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# Fine-scale temporal analysis of genotype-dependent mortality at settlement in the Pacific oyster *Crassostrea gigas*

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A B S T R A C T

Settlement and metamorphosis mark a critical transition in the life cycle of marine invertebrates, during which substantial mortality occurs in both field and laboratory settings. Previous pair-crossing experiments with the Pacific oyster *Crassostrea gigas* have revealed significant selective or genotype-dependent mortality around the metamorphic transition, but the fine-scale nature and timing of this mortality is not known, particularly whether it occurs before, during or after metamorphosis. In this laboratory study, microsatellite marker segregation ratios were followed daily throughout the settlement and metamorphosis of an $F_2$ cross of the Pacific oyster to examine the fine-scale patterns of genotype dependent mortality at this transition and whether settlement timing (early vs. late) might be under genetic control and affect inference of genotype dependent mortality. Settlement occurred over nine days (day 18 to day 27 post-fertilization) with 68% of individuals settling either early (day 19) or late (day 24). Tracking the survival of spat for 40 days after initial settlement revealed almost no mortality and thus no appreciable genetic mortality. Temporal genetic analysis revealed that 3/11 loci exhibited genotype dependent mortality around the metamorphic transition, one of which (*Cg205*) was followed throughout settlement and metamorphosis. Alternative temporal patterns of strong selection against each homozygous genotype at *Cg205* revealed possible defects in both the competency pathway (inability to initiate metamorphosis) and the morphogenesis pathway (mortality during the metamorphic transition). Quantitative trait locus (QTL) mapping of settlement timing identified three individual and one epistatic QTL with significant genetic effects on this trait (29% of the variance explained in total); however, two of these loci were linked to markers exhibiting selective mortality at metamorphosis, potentially confounding their apparent association with settlement timing. Overall, the results of this study highlight the complex nature of mortality and behavior during settlement and metamorphosis in oysters and suggest that endogenous sources of mortality at settlement may play an important role in the recruitment dynamics of oysters and possibly other broadcast spawning marine invertebrates.

## 1. Introduction

The period of settlement and metamorphosis is critical in the life cycle of many marine invertebrates (Gaines and Roughgarden, 1985; Hunt and Scheibling, 1997; Roughgarden et al., 1988; Rodriguez et al., 1993). It is the final developmental hurdle to successful recruitment, wherein larvae go through the dramatic ecological and biological transformation from a free-swimming planktonic larval form to a sessile benthic juvenile. In marine mollusks, metamorphosis is well described, characterized by the complete rearrangement of the body plan, coinciding with the loss of larval features, such as the velum, and the emergence of juvenile or adult characteristics, such as the gills (which first begin to form in the larval stages; e.g. Cole, 1938; Hickman and Gruffydd, 1971; Bonar, 1976; Cannuel and Beninger, 2006; Cannuel et al., 2009). The process of metamorphosis comprises two distinct phases: 1) the attainment of “competency”, the developmental capacity to respond to appropriate settlement cues and to exhibit settlement behavior and 2) the morphogenetic transformation that occurs when larvae attach to the substratum and complete metamorphosis (e.g. Pawlik, 1992; Degnan and Morse, 1995). A suite of genes control an anticipatory, ‘competency’ pathway, which begins to form juvenile structures prior to metamorphosis (e.g. digestive and shell formation pathways; e.g. Jackson et al., 2007), and then the morphogenetic transformation is dictated by the up-regulation of genes related to apoptosis, cell cycling, and calcium flux pathways, among others (the ‘morphogenetic’ pathway; Jackson et al., 2005; Jackson and Degnan, 2006; Jackson et al., 2007; Williams et al., 2009). Though there appear to be clear patterns of gene classes expressed across invertebrate taxa during competency and metamorphosis (e.g. Heylund and Moroz, 2006), most studies have focused on only a few marine gastropods (e.g.

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*Aplysia* and *Haliotus* spp.) and ascidians (e.g. Degnan et al., 1997; Eri et al., 1999; Jackson et al., 2002; Kawashima et al., 2005; Jacobs et al., 2006), all of which have lecithotrophic larvae. Less is known about genetic processes at metamorphosis in broadcast spawning marine invertebrates, such as marine bivalves with planktotrophic larvae.

Metamorphosis in marine invertebrates, and marine bivalves in particular, is accompanied by substantial mortality. High rates of mortality in newly settled juveniles have been observed for a wide variety of marine invertebrates, and, generally, survival curves of new settlers are type III: mortality is initially high but decreases rapidly after the first few days or weeks and then levels off (e.g. Rodriguez et al., 1993; Gosselin and Qian, 1996' reviewed by Gosselin and Qian, 1997; Hunt and Scheibling, 1997). Early mortality in invertebrates has primarily been estimated in field studies that examine rates and patterns of mortality after settlement; however, many of these studies are only able to measure "recruitment", i.e. the survival of a population to a certain time point after settlement, because monitoring mortality during settlement is difficult if not impossible for many species (e.g. Keough and Downes, 1982). Few studies accurately measure larval supply, metamorphosis, and early and late post-settlement periods within the same experiment, thus, a comprehensive understanding of when mortality occurs during settlement and metamorphosis is lacking.

Experimental laboratory studies allow for more detailed analysis of mortality during settlement in marine invertebrates, but few such studies exist. Jones and Jones (1983) found for the flat oyster (*Ostrea edulis*) that only a small fraction (10–30%) of late-stage larvae successfully completed metamorphosis under laboratory conditions. Haws et al. (1993) also noted that substantial mortality occurred during the metamorphic transition in their detailed examination of biochemical and physiological changes during metamorphosis of the Pacific oyster *Crassostrea gigas*. Culture experiments have shown significantly greater post-metamorphic survival associated with improved diet during the larval stages, suggesting that endogenous processes related to energy acquisition prior to metamorphosis are important (e.g. Gallager et al., 1986; Gallager and Mann, 1986; Helm et al., 1991; Coutteau et al., 1994; Pernet and Tremblay, 2004). Recent experimental genetic studies of offspring from inbred crosses of the Pacific oyster have confirmed high mortality at metamorphosis and revealed a large load of deleterious recessive mutations that were 'expressed' (acted) during metamorphosis, which are detected by their negative fitness effects on neutral marker genotypes (microsatellites) carrying alleles that are physically linked to these deleterious loci (i.e. genotype-dependent mortality or GDM; Launey and Hedgecock, 2001; Plough and Hedgecock, 2011). Plough and Hedgecock (2011) determined the stage-specific timing of GDM in two inbred crosses by following temporal changes in genotype frequencies relative to their Mendelian expectation (i.e. tests for distortion of Mendelian segregation ratios; Hedrick and Muona, 1990; Launey and Hedgecock, 2001; Plough and Hedgecock, 2011; Plough, 2012) in daily larval samples (day 1–18), a post settlement sample (day 30), and an adult sample (> 1 yr). The finding of substantial GDM during settlement highlights the potential important physiological and developmental changes associated with this transition, however, there were no temporal genetic data collected during settlement and metamorphosis in Plough and Hedgecock (2011), and thus the fine-scale temporal patterns of selection remain unknown.

In the current study, an inbred, $F_2$ (second generation bi-parental) family was allowed to set on adult shell in the laboratory, and daily samples of spat (recently settled juvenile oysters) and larvae were taken throughout the settlement period to determine the timing of endogenous (i.e. non-environmental) genotype-dependent mortality (GDM) during metamorphosis. While the $F_2$, laboratory-based design limits somewhat the application of results to understand the causes of oyster larval mortality in nature, a laboratory approach is required to eliminate or minimize environmental sources of mortality (e.g. predation, food limitation, etc.), which are difficult to distinguish from the endogenous sources (the focus of this study). Recent work has also shown that many of the deleterious alleles observed in inbred crosses have significant dominance (fitness effects on heterozygotes; Plough and Hedgecock, 2011; Plough, 2012) and thus would likely have fitness effects in wild or outbred crosses (Plough et al., 2016; Plough, 2016). Three hypotheses are proposed to explain how selection and mortality might proceed during settlement based on the differential timing of GDM observed in spat and larvae: 1) larvae are able to advance through metamorphosis and settle, but die soon after, which is reflected as high mortality of, and genotypic shifts in, juveniles after settlement, 2) larvae begin metamorphosis but die sometime during the transition, which is reflected by genotype deficiencies of both larvae and spat during metamorphosis, and 3) larvae with particular genotypes are delayed or fail to initiate metamorphosis, which is reflected by the deficiency of certain genotypes in spat that remain and perhaps accumulate in the larval population. Finally, the effect of genotype on settlement timing (larval duration) was analyzed in a quantitative trait locus (QTL) mapping framework to determine if settlement behavior could potentially affect the inference of loci causing GDM at metamorphosis. Overall, the novel sampling and genotyping strategy carried out in this controlled experiment sheds light on how genotype can influence settlement behavior and mortality at metamorphosis in a family of oysters, setting the stage for future genetic studies of genotype dependent mortality and settlement variation in a broader, population context.

## 2. Materials and methods

### 2.1. Crosses and culturing

Inbred lines *51* and *35* were derived from a naturalized population of *C. gigas* in Dabob Bay, WA, with initial families made from pair crosses of wild individuals in 1996 (Hedgecock and Davis, 2007). These lines were inbred (full-sib mating) for four generations leading up to the $F_1$ hybrid cross that was made in 2007. In 2009, the experimental $F_2$ family was created by mating a pair of male and female full-siblings from the 2007 *51* × *35* $F_1$ hybrid cross. Crosses were performed at the University of Southern California (USC) Wrigley Marine Science Center (WMSC) on Catalina Island, CA. Pedigrees of parents were verified with microsatellite DNA markers (e.g. Hedgecock and Davis, 2007). An inbred cross was used here because deleterious recessive alleles affecting offspring survival (viability selection) can be identified from their characteristic fitness effects on linked alleles in homozygous marker genotypes (homozygote deficiencies; e.g. Launey and Hedgecock, 2001; Plough and Hedgecock, 2011; Plough, 2012).

The cross was performed by stripping ripe gametes from a single male and a single female and combining ~ 1,000,000 eggs and an appropriate amount of sperm in a two-liter beaker of fresh seawater for fertilization (Breese and Malouf, 1975; Hedgecock and Davis, 2007). Observations of fertilizations in 100 μl sub-samples indicated that fertilization success was ~80%, so the record of initial expected stocking density was adjusted to 800,000 embryos. After a one-hour incubation, 800,000 zygotes were stocked in a 200-l vessel (4 embryos·ml$^{-1}$) with fresh seawater. Starting at 24 h post-fertilization, larvae were fed a diet of exponential phase *Isochrysis galbana* (Tahitian Isolate) every two days, at a starting concentration of 30,000 cells·ml$^{-1}$, which was increased up to 120,000 cells·ml$^{-1}$ as larvae grew, following typical larval rearing protocols for the Pacific oyster (Breese and Malouf, 1975; Launey and Hedgecock, 2001; Plough and Hedgecock, 2011). Mean density of larvae ( ± SEM) was calculated every 2–4 days after fertilization, from replicated (n = 4) volumetric counts of 20–100 μl aliquots taken from a concentrated culture (~1 l volume) containing all larvae. These estimates were converted to mean survival from egg (embryo) or the first shelled, prodissoconch I stage, also described as the 'd-hinge' stage.