Bacterial community dynamics during embryonic and larval development of three confamilial echinoids

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ABSTRACT

Development of some animals is influenced by and, in some cases, dependent on the associated microbiota. The timing of when associated bacterial communities are established during the development of marine invertebrates and their subsequent dynamics across stages are known for only a few species. Here, we compare the bacterial communities of three confamilial echinoids from egg to juvenile using sequence-based approaches. Bacterial communities are established on unfertilized eggs and change gradually during embryonic and larval development. Despite the differences amongst these pre-metamorphic stages, approximately thirty-percent of OTUs identified in association with unfertilized egg were present in the juveniles. During embryonic development, host-associated communities diverged from the environmental microbiota but later converged following the onset of larval feeding. Taken together, the data presented here support the hypothesis that bacterial communities are established prior to fertilization and community composition shifts gradually thereafter, all while remaining distinct from the environment. Future work will need to determine the relative influence of the host and bacteria-bacteria interactions in shaping the associated bacterial community to determine the potential functional importance of bacteria during the development of larval sea urchins and benthic marine invertebrates more broadly.

INTRODUCTION

The journey from fertilized egg to adulthood has fascinated developmental biologists for centuries. A growing consensus in the field of animal-microbe symbioses suggests that developmental processes are, at least in part, affected by the presence and activity of associated microbiota (McFall-Ngai & Ruby 2000, McFall-Ngai 2002, Fraune & Bosch 2010, Nyholm & McFall-Ngai 2014). The degree of dependency on these communities is highly variable: the removal of bacterial associates inhibits the differentiation of gut tissues in zebrafish (Bates et al. 2006) while other taxa are seemingly unaffected (Manahan et al. 1993). Nevertheless, several patterns have emerged from comparative studies of animal-bacteria interactions. First, animals tend to associate with developmental stage-specific bacterial communities (Apprill et al. 2012, Mortzfeld et al. 2015, Carrier & Reitzel 2018). Second, changes in morphology and life history stage correlate with shifts in the composition and structure of this community (Apprill et al. 2012, Sharp et al. 2012, Mortzfeld et al. 2015, Fieth et al. 2016). Lastly, during ontogeny, animal-associated bacterial communities can be highly specific and diverge from the environmental microbiota through host-mediated selection (Davidson & Stahl 2008, Fraune et al. 2010, Pradea 2011, Franzenburg et al. 2013, McFall-Ngai et al. 2013).

Development of many marine invertebrates includes several morphologically diverse life-history stages. Regardless of taxonomy, reproduction in most species involves an external fertilization, embryogenesis, and larval development before settling and metamorphosing into a benthic juvenile (*e.g.*, Thorson 1950, Mileikovsky 1971). When microbial communities are established during early development for marine invertebrates appears to vary among species. Larvae of deep-sea vestimentiferans, for example, are

symbiont free until settlement while several species from various taxa are known to vertically inherit microbial symbionts and diversify this community later in development by horizontal (environmental) acquisitions (Nussbaumer et al. 2006, Stewart et al. 2008, Bright & Bulgheresi 2010, Apprill et al. 2012, Sharp et al. 2012, Sipkema et al. 2015). Once established, the location of the bacterial symbionts can also vary. The embryos and larvae of eight echinoderm species have, for example, symbiotic bacteria in the out-pockets of the extracellular matrix surrounding the larval body as well as near or in gut epidermal cells (Cameron & Holland 1983, Bosch 1992, Cerra et al. 1997, Galac et al. 2016, Carrier & Reitzel 2018, Carrier et al. 2018b).

The composition and structure of the bacterial communities associated with echinoderm larvae is highly variable and has been shown to be influenced by food availability (Carrier & Reitzel 2018, Carrier et al. 2018b), phenotype (Carrier & Reitzel 2018), asexual reproduction (Galac et al. 2016), and, potentially, disease (Carrier et al. 2018a). Three confamilial echinoderms (*Strongylocentrotus purpuratus*, *Mesocentrotus franciscanus*, and *S. droebachiensis*), in particular, associate with species-specific bacterial communities that shift according to larval stage (Carrier & Reitzel 2018). When the diverse and dynamic bacterial community associating with these echinoid larvae are established and how this community varies throughout the entirety of pelagic development is unknown.

By sampling each developmental stage from the unfertilized egg to benthic juvenile and assaying the associated bacterial communities using next-generation sequencing, we test two explicit hypotheses. First, we test if stage-specific bacterial communities during larval development result from associations established in earlier embryonic stages and continue after metamorphosis. Second, we test that three confamilial Strongylocentrotids

82 exhibit relatively similar developmental patterning in the bacterial communities due to their
83 phylogenetic relatedness. Implications from these hypotheses are predicted to complement
84 previous studies showing that the biology of echinoid larvae is inseparable from their
85 microbial community and is an informative system to understand the ecology of animal-
86 microbe symbiosis.
87

88 **MATERIALS AND METHODS**

89 **Specimen collection and larval rearing**

90 Adult urchin for each species were collected from populations in the Salish Sea in
91 April 2016 (Carrier & Reitzel 2018). *Strongylocentrotus purpuratus* was hand-collected at
92 low tide at Slip Point, Clallam Bay, WA (48°15'39" N, 124°15'03" W; Figure S1) and
93 transferred overnight to the Friday Harbor Laboratories (FHL; University of Washington;
94 Friday Harbor, WA, USA; Figure S1). Similarly, *S. droebachiensis* were hand-collected at
95 low tide at Cattle Point, San Juan Island, WA (48°27'00" N, 122°57'43" W; Figure S1) and
96 were transferred to FHL within one hour. *M. franciscanus*, on the other hand, were
97 collected by SCUBA off Bell Island, WA (48°35'49" N, 122°58'55" W; Figure S1) and
98 transferred to FHL within two hours. All urchins were suspended in separate sub-tidal
99 cages off the dock at FHL and fed *Nereocystis* spp. (sugar kelp) *ad libitum* until spawning
100 two weeks later.

101 Urchins were spawned by a one to two mL intracoelomic injection of 0.50 M KCl.
102 Gametes from *S. purpuratus* (n=3 for males and females), *M. franciscanus* (n=1 for males
103 and n=3 for females), and *S. droebachiensis* (n=3 for males and females) were collected,
104 rinsed, and pooled separately for each species. Fertilization of eggs and larval rearing

105 followed Strathmann (1987), with the exception that embryos and larvae were reared using
106 5.0-µm filtered seawater (FSW) to include the environmental microbiota.
107 Embryos were incubated in one-liter of FSW at ambient conditions (~10ºC and
108 12ºC; Carrier & Reitzel 2018). Two hours post-fertilization, embryos were transferred to
109 triplicate one-liter jars at a density of two individuals•mL^{-1}. Beginning at the prism stage,
110 larvae were provided *Rhodomonas lens* at 10,000 cells•mL^{-1}, for which was made growth
111 medium-free by centrifugation and resuspension in FSW prior to the introduction to larval
112 cultures. Larval cultures were subsequently diluted to one larvae per two mL at advanced
113 developmental stages. Larval cultures were given 90 to 95% water changes every other day
114 and *R. lens* was replenished at 10,000 cells•mL^{-1}.
115 Monocultures of *R. lens* were grown in f/2 media at room temperature with a
116 combination of ambient and artificial lighting for 24 hours per day (Guillard 1975).
117
118 **Sampling across development**
119 Each urchin species was cultured from egg to juvenile. We sampled ten stages: (i)
120 unfertilized eggs, (ii) 16-cell embryo (referred to hereafter as 'cleavage'), (iii) pre-hatched
121 blastula, (iv) hatched blastula, (v) gastrula, (vi) pre-feeding prism, (vii) 4-, (viii) 6-, and (ix)
122 8-armed larvae, and (x) post-metamorphic juvenile (see, Table S1 for biological replication
123 for each species at each developmental stage). Developmental stages were identified using
124 a compound microscope (Nikon Eclipse E600; camera: QImaging MicroPublisher 5.0
125 RTV). At each developmental stage, 100 individuals per jar (n=3) were sampled at random
126 for each species. Immediately after collections, samples were concentrated into a pellet
127 using a microcentrifuge, the FSW was removed with a sterile glass pipette, and pelleted

128 embryos, larvae, or juveniles were then preserved in RNAlater (Thermo Scientific,
129 Massachusetts, USA) and stored at -20°C before DNA extractions.
130 Complementary to sampling of the developmental stages, the environmental
131 microbiota from the seawater was also sampled. When cultures were sampled, triplicate ~1
132 L of 5.0-µm FSW was filtered onto a 0.22-µm Millipore filter to retain the environmental
133 microbiota. Full filter disks were then preserved in RNAlater and stored at -20°C before
134 DNA extractions.
135
136 **Assaying bacterial communities**
137 Total DNA was extracted from larval samples using the GeneJet Genomic DNA
138 Purification Kit (Thermo Scientific, Massachusetts, USA). For FSW samples, we extracted
139 eDNA using the FastDNA Spin Kit for Soil (MP Biomedical, Illkirch, France). DNA was
140 then quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific,
141 Massachusetts, USA) and diluted to 5 ng•μL^{-1} using RNase/DNase-free water.
142 Bacterial sequences were amplified using universal primers for the V3/V4 regions
143 of the 16S rRNA gene (Forward: 5′ CTACGGGNGGCWGCAG, Reverse: 5′
144 GACTACHVGGGTATCTAATCC; Klindworth et al. 2013). Products were purified using
145 the Axygen AxyPrep Mag PCR Clean-up Kit (Axygen Scientific, New York, USA),
146 indexed via PCR using the Nextera XT Index Kit V2 (Illumina, California, USA), and then
147 purified again. At each of these three steps, fluorometric quantitation was performed using
148 a Qubit (Life Technologies, California, USA) and libraries were validated using a
149 Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, California, USA). Illumina

150 MiSeq sequencing (v3, 2x300 bp paired-end reads) was performed at the University of
151 North Carolina at Charlotte. PCR recipe and thermal profiles are available in Table S2.
152 Forward and reverse sequences were paired and trimmed using PEAR (Zhang et al.
153 2014) and Trimmomatic (Bolger et al. 2014), respectively, converted from fastq to fasta
154 using a custom script, and chimeric sequences were detected using USEARCH (Edgar et
155 al. 2011) and removed using filter_fasta.py prior to the analysis of bacterial 16S rRNA
156 sequences. Using QIIME 1.9.1 (or Quantitative Insights Into Microbial Ecology; Caporaso
157 et al. 2010), bacterial 16S rRNA sequences were analyzed and grouped into operational
158 taxonomic units (OTUs) based on a minimum 97% similarity. The biom table generated
159 by the pick_open_reference_otus.py script was filtered of OTUs with ten or less reads as
160 well as sequences matching the chloroplast of cryptophytes (*i.e.*, *R. lens*; Carrier & Reitzel
161 2018).
162 Using the filtered biom table and "biom summarize-table" function to count total
163 sequences per sample, the rarefaction depth of 25,396 was determined and applied to all
164 subsequent analyses (Figure S2). Alpha diversity estimates were calculated using the
165 alpha_diversity.py script and compared statistically by a two-way analysis of variance
166 (ANOVA) in JMP (Pro v. 13). Beta diversity was calculated using unweighted and
167 weighted UniFrac (Lozupone & Knight 2005) as part of jackknifed_beta_diversity.py, and
168 compared using principal coordinate analyses (PCoA) that were then visualized in
169 EMPeror (Vazquez-Baeza et al. 2013) and stylized in Adobe Illustrator CS6. UPGMA-
170 based microbial dendrograms were generated using jackknifed_beta_diversity.py as part
171 of the PCoA analyses. Community similarity across species and developmental stages were
172 compared statistically using an analysis of similarity (ANOSIM) in QIIME as part of the

173 compare_categories.py script, with $p<0.05$ considered significant. Community
174 composition was generated using summarize_taxa_through_plots.py script and visualized
175 using Prism 7 (GraphPad Software, California, USA). Lastly, observed OTUs were
176 calculated using the alpha_diversity.py script, shared OTUs were calculated using the
177 shared_phylotypes.py script, and the Bray-Curtis index was calculated using the
178 beta_diversity.py script, and compared statistically using ANOVA in JMP (Pro v. 13).
179 A step-by-step listing of QIIME scripts used to convert raw reads to OTUs for
180 visualization of the data is located in Supplemental Note 1. The raw sequence reads as part
181 of this dataset are available on Dryad.
182
183 **RESULTS**
184 **Community-level shifts across development**
185 Urchin-specific bacterial communities (ANOSIM, unweighted: $p=0.0011$; weighted:
186 $p<0.001$; Figure 1) shifted significantly in OTU membership (ANOSIM, $p=0.0013$; Figure
187 1A; Figures S3-4) and composition (ANOSIM, $p=0.0011$; Figure 1B; Figures S2-3) during
188 embryonic and larval development. Moreover, community evenness (Shannon and
189 Robbins), diversity (Simpson), richness (Chao1), phylogenetic distance (Faith's), and
190 Fishers alpha varied significantly across developmental stages ($p<0.0001$ for all tests) and
191 amongst urchin species ($p<0.0001$ for all tests) (Figure S5; Table S2).
192 Unweighted and weighted UniFrac comparisons of the associated bacterial
193 communities for each urchin species suggests that community similarity largely group by
194 developmental stage (Figure 2). Bacterial communities were grouped largely by sequential
195 developmental stages with some specific clusters for: (i) early embryological stages, (ii)

196 late embryogenesis and early larval development, and (iii) later larval stages. The benthic
197 juveniles had more variable positioning on the microbial dendrograms: the bacterial
198 community of *S. purpuratus* juveniles were intermediate to early- and late-stage larvae, *M.*
199 *franciscanus* juvenile was most similar to eggs, and the *S. droebachiensis* juvenile were
200 most similar to late-stage larvae (Figure 2).

201 A total of nine classes of bacteria represented at least 1% of the community (Figure
202 2). Of these, the relative abundance of the γ-proteobacteria (Phylum: Proteobacteria), α-
203 proteobacteria (Phylum: Proteobacteria), and Flavobacteriia (Phylum: Bacteroidetes) were,
204 on average, each more than 10% of the community (Figure 2). For the γ-proteobacteria, the
205 relative abundance ranged from 26.6% to 61.0% for *S. purpuratus*, 12.2% to 62.9% for *M.*
206 *franciscanus*, and 39.6% to 88.7% for *S. droebachiensis* (Figure 2). Next, for the α-
207 proteobacteria, the relative abundance ranged from 7.7% to 42.4% for *S. purpuratus*,
208 11.0% to 31.1% for *M. franciscanus*, and 5.1% to 22.7% for *S. droebachiensis* (Figure 2).
209 Lastly, for the Flavobacteriia, the relative abundance ranged from 4.9% to 30.5% for *S.*
210 *purpuratus*, 5.1% to 36.1% for *M. franciscanus*, and 1.5% to 34.2% for *S. droebachiensis*
211 (Figure 2).

212

213 **Dynamics of the associated bacterial species**

214 Total OTUs in association with these confamilial echinoids differed significantly
215 between developmental stages (ANOVA, $p<0.0001$) for each species (ANOVA, *S.*
216 *purpuratus*: $p<0.0001$; *M. franciscanus*: $p<0.0001$; *S. droebachiensis*: $p<0.0001$; Figure
217 3A; Table S1). Unfertilized eggs, on average, associated with a similar number of OTUs
218 amongst species (~1,400 OTUs; ANOVA, $p=0.508$), which increased during embryonic

219 development (Figure 3A). Following the onset of feeding and larval development, the
220 number of OTUs subsequently decreased until metamorphosis (Figure 3A). The maximum
221 number of associated OTUs during development varied amongst species (*S. purpuratus*:
222 1,839; *M. franciscanus*: 1,723; *S. droebachiensis*: 1,864) and at which stages this occurred
223 (*S. purpuratus*: gastrula; *M. franciscanus*: 4-armed larva; *S. droebachiensis*: pre-hatched
224 blastula (Figure 3A).

225 A significant proportion of the OTUs associated with echinoid eggs did not remain
226 in association throughout embryonic and larval development for each urchin species
227 (ANOVA, *S. purpuratus*: $p<0.0001$; *M. franciscanus*: $p<0.0001$; *S. droebachiensis*:
228 $p<0.0001$; Figure 3B; Table S1). Following fertilization and early embryogenesis, ~52%
229 of OTUs from the egg remained in association while ~48% were lost (Figure 3B). In the
230 subsequent larval and juvenile stages, egg-associated OTUs were lost gradually, resulting
231 in juvenile urchins with ~29% of the egg-associated OTUs (Figure 3B). Moreover, when
232 transitioning between any two sequential developmental stages, ~48% of OTUs retained
233 while the rest were specific to either stage (Figure 3C). This result was not simply due to a
234 high diversity of OTUs in the egg samples because OTU number increased after the egg
235 stage for all species (Figure 3A).

236 Community dissimilarity (Bray-Curtis) between the sea urchin host and
237 environmental microbiota showed significant differences during embryonic and larval
238 developmental for each species (ANOVA, *S. purpuratus*: $p<0.0001$; *M. franciscanus*:
239 $p<0.0001$; *S. droebachiensis*: $p<0.0001$; Figure 3D, Figure S6, Table S1). During
240 development from egg to hatched blastula, the associated bacterial community became
241 more dissimilar from the environmental microbiota (Figure 3D), with the magnitude of

242 dissimilarity varying amongst species (Figure 3D; ANOVA, egg through hatched blastula,
243 each $p \leq 0.0001$). Furthermore, following development of the mouth and the onset of
244 feeding, late embryos and early stage larvae became more similar to the environmental
245 bacterial community (Figure 3D). The relative dissimilarity converged in the early larval
246 stages (Figure 3D; ANOVA, 4-armed larvae: $p=0.406$), which was the last sampled time
247 point for which we determined the bacterial community of the FSW. During divergence
248 and convergence events between the larval host and the environmental microbiota, the
249 environmental bacterial community was consistent throughout the experiment and did not
250 cluster with bacterial communities associated with the urchin (Figure S6).
251

252 ***Psychromonas* and echinoid eggs**

253 Bacteria in the genus *Psychromonas* showed the highest abundance for any
254 bacterial genus for each urchin species at any one of the sampled stages, in contrast to their
255 relative rarity (0.7%) in the seawater (Figure 4; Tables S6-7). For each urchin species, the
256 abundance and diversity (Figure S7; ANOVA, $p<0.0001$ for each) of *Psychromonas* was
257 highest in the unfertilized eggs and declined significantly following fertilization (ANOVA,
258 $p<0.0001$ for each), until being in very low abundance in 4-armed larvae and the
259 subsequent developmental stages (Figure 4). The magnitude of relative abundance varied
260 amongst urchin species (ANOVA, $p<0.0001$) and was directly proportion to egg diameter
261 ($R^2=0.833$). The OTUs within *Psychromonas* totaled 96 for *S. purpuratus*, 100 for *M.*
262 *franciscanus*, and 116 for *S. droebachiensis*, while the FSW had 81 (Figure 4). Of those,
263 nine of the ten most abundant *Psychromonas* OTUs for each urchin species were shared
264 amongst urchin species.

265

266 **DISCUSSION**

267 Like many marine invertebrates, echinoid life cycles involve a distinct embryonic,
268 larval, and juvenile stage. Our assay of the bacterial communities of three confamilial
269 echinoids supports the hypothesis that the bacterial consortium is highly dynamic across
270 developmental stages, with taxonomic membership and the relative abundance of those
271 taxa shifting gradually from egg to juvenile. Furthermore, over the course of development,
272 the bacterial community of urchin embryos remains distinct and diverges from the
273 environmental microbiota until larvae begin feeding. Lastly, eggs of these echinoids appear
274 to have a specific association with diverse OTUs that match the genus *Psychromonas*.

275 The bacterial communities associated with echinoplutei are highly dynamic when
276 the larval holobiont faces variation in the abiotic and biotic environment (Carrier & Reitzel
277 2018, Carrier et al. 2018b). For example, following diet-restriction the bacterial
278 communities of *S. purpuratus*, *M. franciscanus*, and *S. droebachiensis* each shift
279 considerably from their well-fed counterparts, to potentially cope with reduced exogenous
280 resources (Carrier & Reitzel 2018). When diet-restriction is prolonged and the larval host
281 expresses phenotypic plasticity, the composition and structure of the bacterial community
282 is specific to phenotype, a response correlated with the magnitude in which phenotypic
283 plasticity is expressed (Carrier & Reitzel 2018).

284 Phenotypic plasticity for echinoid larvae, more broadly, is the interaction between
285 phenotype, nutritional state, and the environment. Characterization of bacterial
286 communities across developmental stages for these three confamilial echinoids extends the
287 understanding of the complex interplay between developmental processes and the

288 surrounding environment in which these species develop. Particularly, after unfertilized
289 eggs establish a bacterial community, the composition of these prokaryotic species shift
290 gradually during embryogenesis and during transitions towards a feeding larva. In these
291 gradual community transitions, it was surprising that more coarse shifts were not observed
292 between major developmental milestones, such as when blastula hatch and shed the
293 fertilization envelope, larvae begin feeding, and/or when larvae metamorphose into benthic
294 juveniles. The latter may be because echinoid larvae undergoing metamorphosis reabsorb
295 some of the larval digestive tract (Chia & Burke 1978). The juveniles therefore may have
296 retained, instead of shed (Fieth et al. 2016), a portion of their bacterial community present
297 in late larval stages.

298 The influence of particular bacteria and bacterial communities on the development
299 of aquatic and terrestrial invertebrates is well-studied in few species (McFall-Ngai & Ruby
300 2000, Fraune & Bosch 2010, McFall-Ngai 2014, Nyholm & McFall-Ngai 2014).
301 Established invertebrate systems where bacteria play a significant functional role include
302 *Vibrio fisheri*-mediated maturation of the light organ in the bobtail squid *Euprymna*
303 *scolopes* (McFall-Ngai & Ruby 2000, McFall-Ngai 2014), immune regulation relating to
304 the epithelial bacteria associated with the freshwater cnidarian *Hydra* spp. (Fraune & Bosch
305 2010, Bosch 2013, Franzenburg et al. 2013), and the nutritional endosymbionts (*e.g.*,
306 *Buchnera*) of aphids (Douglas 1998). Provided the vast genomic and molecular foundation
307 established over the past several decades for the echinoid clade used in this study
308 (Kudtarkar & Cameron 2017), these confamilial urchins have the potential to be established
309 as a comparative system for beneficial animal-microbe interactions during development
310 (Buckley & Rast 2017, Heyland et al. 2018) and in bi-phasic life-histories. One route for

311 testing the impact of microbial symbionts on echinoid development may be through the
312 egg-associated *Psychromonas*.

313 Eggs of echinoids have been used as a metric in larval biology, ecology, and
314 evolution (Vance 1973, Wray & Raff 1991, Jaeckle 1995, Moran & McAlister 2009,
315 Marshall et al. 2012) to make inferences about life history mode and transitions (Vance
316 1973, Collins & Moran 2018), fertilization ecology (Pennington 1985, Levitan 1995), and
317 maternal inheritance (Byrne et al. 1999, Villinski et al. 2002). The high abundance of egg-
318 associated *Psychromonas* and their loss and replacement may imply that this network of
319 bacterial symbionts are functionally connected to early developmental processes.

320 Interestingly, egg size of echinoids is strongly correlated with total lipid content
321 (Jaeckle 1995, Moran & McAlister 2009) and data here support that egg size is also highly
322 correlated with the abundance of *Psychromonas*. Maternally-inherited lipids for
323 *Dendraster excentricus*, a related echinoid found in the same geographical region of this
324 study, are depleted by the same developmental stage that *Psychromonas* was undetectable
325 here (Rendleman et al. 2018). Moreover, symbiotic bacteria associated with developing
326 embryos of the deep-sea clam *Calyptogena okutanii* is spatiotemporally regulated and has
327 the genetic components to process lipopolysaccharides (Ikuta et al. 2016). *Psychromonas*
328 may, therefore, aid in lipid metabolism (Riley et al. 2008) during embryonic development
329 or serve as a commensal symbiont that relies on the lipids leaching out maturing embryos.

330 An additional pattern we observed during embryonic development was that urchin-
331 associated bacterial communities diverged from the environmental microbiota. Divergence
332 of host-associated microbiota from the environmental community suggests that urchin
333 embryos and larvae may select, at least, part of their microbial symbiont community.

334 Maintenance of host-specific symbionts may further suggest that these communities are
335 functionally important. To explicitly test how urchin larvae maintain a specific bacterial
336 community and their functional relevance during embryonic and larval development, a
337 multifaceted molecular and -omic (*e.g.*, meta-genomic and -transcriptomic) approach
338 would be needed (Williams & Carrier 2018). Approaches for characterizing the
339 mechanisms that establish and regulate host specificity may be adopted from well-
340 established biological systems, such as *Hydra* (Fraune et al. 2010, Fraune & Bosch 2010,
341 Franzenburg et al. 2013) or the winnowing of *Euprymna scolopes* to the luminescent
342 mutualist *Vibrio fisheri* (McFall-Ngai & Ruby 2000, McFall-Ngai 2002, Nyholm &
343 Mcfall-Ngai 2004).

344 Taken together, data presented here support the hypothesis that the bacterial
345 community associated with the developmental stages of three sea urchin species are
346 established prior to fertilization and shift gradually thereafter. The functional importance
347 and evolutionary significance of this bacterial community and the mechanisms for these
348 corresponding shifts remains unknown. Future experiments involving germ-free culturing
349 as well as manipulations of the host, members of the microbial community, and variable
350 culturing conditions will be needed to determine roles of associated microbiota. Results
351 from these experiments would serve as a key piece towards establishing echinoids as a
352 comparative system for understanding the interdependence of animal development and the
353 associated bacterial communities.

354

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366 **FIGURE LEGEND**

367

368 **Figure 1**. Similarity amongst the associated bacterial community across development for
369 three species of echinoids. Community similarity for *Strongylocentrotus purpuratus*
370 (triangle), *Mesocentrotus franciscanus* (square), and *S. droebachiensis* (circle) for ten
371 developmental stages when considering the presence/absence of taxa (A) and their relative
372 abundance (B).

373

374 **Figure 2**. Relatedness of bacterial communities during development. Weighted UniFrac
375 dendrograms and corresponding class-level taxonomic plots for the bacterial communities
376 of *Strongylocentrotus purpuratus* (left), *Mesocentrotus franciscanus* (center), and *S.*
377 *droebachiensis* (right) across ten developmental stages.

378

379 **Figure 3**. Measures of OTU shifts during development. OTU dynamics across embryonic
380 and larval development for *Strongylocentrotus purpuratus* (purple), *Mesocentrotus*
381 *franciscanus* (red), and *S. droebachiensis* (green), and the average of the urchin species
382 (black), when considering (A) total observed OTUs, (B) percent of OTUs shared with
383 unfertilized eggs, (C) proportion of OTUs shared between developmental stages, and (D)
384 similarity with the environmental microbiota.

385

386 **Figure 4**. Decay in abundance of *Psychromonas* during development. Relative abundance
387 of the bacterial genus *Psychromonas* across embryonic and larval development for

388 *Strongylocentrotus purpuratus* (purple), *Mesocentrotus franciscanus* (red), *S.*
389 *droebachiensis* (green), and the seawater (blue) with the number of total OTUs.
390
391

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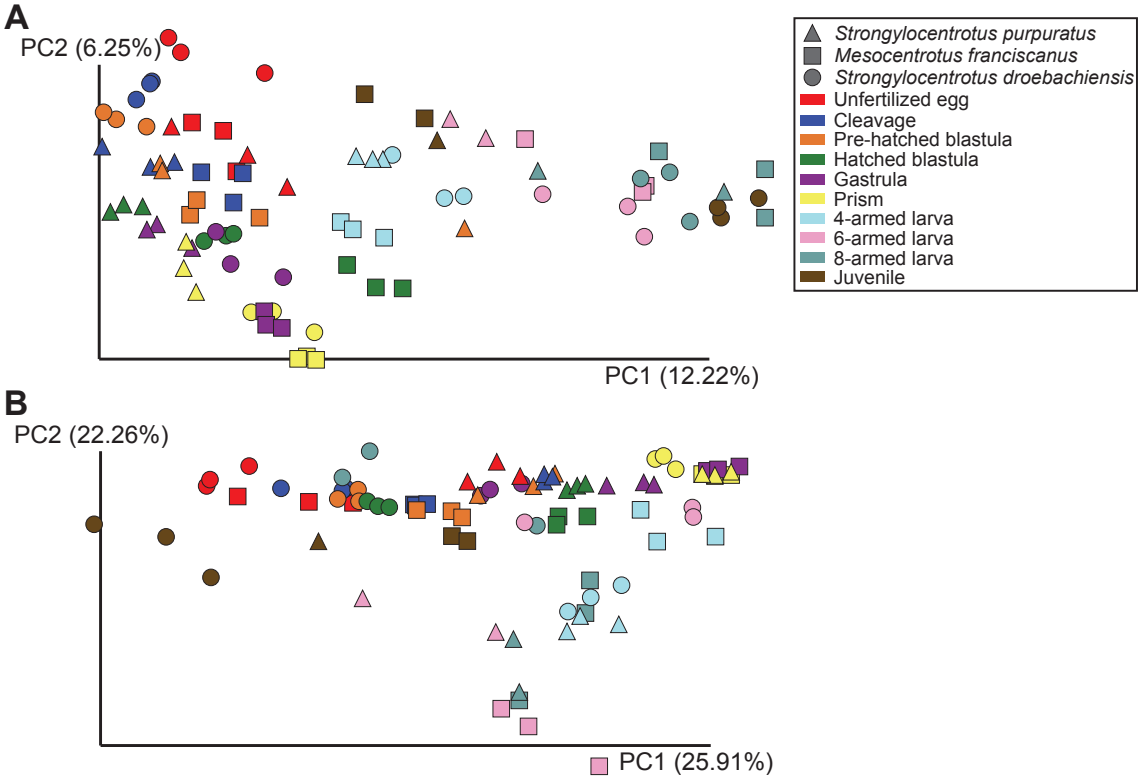
Figure 1


A
PC2 (6.25%)
PC1 (12.22%)
B
PC2 (22.26%)
PC1 (25.91%)
Strongylocentrotus purpuratus
Mesocentrotus franciscanus
Strongylocentrotus droebachiensis
Unfertilized egg
Cleavage
Pre-hatched blastula
Hatched blastula
Gastrula
Prism
4-armed larva
6-armed larva
8-armed larva
Juvenile

6 **Figure 2**

7

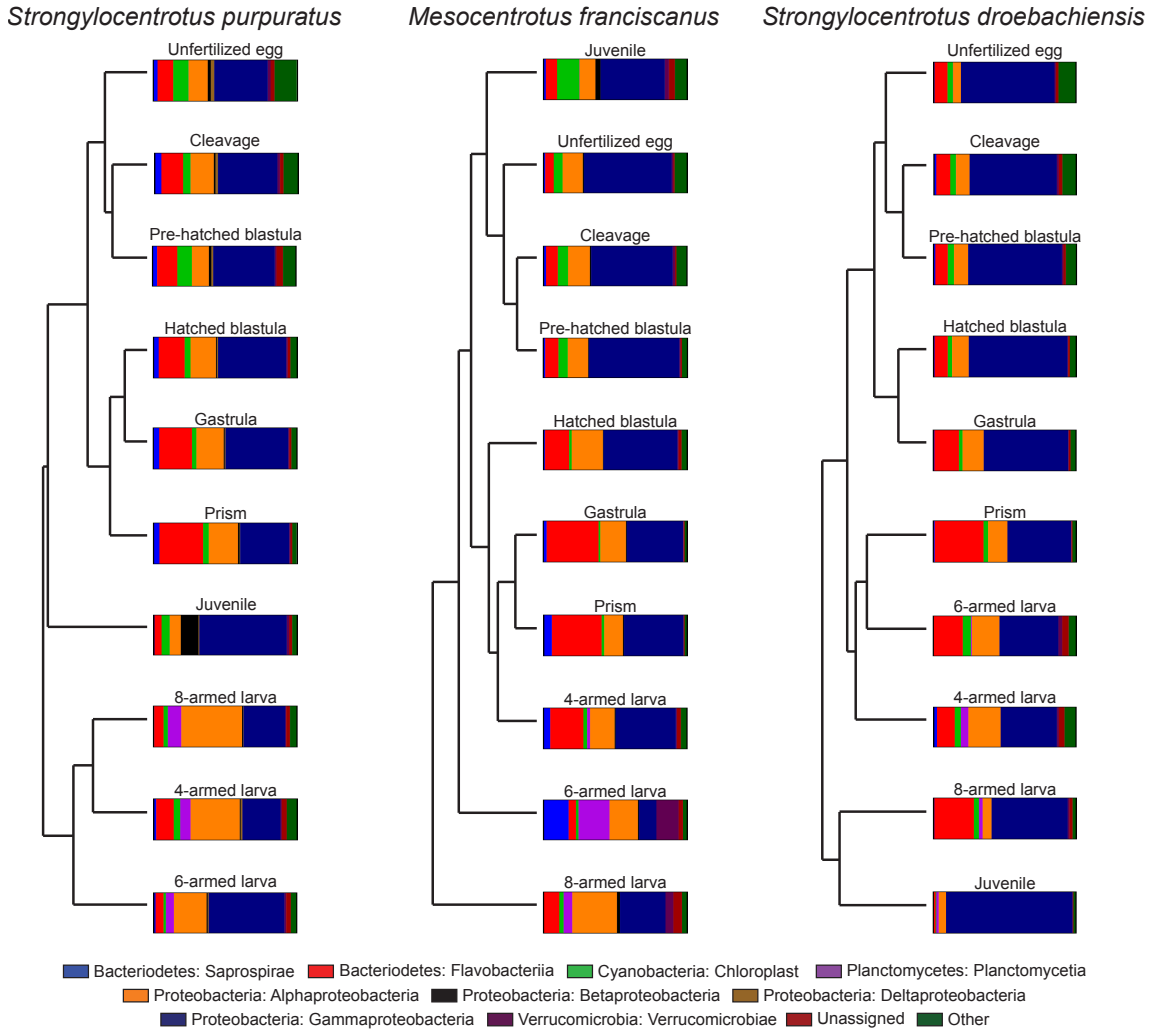


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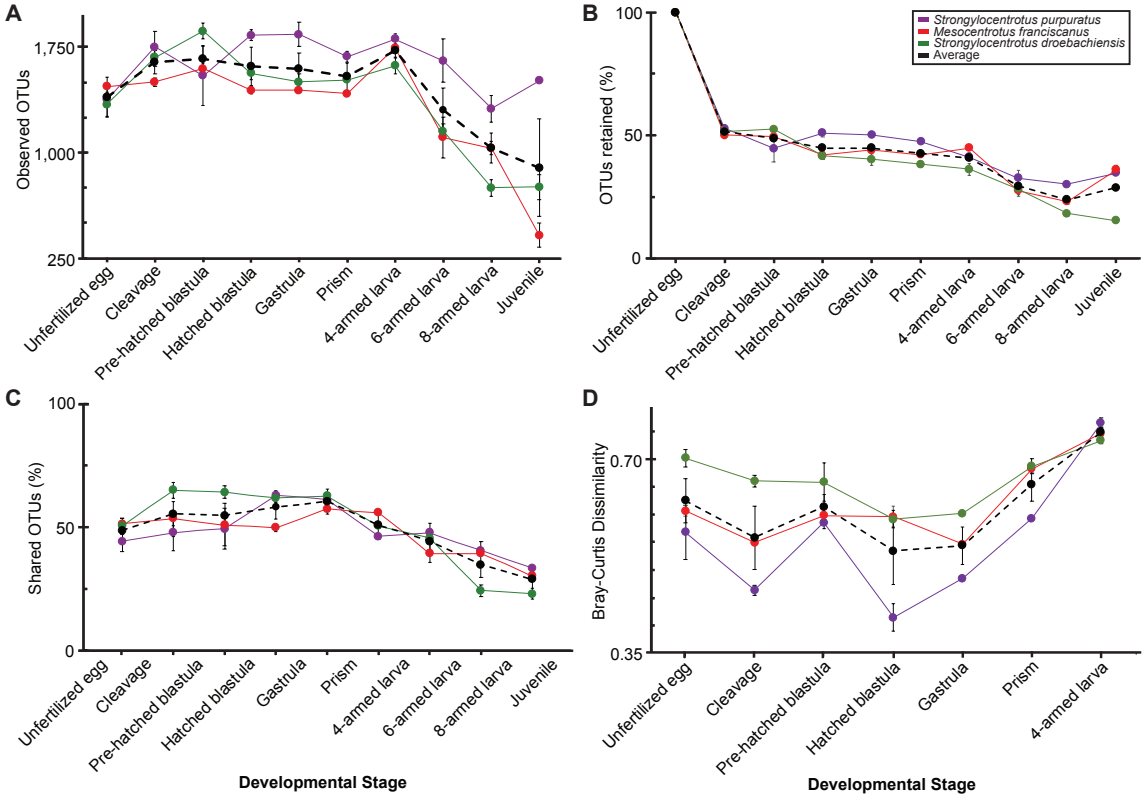
Figure 3


A
Observed OTUs
1,750
1,000
250
B
OTUs retained (%)
100
50
0
Strongylocentrotus purpuratus
Mesocentrotus franciscanus
Strongylocentrotus droebachiensis
Average
C
Shared OTUs (%)
100
50
0
D
Bray-Curtis Dissimilarity
0.70
0.35
Unfertilized egg
Cleavage
Pre-hatched blastula
Hatched blastula
Gastrula
Prism
4-armed larva
6-armed larva
8-armed larva
Juvenile
Developmental Stage

16 **Figure 4**
17

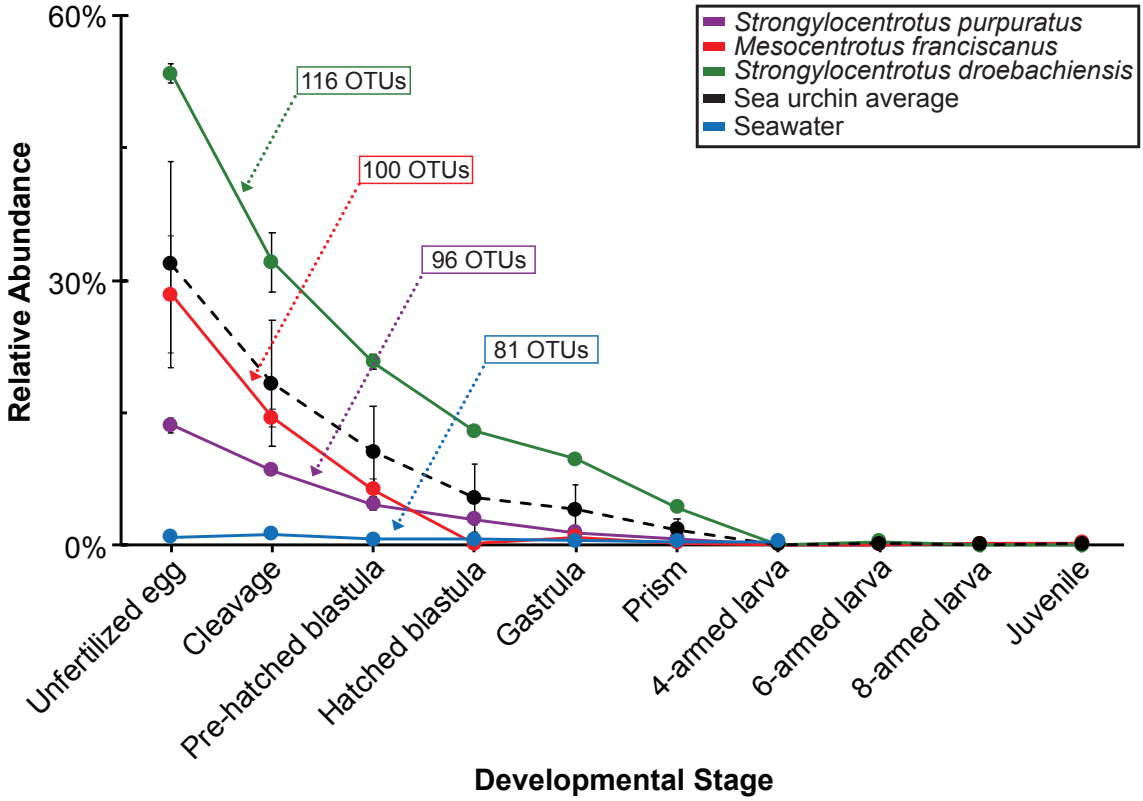


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Figure S1. Locations of adult urchin collections throughout the Salish Sea with geographic reference points. Specifically, *Strongylocentrotus purpuratus* were hand-collected at Slip Point, Clallam Bay, WA; *S. droebachiensis* were hand-collected at low tide at Cattle Point, San Juan Island, WA; and, *M. franciscanus* were collected by SCUBA off Bell Island, WA.

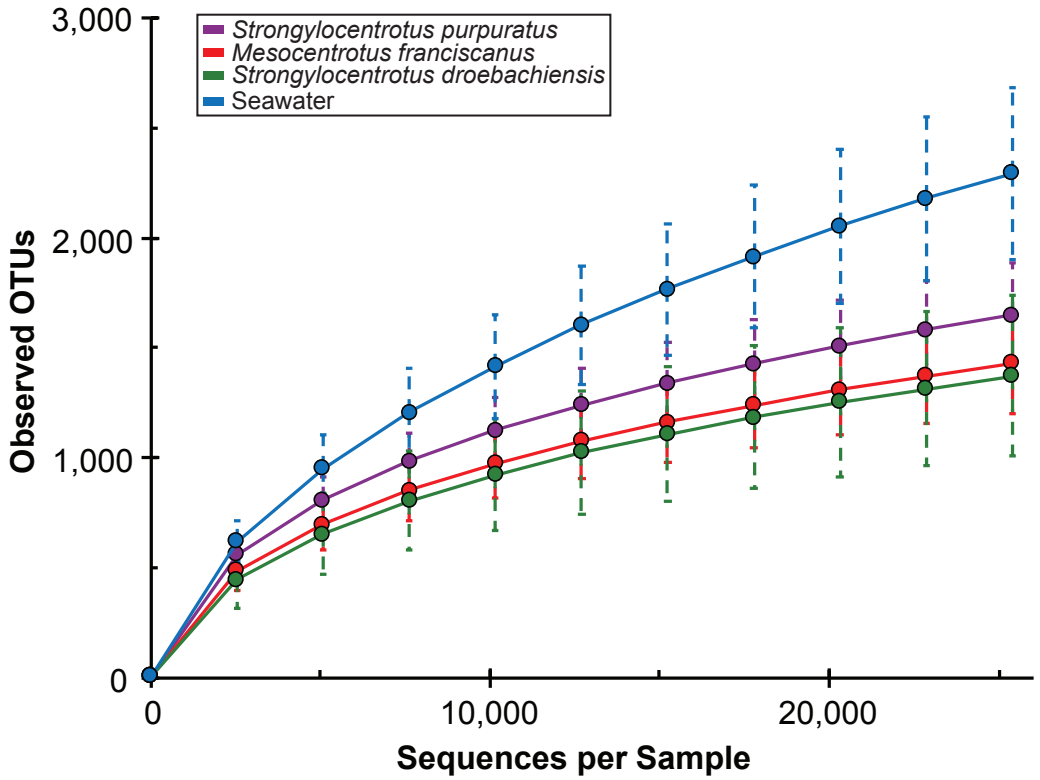

Figure S2. Alpha rarefaction curves for three species of echinoids and of the seawater. Alpha rarefaction curves for *Strongylocentrotus purpuratus* (purple), *Mesocentrotus franciscanus* (red), and *S. droebachiensis* (green) and seawater (blue) based on rarefaction depth of 25,396.

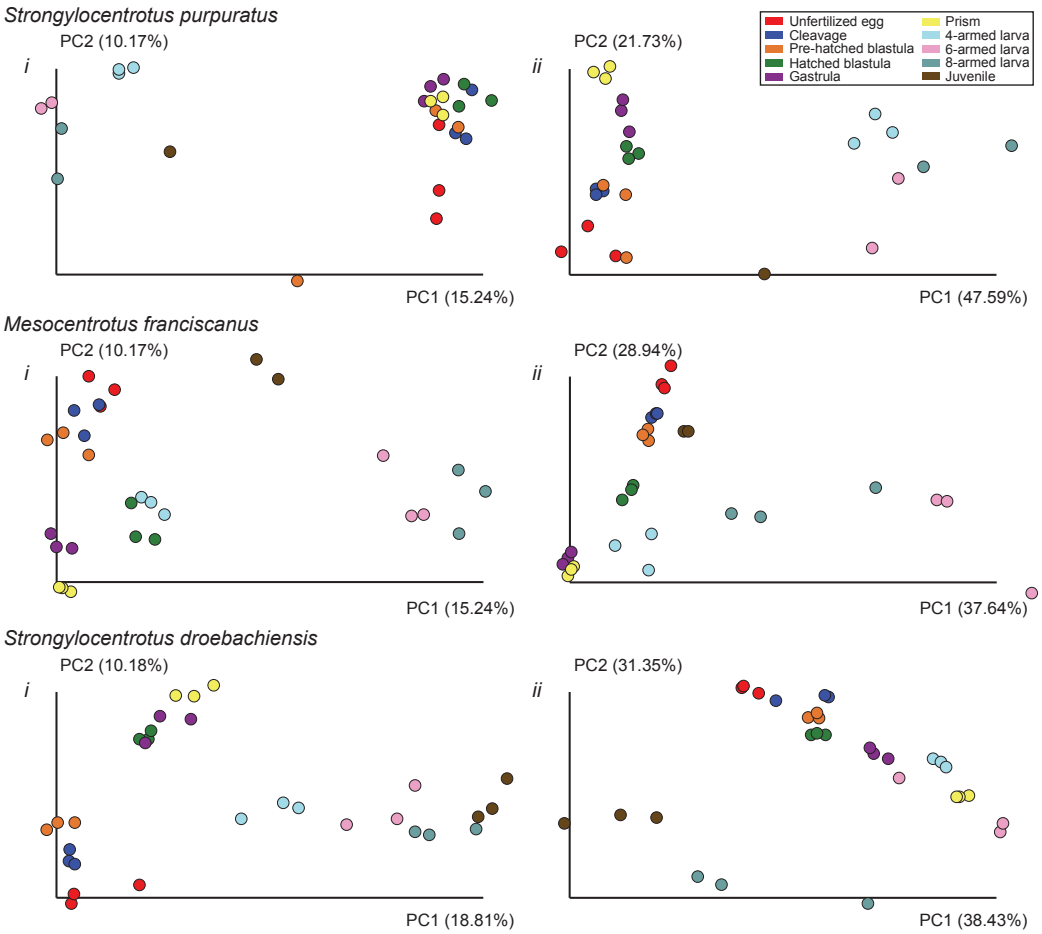

Figure S3. Similarity between the associated bacterial community across development for three species of echinoids. Community similarity for *Strongylocentrotus purpuratus* (top), *Mesocentrotus franciscanus* (middle), and *S. droebachiensis* (bottom) for ten developmental stages when considering the presence/absence of taxa (*i*) and their relative abundance (*ii*).

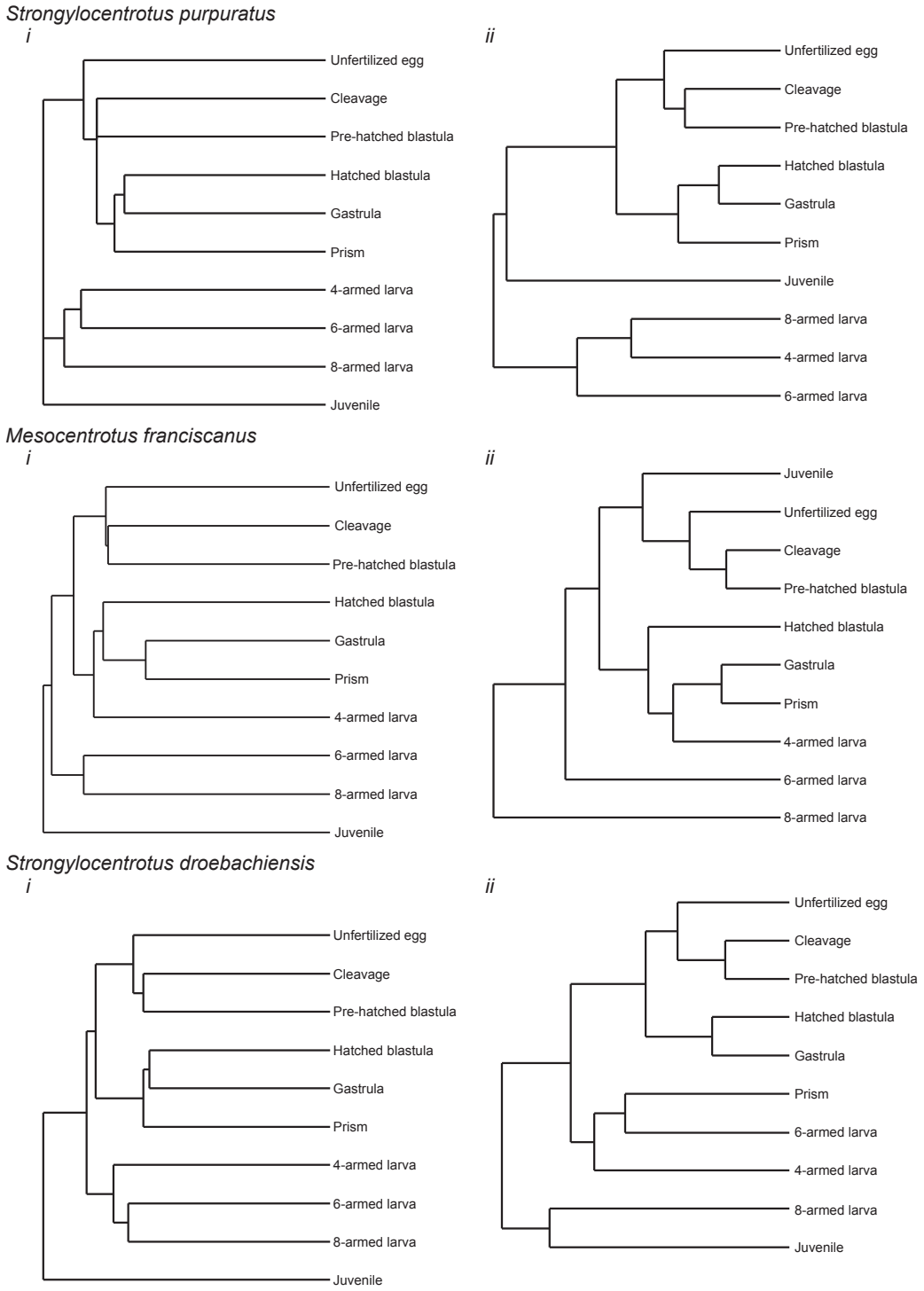

Figure S4. Bacterial community dendrograms across development for three echinoid species. Unweighted (*i*) and weighted (*ii*) bacterial community dendrograms for *Strongylocentrotus purpuratus* (top), *Mesocentrotus franciscanus* (middle), and *S. droebachiensis* (bottom).

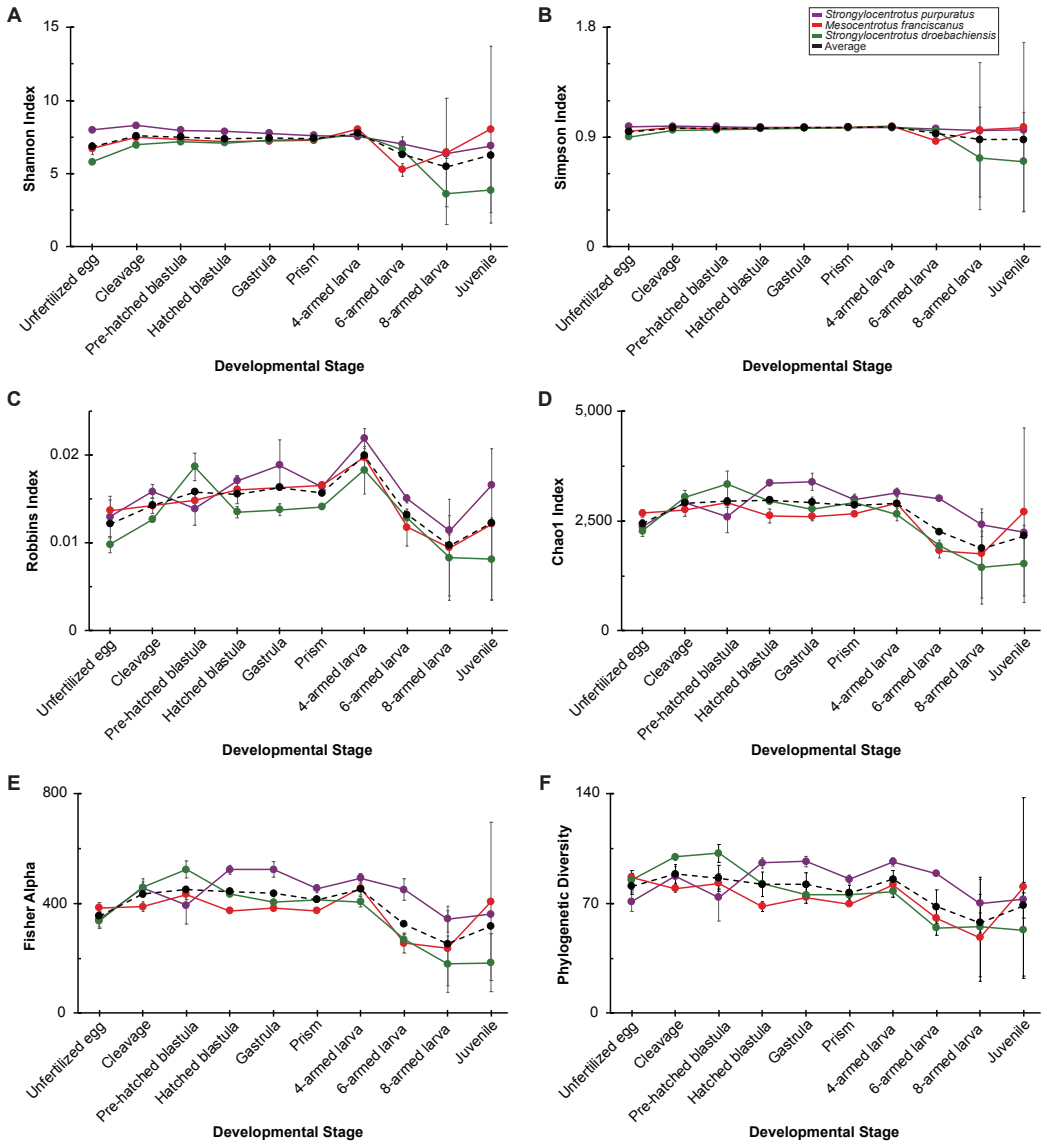

Figure S5. Various alpha diversity indices for three echinoid species across development. Shannon (A), Simpson (B), Robbins (C), Chao1 (D), Fisher (E), and Faiths (F) indices for *Strongylocentrotus purpuratus* (purple), *Mesocentrotus franciscanus* (red), *S. droebachiensis* (green), and the average (black) at ten developmental stages.

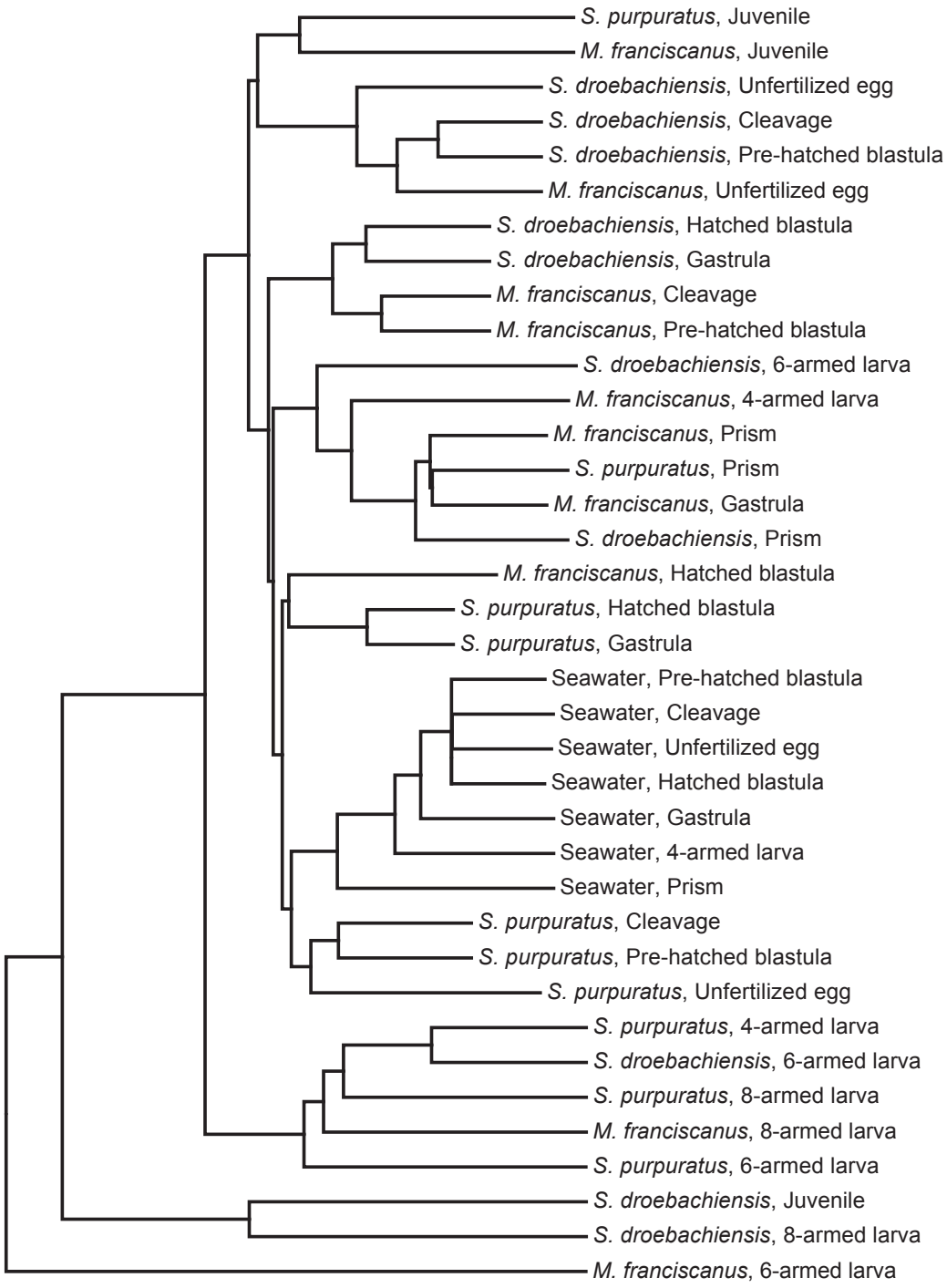

Figure S6. Microbial dendrogram of developmental stages and seawater. Dendrogram of the bacterial communities based on weighted UniFrac for the developmental stages of *Strongylocentrotus purpuratus*, *Mesocentrotus franciscanus*, and *S. droebachiensis* as well as the respective environmental bacterial communities.

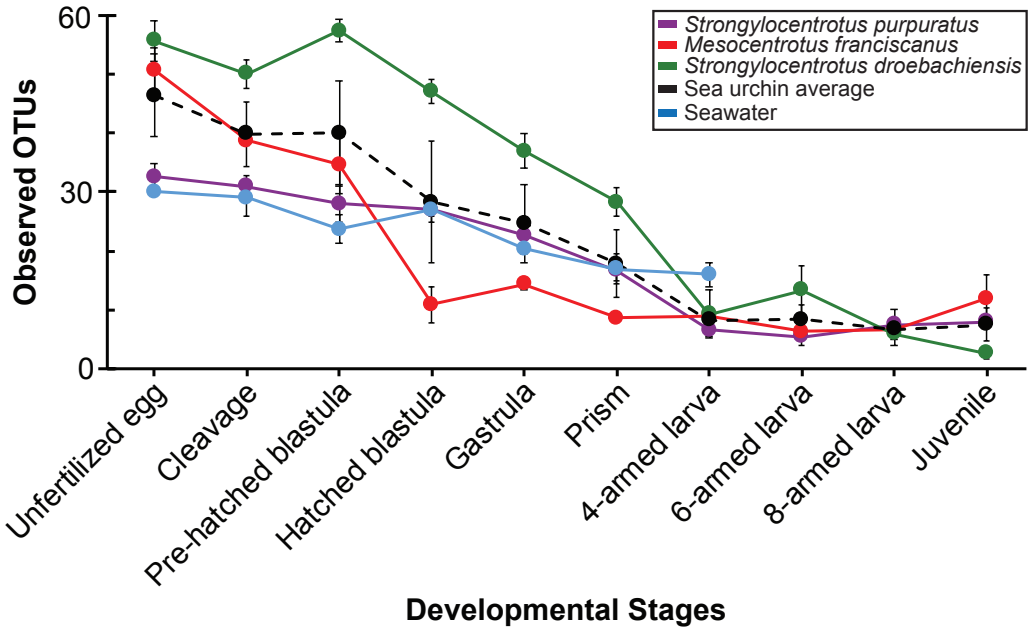

Figure S7. Diversity of *Psychromonas* during development. Total operational taxonomic units (OTUs) within the bacterial genus *Psychromonas* across embryonic and larval development for *Strongylocentrotus purpuratus* (purple), *Mesocentrotus franciscanus* (red), *S. droebachiensis* (green) as well as the average for the three sea urchin species and the in the seawater.