here could dramatically speed progress towards developing true-breeding light- or dark-shelled strains of Pacific oysters.

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