Molecular and biochemical approaches to understanding conspecific cue mediated larval settlement induction in Pacific oysters *Crassostrea gigas*

（同種によるマガキ（*Crassostrea gigas*）幼生の付着誘起機構の解明に関する分子的及び生化学的研究）

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The Pacific oyster *Crassostrea gigas* is a benthic bivalve mollusk with a biphasic life cycle. It is one of the most well-studied organisms due to its biology and aquaculture importance. A variety of chemical cues from the natural environment have been implicated to play a role in the induction of larval settlement in many species. Surface-bound chemical cues from conspecifics such as those from oyster shells have been reported to induce larval settlement in *C. gigas*. These studies show that chemical cues from conspecifics may play a role in the gregarious behavior exhibited by oyster larvae upon settlement. However, the exact nature of these cues is not yet fully elucidated. This study elucidates the identity, characteristics, and mechanisms underlying the conspecific cue, CgSPPC, mediated induction of larval settlement in *C. gigas*.

In Chapter II, oyster larvae were exposed to 12 types of sugars, surfaces coated with a conspecific cue from *C. gigas* adult shell extract and non-coated surfaces, and under varied sugar exposure times, in order to understand how the presence of different chemical cues regulate settlement behavior. Lectin-glycan interaction effects on settlement and its localization on oyster larval tissues were investigated. The results showed that the conspecific cue elicited a positive concentration dependent settlement inducing trend. Sugars in the absence of a conspecific cue, *C. gigas* adult shell extract, did not promote settlement. Whereas, in the presence of the cue, showed varied effects, most of which were found inhibitory at different concentrations. Sugar treated larvae exposed for 2 h showed significant settlement inhibition in the presence of a conspecific cue. Neu5Ac, as well as GlcNAc sugars, showed a similar interaction trend with wheat germ agglutinin (WGA) lectin. WGA-FITC conjugate showed positive binding on the foot, velum, and mantle when exposed to GlcNAc sugars. This study suggests that a WGA lectin-like receptor and its endogenous ligand are both found in the larval chemoreceptors and the shell Ethylenediaminetetraacetic acid (EDTA) extract that may complementarily work together to allow the oyster larva greater selectivity during site selection.

In Chapter III, the isolated and characterized dominant EDTA-soluble shell matrix proteins were shown to be interacting synergistically to form a macromolecular assembly capable of creating a stable and strong aggregating signal for gregarious settlement. These identified matrix proteins include Gigasin-6 isoform X1/X2; a renamed *Crassostrea gigas* Pheromone Protein-17 (CGPP-17) from Surface protein P12p-like; and several stains-all stainable proteins (intrinsically disordered proteins), the most dominant of which was a ~48 kDa band, putatively identified as *Crassostrea gigas* Dentin sialophosphoprotein (CGDSP). I collectively named this chemical cue as the *Crassostrea gigas* Settlement Pheromone Protein Complex (CGSPPC). These results highlight a novel function of shell matrix proteins as an aggregating settlement pheromone.

One common factor that is attributed to their ability to induce settlement, either individually or collectively, is due to post-translational modifications that may play important roles in oyster chemical signaling for conspecific recognition. Further discussion is made on the role of post-translational modifications, the properties and contribution of each protein component to settlement induction, and lastly, on the molecular basis of the conspecific cue-mediated larval settlement mechanism.

In Chapter IV, a transcriptomics analysis was applied to evaluate the oyster larval response before and after it recognizes the CgSPPC; and when it is induced to undergo larval settlement by comparing the transcriptomes of the pediveliger (Pedi) and conspecific cue-induced postlarvae (PL). A total of 2,383 candidate genes were identified: 1,643 in the Pedi transcriptome and 740 in the PL transcriptome. Gene Ontology analysis revealed active chitin binding, calcium ion binding, and extracellular region processes in both stages. A verification experiment using a real-time PCR assay confirmed six examined genes exhibited the same trends at the RNA-seq and mRNA gene expression levels. The differentially expressed genes related to shell formation shows closely linked dynamics with a gene regulatory network that may involve the interplay of various together in a concerted way in both developmental stages. These results expand our understanding of the molecular mechanisms underlying the settlement of oysters on conspecific adult shells and demonstrate the potential use of this cue as an attractant for wild and hatchery-grown oyster larval attachment on artificial substrates. It also suggests the possible involvement of an ecdysone signal pathway that may be linked to neuroendocrine-biomineralization crosstalk in *C. gigas* settlement.

In conclusion, this study presents the regulatory role of sugars and post-translational modifications in chemical sensing governing *C. gigas* substrate selection. This study provides evidence to *Crassostrea gigas* Settlement Pheromone Protein Complex (CGSPPC) as the biological cue and key molecule(s) responsible for gregarious settlement in *C. gigas*. Hence, a novel role of shell matrix proteins as an aggregating settlement pheromone is demonstrated for the first time. New data on Gigasin-6 isoform X1/X2 characteristics and functional role as the major settlement inducing cue in CGSPPC is presented. Among others, this includes the localization of actual N-glycan modifications in this protein wherein a newly detected mass spectrometry signal of m/z 2525.21 spectra is reported. CGPP-17 and CGDSP together with other isolated stains-all stainable proteins are also co-factors that could contribute to strengthening the settlement inducing signal via phosphorylation crosstalk with a CGSPPC recognizing larval receptor. A structural, functional, and biological characterization is newly annotated on these two identified proteins in *C. gigas*. Furthermore, this study records for the first time a transcriptomic analysis on the specific cellular and physiological processes involved in the exposure of the competent pediveliger larvae to CGSPPC and their transition into postlarvae. A possible involvement of an ecdysone signal pathway that may be linked to neuroendocrine-biomineralization crosstalk in *C. gigas* settlement is suggested. Lastly, oysters may seem simple, but this study provides insights on the complex molecular mechanisms governing oyster substrate selection that may indicate their evolutionary success as benthic organisms.