WILEY ECOLOGICAL APPLICATIONS

Effects of winter conditions on Olympia oyster reproduction and larval yield

Abstract:	For marine invertebrates that live in temperate regions, reproductive processes are tightly linked to seasonal temperature changes, yet we know little about how reproduction will shift as winters become milder. This study examined effects of winter temperature on spring reproduction in the Olympia oyster, *Ostrea lurida*. Adults were exposed to two winter temperatures (7°C, 10°C) in the presence of two feeding regimes, high (50k cells/mL) and low (5k cells/mL) algal density, for either 7 weeks or 12 weeks. Following treatments, adults were induced to spawn in common conditions using hatchery techniques, and larvae were reared through settlement to assess viability. Adults overwintered in elevated temperature contained larger oocytes, and those also held in elevated algal density contained more developed sperm. Elevated temperature (10°C) under both feeding regimes resulted in larvae that tended to be larger upon release from the maternal brood chamber. However, winter temperature did not impact fecundity, larval release timing, or larval viability, nor was larval viability related to larval size upon release. In the wild, more developed gametes and larger larvae following milder winters could greatly impact recruitment patterns. When

larvae are reared in the hatchery, however, elevated winter temperature will not likely impact larval viability or yield. Interestingly, overwintering duration greatly impacted broodstock survival and larval production. Regardless of winter temperature or feeding rate, broodstock overwintered in the hatchery for 12 weeks produced fewer larvae and had higher mortality during spawning compared to those held for only 7 weeks. Furthermore, broodstock overwintered in the low temperature treatment (7°C) with high algal density (50k cells/mL) experienced high mortality during spawning. Broodstock mortality is disadvantageous for hatcheries, can hinder larval production, and decrease genetic diversity of offspring. We therefore recommend that hatcheries overwinter *O. lurida* broodstock in slightly warmer temperatures and minimize the amount of time they are held in captivity prior to spawning. Finally, because algal density during winter treatments did not impact broodstock survival or spring larval production, hatcheries may restrict feeding without impacting production, given broodstock are in good condition upon collection.

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1   *Effects of winter conditions on Olympia oyster reproduction and larval yield*

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15 Abstract

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17 to seasonal temperature changes, yet we know little about how reproduction will shift as winters

18 become milder. This study examined effects of winter temperature on spring reproduction in the

19 Olympia oyster, *Ostrea lurida*. Adults were exposed to two winter temperatures (7°C, 10°C) in

20 the presence of two feeding regimes, high (50k cells/mL) and low (5k cells/mL) algal density,

21 for either 7 weeks or 12 weeks. Following treatments, adults were induced to spawn in common

22 conditions using hatchery techniques, and larvae were reared through settlement to assess

23 viability. Adults overwintered in elevated temperature contained larger oocytes, and those also

24   held in elevated algal density contained more developed sperm. Elevated temperature (10°C)

25   under both feeding regimes resulted in larvae that tended to be larger upon release from the

26   maternal brood chamber. However, winter temperature did not impact fecundity, larval release

27   timing, or larval viability, nor was larval viability related to larval size upon release. In the wild,

28   more developed gametes and larger larvae following milder winters could greatly impact

29   recruitment patterns. When larvae are reared in the hatchery, however, elevated winter

30   temperature will not likely impact larval viability or yield. Interestingly, overwintering duration

31   greatly impacted broodstock survival and larval production. Regardless of winter temperature or

32   feeding rate, broodstock overwintered in the hatchery for 12 weeks produced fewer larvae and

33   had higher mortality during spawning compared to those held for only 7 weeks. Furthermore,

34   broodstock overwintered in the low temperature treatment (7°C) with high algal density (50k

35   cells/mL) experienced high mortality during spawning. Broodstock mortality is disadvantageous

36   for hatcheries, can hinder larval production, and decrease genetic diversity of offspring. We

37   therefore recommend that hatcheries overwinter *O. lurida* broodstock in slightly warmer

38   temperatures and minimize the amount of time they are held in captivity prior to spawning.

39   Finally, because algal density during winter treatments did not impact broodstock survival or

40   spring larval production, hatcheries may restrict feeding without impacting production, given

41   broodstock are in good condition upon collection.

2

1. Introduction

1.1 Background

Temperature regulates many reproductive processes in marine invertebrates (Newell and Branch 1980; Hoegh-Guldberg and Pearse 1995). For species that live in temperate regions, reproductive cycles are tightly linked to seasonal temperature changes (Orton 1920). Gametogenesis onset, gamete growth rate, time to ripening, and the act of spawning are all believed to be a function of temperature, along with other environmental drivers such as nutrient availability and photoperiod (Olive 1995; Bates 2005). The precise timing, duration, and frequency of reproductive processes varies among species, but many follow a general seasonal pattern: rapid gametogenesis occurs in the spring; spawning occurs in the late spring and summer; recovery and resorption of residual gametes occurs in autumn, with some early differentiation of next season's gametes; and reproductive activity slows or ceases in the winter when temperatures drop below a minimum breeding temperature (Olive 1995; Orton 1920).

As global temperatures rise due to anthropogenic inputs, milder winters are anticipated due to increased sea surface temperature and more frequent marine heat waves (Gentemann, Fewings, and García-Reyes 2017; IPCC 2019). Ocean warming will invariably interfere with marine invertebrate reproductive cycles, possibly by altering reproductive timing, synchronicity, and productivity (Lawrence and Soame 2004; Edwards and Richardson 2004). For instance, we know that elevated temperature during spawning alters fertilization rates (Byrne 2011; Rogers-Bennett et al. 2010), and high adult mortality can occur when spawning coincides with heat stress (Mori 1979; Samain et al. 2007; Sastry 1966). We know little about how changes to winter conditions will impact reproductive processes.

67   Many temperate species are thought to enter reproductive diapause in the winter, until

68   temperatures exceed the physiological minimums for breeding in the spring (Orton 1920; Giese

69   1959; Pearse 1968; B. L. Bayne 1976). Winter warming could therefore result in uninterrupted

70   gametogenesis, precocious spawning, or asynchronous spawning if spermatogenesis and

71   oogenesis respond differently to winter warming (Philippart et al. 2003; Chevillot et al. 2017).

72   Warming could also impact offspring phenotype by direct impacts to gametes, or indirectly via

73   impacts to progenitors. Egg size, which is associated with lipid content and egg quality, typically

74   correlates negatively with maternal environmental temperature (Moran and McAlister 2009;

75   Gosselin et al. 2019; Atkinson et al. 2001). Maternal RNAs and lipid composition, which are

76   utilized by embryos and larvae during development, could differ if their structure or production

77   is sensitive to winter temperature (Leroy et al. 2018; Krisher 2013). It is also possible that

78   elevated metabolic demand during warmer winters will drain glycogen reserves, resulting in

79   smaller or poor-quality oocytes in the spring (Mathieu and Lubet 1993). Elevated winter

80   temperature therefore has capacity to alter wild populations and cultured stocks through wide-

81   scale shifts in reproductive timing and capacity, and offspring viability.

82   Impacts of warming on aquaculture species is particularly concerning, given the

83   increased global reliance on cultured fishes that is needed to meet growing demand for animal

84   protein (Hall 2011; U.S. Department of Commerce 2015). Aquaculture industries that require

85   reliable sources of wild-caught broodstock, larvae, or juveniles, or that grow their animals in

86   natural settings, all may be impacted by warming-induced reproductive changes (Cochrane et al.

87   2009; Stickney and McVey 2002). Here, we explore the effects of elevated winter temperature

88   on spring larval production in *Ostrea lurida,* the Olympia oyster. *Ostrea lurida* is native to the

89   Northeast Pacific Ocean, is cultured for restoration and the commercial market, and is related to

4

90   the widely cultured European flat oyster *Ostrea edulis* and Chilean flat oyster *Ostrea chilensis*

91   (Baker 1995). Adult *O. lurida* were overwintered at two temperatures (7°C, 10°C), both below

92   the reported minimum breeding temperature for the region (12.5°C) and selected to represent

93   historic and elevated winter temperatures, and larval production was subsequently monitored.

94   Two feeding regimes were tested to assess effects of temperature under high (50k cells/mL) and

95   low (5k cells/mL) algal density. To examine whether duration of temperature exposure impacts

96   reproduction, the four treatments were applied for two exposure durations (7 weeks, 12 weeks).

97   This study sought to examine whether winter warming will impact *O. lurida* reproduction and

98   offspring viability, and to improve *O. lurida* broodstock pre-conditioning practices to optimize

99   hatchery larval production.

100 1.2 Predictions

101 Observations of poor larval production in an *O. lurida* restoration hatchery following the

102 anomalously warm winter (10°C) as experienced during 2014-2016 (Gentemann, Fewings, and

103 García-Reyes 2017) suggested possible negative effects of elevated winter temperature on

104 fecundity and larval vigor. This prediction was based on energetic models in marine ectotherms,

105 which allocate energy for growth, reproduction, and maintenance (Sokolova et al. 2012). In

106 theory, elevated winter temperature increases an organism's metabolic rate, and in the absence of

107 increased food results in less energy available for reproduction (i.e. gamete development) and

108 growth (i.e. glycogen/lipid storage, calcification). Additionally, it was previously understood that

109 Olympia oysters enter reproductive quiescence in winter months due to cold temperatures (Oates

110 2013; Hopkins 1936; W. Coe 1932; W. R. Coe 1931b). Winter quiescence is presumably an

111 important phase for resorption of remnant gametes from the previous season, needed to maintain

112 adequate glycogen reserves to fuel spring gamete production. Based on these observations and

theories, elevated winter temperature (+3°C) was predicted to result in poor fecundity,

particularly in a food-limited environment (5k cells/mL). We report that despite signals of

overwintering temperature and algal density in gametes and larval size, neither treatment

impacted fecundity or larval viability. Unexpectedly, the amount of time broodstock were held

most significantly impacted larval production, as lower fecundity and higher broodstock

mortality was observed when broodstock were held for longer before spawning.

2. Methods

2.1 Broodstock treatments

Adult *Ostrea lurida* (3.80±0.50 cm) were collected from Mud Bay in Dyes Inlet in Bremerton,

WA on November 6, 2017, acclimated to hatchery conditions in filtered (5 um), flow-through

ambient seawater and fed live algae *ad libitum*. On December 8th, the broodstock were divided

among eight flow-through tanks (50-L), each with four bags of 50 animals for a total of 200

oysters per tank. Adults were treated in a factorial design to two temperatures (Cold: 7°C, Warm:

10°C) and two feeding levels (High: 50,000 cells/mL, and Low: 5,000 cells/mL), with two

replicate tanks per treatment (400 oysters per treatment in total). In addition to the four

treatments (7°C+high-food, 7°C+low-food, 10°C+high-food, 10°C+low-food), two treatment

durations were tested, 7-week and 12-week exposures (Figure 1).

To establish experimental conditions, temperatures were reduced by 0.5°C/day over one

and two weeks for the 10°C and 7°C groups, respectively, then maintained for the 7-week or 12-

week exposure durations. Temperatures were maintained by recirculating seawater from a

reservoir (50-L) through an aquarium chiller (Teco TK-2000 Tank Chiller, 1/3 HP, with built-in

heater), before seawater was distributed to experimental tanks at a flow rate of 8L/hr. Feeding

135 rates were maintained using Iwaki Metering pumps from a common algae mixture (cocktail of

136 *Tisochrysis lutea*, *Tetraselmis suecica*, and *Chaetoceros* spp). Algae cocktail concentration was

137 estimated daily by manual cell counts and adjusting metering pump rates to achieve 50,000 and

138 5,00 cells/mL for high and low feeding rates, respectively. Temperature was monitored

139 continuously with Avtech temperature probes and recorded with HOBO Pendant Temperature

140 Data Loggers (UA-002-64). Broodstock were cleaned twice weekly and monitored for mortality.

141 On January 24th (7-week exposure) half of the broodstock were removed and returned to

142 common conditions for reproductive conditioning and spawning. The other half of the

143 broodstock remained in temperature and diet treatments until February 28th (12-week exposure),

144 then were similarly conditioned and spawned. Tissue sampling occurred on a regular basis

145 (Figure 1).

146 2.2 Broodstock tissue sampling

147 Approximately twice monthly during broodstock treatments (November 30 - February 27, Figure

148 1), 10 oysters per treatment were sacrificed to assess growth and gonad development, sampled

149 evenly across treatment replicates and bags within replicates. An additional 10 oysters were

150 collected directly from the source location (Dyes Inlet, Washington, henceforth "wild") on each

151 sampling day to assess winter growth and gonad development in the natural setting. Broodstock

152 were also sampled twice during reproductive conditioning for the 12-week experimental animals

153 only (n=12 per treatment, March 13 and 23). Upon sampling, shell height was measured as the

154 distance from hinge to margin, perpendicular to the hinge, and tissue wet weight was estimated

155 by subtracting shell weight from whole wet weight. Oyster plumpness, an estimate of condition

156 index, was calculated as the ratio of wet tissue weight to shell height. Gonad tissue was excised

157 by opening the oyster at the umbo, discarding gill tissue, then preserving the whole visceral mass

158   in the PAXgene Tissue FIX system (PreAnalytiX, Hombrechtikon, Switzerland). Fixed gonad

159   tissues were processed for gonad analysis by Histology Consulting Services (Everson, WA).

160   2.3 Gonad stage and sex designations

161   The sex and stage of sampled oysters were determined from preserved gonad histology sections,

162   using designations adapted from (da Silva, Fuentes, and Villalba 2009). As per da Silva, sex was

163   assigned as indeterminate (I), male (M), hermaphroditic primarily-male (HPM), hermaphroditic

164   (H), hermaphroditic primarily-female (HPF), and female (F). Due to the high frequency of

165   hermaphroditism (41.3% of the 386 sampled oysters), male and female gametes within the same

166   oyster were assigned separate developmental stages, then a dominant gonad stage was assigned

167   for each oyster based on the predominant sex. The da Silva designations were applied for stages

168   1-3 (1: early gametogenesis; 2: advanced gametogenesis; 3: ripe). Departures from da Silva's

169   stage 0, stage 4 (partially spawned), and stage 5 (fully spawned/resorbing) are as follows: stage 0

170   in this study represents empty follicles, or no presence of male or female gonad tissue. Stage 4

171   represents both spawned and resorbing gonad. This method does not include a separate stage 5,

172   due to the very high frequency of residual gametes, and no distinct partially spawned oysters.

173   Impacts of treatments on gonad development were assessed using Chi-Square or Fisher

174   Exact tests on contingency tables (depending on sample size), which were constructed from

175   counts of gonad sex, male gamete stage, and female gamete stage. Prior to statistical testing, the

176   six sex categories (I, M, HPM, H, HPF, F) were collapsed into four categories by combining

177   HPF and F into one female designation (F), and HPM and M into one male designation (M).

178   Tests were performed for each sampling date individually, for all treatment weeks combined

179   (December 20 - February 27), and reproductive conditioning weeks combined (March 13 & 23).

180   To account for multiple comparisons (nine tests per gonad metric), significance was designated

181   as α=0.0056. To determine pairwise differences Fisher Exact post-hoc tests were run using the

182   pairwiseNominalIndependence function from the rcompanion package (vs. 2.3.7).

183   2.4 Ripe oocyte size

184   To assess impacts of treatments on maternal provisioning, ripe oocytes were measured just prior

185   to and during spawning (February 27, March 13 & March 23) for the 12-week exposed oysters.

186   The maximum oocyte length was measured for 24 of the largest oocytes in oysters determined to

187   be stage 3 (ripe/gravid) females. Measurements were taken using a Nikon eclipse Ni microscope

188   and the NIS-Elements BR imaging and measuring software (version 4.60). The maximum length

189   was assessed due to elongated oocyte shape, and the varying orientation of oocytes in mounted

190   histology sections. The number of stage 3 females varied among treatments, and was 7, 6, 10,

191   and 14, for 7°C+high-food, 7°C+low-food, 10°C+high-food, and 10°C+low-food, respectively.

192   Mean oocyte length was calculated for each oyster, then compared among treatments using Two-

193   Way Analysis of Variance.

194   2.5 Larval production

195   After terminating treatments, broodstock were spawned to assess impacts of treatments on larval

196   production. Following hatchery operating procedures, continuous, volitional spawning was

197   induced by holding broodstock in elevated temperature and nutrition. Reproductive conditioning

198   began on January 24 for the 7-week trial, and on February 28 for the 12-week trial. Broodstock

199   were divided into flow-through spawning tanks (20-L at 26-L/hr), with three treatment replicates

200   of ~50 oysters for the 7-week trial, and four treatment replicates of ~25 oysters for the 12-week

201   trial. Temperature was increased 0.5°C/day for 7°C treatments to 10°C (7 days), then all groups

202   increased 1°C/day to 18°C and fed live algae cocktail ad libitum. Tanks were checked daily for

203   veliger larvae, which are released from the maternal brood chamber approximately 10-14 days

204   after fertilization (Coe 1931a, Hopkins 1937). Once larval release began, larvae were collected

205   daily for four weeks and counts were estimated by hand-counting larvae in triplicate subsamples.

206   Twice weekly tanks were cleaned and broodstock were inspected for mortality.

207   Larval production timing and magnitude were compared among treatments (temperature

208   x food level) and treatment duration (7-week, 12-week). Release timing was assessed by

209   comparing the date of onset and date of maximum release using Kruskal-Wallis rank sum tests.

210   Release magnitude was assessed by comparing the average number of larvae released each day

211   and the total number of larvae released over the 4-week collection period by Analysis of

212   Variance (ANOVA) of a fitted linear model with aov().

213   2.6 Larval size upon release

214   To assess impacts of broodstock treatment on larval size, larvae were measured upon release

215   from the maternal brood chamber. After larvae were collected, counted, and aliquoted to rearing

216   tanks, excess larvae were preserved directly in -80°C, then measured using a Nikon eclipse Ni

217   microscope and the NIS-Elements BR imaging and measuring software (version 4.60). Mean

218   shell height (distance from hinge to margin, perpendicular to hinge), and mean shell width

219   (longest distance parallel to hinge) were estimated from at minimum 40 larvae per group. Mean

220   height and width were compared among treatments using Two-Way Analysis of Variance.

221   2.7 Larval survival

222   To assess impacts of broodstock treatments on larval vigor, a subset of larvae collected from the

223   12-week trial were reared through settlement. In total, 48 pulses of larvae were reared, 12 per

224   broodstock treatment, which were collected over a 19-day period. As multiple females can

225 release larvae on the same day, some larval pulses may represent more than one male x female

226 mating pair, and thus each pulse is henceforth referred to as a "group" (as opposed to "family").

227 To ensure genetically diverse larval groups within treatments, three groups were collected from

228 each of the four replicate spawning tanks. Upon collecting a larval group, larvae were cleaned of

229 debris using nylon mesh (224 μm) and soaked in fresh water (18°C) for 1 minute. For each larval

230 group, approximately 2,400 larvae were reared in triplicate (800 larvae per tank). Larval stocking

231 error rate (3.1%, mean 824±54 SD larvae) was determined for 12 for the 48 groups by hand

232 counting triplicate samples, taken simultaneous to tank preparation. Larval tanks were

233 constructed from thin-walled (7.6 cm) polyvinyl chloride pipe and nylon mesh (100 μm) placed

234 in individual containers with static seawater (800±50 mL). Water was changed daily from a

235 common mixture of filtered seawater (<1 μm) and live algae, which consisted of a 1:1 mix by

236 volume of *Chaetoceros muelleri* and *Pavlova pinguis*, for a combined concentration of 100,000

237 cells/mL. Fourteen days after collecting each larval group, oyster shell fragments (0.5-mL of 224

238 μm) were sprinkled into larval tanks to serve as settlement substrate. After thirty-five days, post-

239 settlement survival rate was estimated by hand-counting the number of metamorphosed larvae.

240 Factors influencing larval survival were assessed using quasibinomial generalized linear models

241 (GLM) and Pearson's Chi-squared tests. Factors tested included broodstock temperature

242 treatment, broodstock feeding level, larval size upon release (height, width), the number of

243 larvae released in a group (i.e. brood size), and the date larvae were collected.

3. Results

3.1 Broodstock reproductive development

3.1.1 *Gonad stage and sex*

On the final day of the 12-week trial (February 27), sperm developmental stage differed

significantly among experimental treatments ($\chi^2 = 32.5$, p=0.0052). More of the 10°C+high-food

oysters contained advanced or ripe male gametes (60%) than the 10°C+low-food (0%) and

7°C+low-food oysters (0%) (Table 1, Figure 2). Sperm stage also differed among the 12-week

trial during the spawning phase (March 13 & 23, $\chi^2 = 32.2$, p=0.0010). The 10°C+high-food

group contained more late-stage spermatocytes (58% male tissue was advanced or ripe) and only

2 oysters fully lacked sperm (8%), compared the other treatments which had a higher proportion

without sperm (21%, 42%, and 63% for 7°C+low-food, 10°C+low-food, 7°C+high-food,

respectively, Table 1, Figure 2). No differences in gonad sex, male stage, or female stage were

detected during the 7-week trial.

　　　No brooded larvae were found in sampled oysters during experimental treatments. For

the 12-week trial, brooded embryos were first observed on March 23, 24 days after beginning the

spawning phase, and 8 days after reaching the maximum spawning temperature (18°C). Of the

12 oysters sampled per treatment on March 23, there were six broods in 10°C+high-food (white

sic only), five in 10°C+low-food (three white sic, two gray sic), one in 7°C+low-food (white

sic), and zero in 7°C+high-food. Broadly, there were few obvious instances of oocyte atresia,

indicating that winter oocyte resorption was not common.

264   3.1.2 *Ripe oocyte size*

265   Broodstock held in elevated temperature had larger ripe oocytes (stage 3) prior to and during

266   spawning (Feb 27, Mar 13 & 23) than broodstock held in cold temperature ($F_{(1,33)}=6.06$,

267   p=0.019, Figure 5). Feeding level had no effect on ripe oocyte size (p=0.32), nor was there an

268   interaction between temperature and food treatments (p=0.77). Mean oocyte length (maximum

269   length) of stage 3 oocytes was 91±14 μm and 83±13 μm for 10°C and 7°C oysters, respectively.

270   3.2 Larval production

271   3.2.1 *Larval release timing*

272   Broodstock exposed to treatments for less time (7-weeks) released larvae earlier than those

273   exposed for longer (12-weeks, $\chi^2 = 5.61$ p=0.018, release onset in Table 2). Larvae were first

274   observed 26 days after entering spawning conditions in the 7-week trial, and 31 days in the 12-

275   week trial. Release onset was also more synchronized in broodstock exposed for 7-weeks; larvae

276   were collected from all 7-week spawning groups within 2 days of each other, while 12-week

277   spawning groups released larvae up to 8 days apart. Peak larval release occurred sooner in the

278   oysters held for 7-weeks (5.8±2.8 days) than those held for 12-weeks (10.2±6.4 days,

279     $F_{(1,20)}=6.14$, p=0.022).

280   Broodstock exposed to low levels of food began releasing larvae on average 2 days

281   earlier than those exposed to high food levels ($\chi^2 = 6.84$, p=8.9e-3, Figure 3). There was also

282   significant interaction between food level and exposure duration (Trial:Food, onset $\chi^2 = 18.1$

283   p=4.1e-4). The broodstock exposed to low-food for 12-weeks began to release larvae on average

284   3.75 days earlier than those exposed for 12-weeks to high-food. All three factors influenced

285   larval release timing (Trial:Temp:Food: onset $\chi^2 = 18.9$ p=8.6e-3, peak F=5.95 p=0.024, Table

286   2). The 3-factor interaction was influenced predominantly by the 12-week 7°C+high-food

287   treatment, which released larvae much later than the other groups (up to one week, Figure 3).

288   Temperature did not influence onset or peak release timing as a sole factor.

289   3.2.2 *Larval release magnitude*

290   Broodstock held for 7-weeks released more larvae than those held for 12-weeks (Figure 4). The

291   cumulative number of larvae, normalized by the number of broodstock and averaged across

292   treatments and replicates, was 164,000±40,000 from 7-week broodstock and 92,000±29,000

293   from 12-week broodstock (F(1,20)=29.4, p=2.65e-5). The magnitude of larvae released did not

294   differ among temperature or food treatments (Table 2).

295   3.3 Larval viability

296   3.3.1 *Larval shell height and width at release*

297   Broodstock exposed to elevated food levels produced larger larvae, with larger mean shell width

298   (F(1,120)=3.92, p=0.050). Broodstock exposed to elevated temperature also tended to release

299   larvae with larger shell width (F(1,120=3.00, p=0.086, Figure 5). Of the larvae reared through

300   settlement, those released from high-food exposed broodstock were larger (shell width:

301   F(1,36)=4.66, p=0.038; shell height: F(1,36)=4.63, p=0.038).

302   3.3.2 *Larval Survival*

303   Larval survival through metamorphosis (assessed 5 weeks after release) was not significantly

304   influenced by broodstock temperature (χ^2=2.7, p=0.098) or food treatment (χ^2=0.012, p=0.89),

305   nor was there a significant interaction between the two treatments (χ^2=3.36, p=0.067, evaluated

306   using quasibinomial GLM). Larval survival was not associated with larval shell height (χ^2=2.65,

14

307   p=0.10) or width (χ^2=0.29, p=0.59, Figure 6) upon release, but survival was associated with the

308   number of larvae released on the day of collection (χ^2=6.93, p=0.0085), and the date of release

309   (χ^2=15.5, p=8.5e-5). Survival tended to be higher from larger pulses of larvae, and at later

310   collection dates (Supplemental Figure 2).

311   Broadly, larval survival was highly variable among the 48 groups, which were collected

312   from distinct larval pulses to maximize genetic diversity (3 groups from each of the 12 spawning

313   tanks). Mean and median survival was 10.6% and 7.2% across all groups, and ranged from

314   0.3±0.3% to 46.1±5.6%. Survival exceeded 20% in 9 of the 48 groups (Table 3).

315   3.4 Broodstock survival and size

316   During reproductive conditioning, high broodstock mortality occurred in both 7°C groups.

317   Cumulative survival at the end of the experiment was 50% and 70% in the 7°C+high-food and

318   7°C+low-food groups, and 81% in both 10°C+high-food and 10°C+low-food groups (Figure 7).

319   Oyster plumpness (wet tissue weight normalized by shell height) decreased over time in all

320   experimental broodstock (F(1,280)=11.8, p=7.5e-4), and in wild-collected oysters (F(1,68)=29.5,

321   p=8.3e-7). Shell height, which was on average 38.0±5.0 mm, did not change significantly in

322   experimental broodstock (F(1,294)=3.55, p=0.061), but increased in the wild-collected oysters

323   (F(1,68)=7.88, p=06.5e-3). Broodstock shell height and wet tissue weight did not differ by

324   temperature or feeding level during treatment.

325   **4. Discussion**

326   This study investigated impacts of over-wintering conditions on *O. lurida* larval production.

327   Broodstock were simultaneously exposed to different temperatures (7°C, 10°C), and algal

328   densities (low-food=5k cells/mL, high-food=50k cells/mL) for 7-weeks or 12-weeks in winter

329   prior to spawning. We predicted that elevated winter temperature would result in fewer larvae or

330   poorly provisioned larvae (i.e. smaller, poor survival), which would be amplified in the presence

331   of low food and after prolonged treatment (12-weeks). This hypothesis was based on

332   observations of poor larval production in an *O. lurida* hatchery the spring following the winter

333   2015 marine heat wave in the Northeast Pacific Ocean (Gentemann, Fewings, and García-Reyes

334   2017). Elevated winter temperature was posited to interfere with seasonal reproductive

335   quiescence, which is considered an important component of the *O. lurida* reproductive cycle, and

336   deplete glycogen and lipid reserves due to increased metabolic demand.

337   While winter temperature and algal density did influence gamete development and larval

338   release timing, treatments did not ultimately influence larval production. Counter to predictions,

339   larvae tended to be larger from broodstock exposed to elevated temperature, however larval size

340   did not correlate with larval survival. Surprisingly, high broodstock mortality occurred following

341   exposure to low temperature, and fecundity was substantially lower after prolonged exposure to

342   all treatments. We expand on these findings below, and include recommendations for the

343   handling of *O. lurida* broodstock during the winter for restoration and commercial hatcheries.

344   4.1 Effects of overwintering temperature and algal density

345   4.1.1 *Larval production*

346   Larval production was unaffected by winter temperature and algal density, regardless of

347   exposure time (7 or 12 weeks). The results are in contrast with a complementary study, Spencer

348   et al. (2020), in which elevated temperature exposure prior to spawning resulted in more larvae.

349   The present study specifically expands Spencer et al. (2020), to use a new *O. lurida* population

350   collected from the wild rather than oysters that were bred in captivity. The response of *O. lurida*

351   reproduction to elevated winter temperature may be conditional upon gonad stage prior to

352   treatment, and population-specific reproductive traits (Barber, Ford, and Wargo 1991; Silliman,

353   Bowyer, and Roberts 2018). For instance, in this study the percentage of female broodstock was

354   unusually high upon collection (63%), and many already contained late-stage oocytes (55% of

355   females). In comparison, only 28% of all oysters sampled in Spencer et al. (2020) were female,

356   33% of which contained late-stage oocytes. Oysters that enter the winter with late-stage oocytes

357   may be less influenced by winter conditions, and may require less time and energy for

358   maturation in the spring. The developmental status of Olympia oyster oocytes entering the winter

359   season may therefore influence how winter temperature affects spring reproduction. That larval

360   production was unaffected by overwintering treatments suggests that *O. lurida* are capable of

361   withstanding a range of winter conditions without compromising spring larval production.

362   4.1.2 *Larval survival*

363   Larval survival was not influenced by parental exposure to elevated winter temperature. This

364   indicates that the quality of *O. lurida* larvae produced in the hatchery is not sensitive to winter

365   conditions. The broodstock collected for this study may have had sufficient energy stores to

366   endure winter treatments and subsequently reproduce. Alternatively, *O. lurida* may modulate

367   energy allocation to prioritize reproductive processes over other processes such as growth. While

368   these results contrast expectations they are in alignment with the related study, Spencer et al.

369   (2020), which also did not detect temperature effects on larval survival. Together, these studies

370   indicate that elevated winter temperature does not compromise larval quality when larvae are

371   reared in a hatchery setting. Interestingly, broodstock that were exposed to elevated temperature

372   contained larger oocytes, and tended to produce larger larvae. Both oocyte and larval size are

373   typically associated with maternal provisioning and can correlate with larval survival (Gallager

374   and Mann 1986; Fukazawa et al. 2005; Kraeuter, Castagna, and van Dessel 1981; Wilson,

375   Chaparro, and Thompson 1996; Millican and Helm 1994). Larger oocytes and larvae following

376   elevated winter temperature did not translate to higher larval survival, which may reflect the

377   larval rearing conditions (*e.g.* ample food). In the wild, larger larvae may reflect more maternal

378   provisions or a faster growth rate, such that a warmer winter could result in higher recruitment

379   rates. Oocyte and larval size could also be a signal of winter temperature in wild-collected

380   larvae. These hypotheses, however, should be tested with unfed larvae to determine whether

381   larger oocytes and larvae are in fact more viable.

382   Overwintering broodstock with varying algal density also did not affect larval survival.

383   We know from prior work that parental nutrition at various phases can be critical for gamete and

384   larval quality (Marshall and Keough 2007). In *Ostrea*, a single-algal species fed to *Ostrea edulis*

385   during conditioning results in smaller larvae and low survival through metamorphosis, when

386   compared to a mixed algal diet (Millican and Helm 1994; Gonzalez Araya et al. 2012). Similarly,

387   starved *Ostrea chilensis* and those fed a single algae diet produce smaller and slower-growing

388   larvae than those fed a diverse estuarine diet (Wilson, Chaparro, and Thompson 1996). Berntsson

389   et al. (1997) found that broodstock diet during conditioning did not affect *O. edulis* larval

390   survival, but did influence growth rate. Here, higher algal density tended to result in larger larvae

391   upon release, but did not influence larval survival. Broodstock diet was only limited during

392   treatment, not during spawning, which could have obfuscated effects of winter starvation.

393   Alternatively, the animals tested may have been buffered by endogenous energy reserves,

394   perhaps acquired from fall phytoplankton blooms. Starving broodstock through the spawning and

395   brooding phases would expose whether *O. lurida* rely on endogenous glycogen to provision

396   gametes, or can exploit exogenous energy when available.

397   4.1.3 *Gamete development*

398   Broodstock exposed to both elevated temperature and food for 12 weeks contained more

399   developed sperm compared to low-food treatments (Figure 2, Table 1). Elevated food and

400   temperature could have resulted in persistent, low-levels of spermatogenesis throughout the

401   winter, which may not have occurred in the low-food treatments. While food availability likely

402   plays a significant role in *O. lurida* reproductive cycles, a foundational study is needed to

403   determine whether supplemental algae is a necessary cue for *O. lurida* spermatogenesis, or if it

404   simply accelerates it when combined with elevated temperature.

405   Overwintering treatments also impacted oocyte growth. Based on observations of slightly

406   larger larvae from broodstock exposed to elevated temperature, we measured ripe oocytes to see

407   whether oocyte size explained larval size. Indeed, broodstock that were exposed to elevated

408   temperature contained significantly larger ripe oocytes after 12-weeks and during spawning, but

409   the cause is not clear. In ectothermic species, egg size often correlates negatively with

410   environmental temperature during oogenesis, both between and within ectothermic species

411   (Moran and McAlister 2009; Fischer, Brakefield, and Zwaan 2003). We observed the opposite -

412   larger eggs under warmer temperature. This could be explained by low-level vitellogenesis

413   persisting in elevated temperature, resulting in oocyte growth throughout the winter.

414   Alternatively, broodstock held at elevated winter temperature may have experienced less thermal

415   stress when temperature was increased rapidly to induce spawning, resulting in more energy

416   available for vitellogenesis. Under experimental conditions, the observed gamete differences had

417   no bearing on larval production or viability. In a natural setting, slight changes in reproductive

418   development and larval size could alter spawn timing and larval recruitment, factors which

419   greatly influence population dynamics.

19

420   *4.1.4 Broodstock survival*

421   Poor survival was observed in broodstock that had been exposed to low temperature (7°C) in the

422   presence of high food, particularly those exposed for 12-weeks (Figure 7). The majority of

423   mortality occurred when broodstock from all treatments were in common conditions and

424   undergoing a 1°C/day increase to induce spawning. The added 3°C increase for the low

425   temperature exposed oysters may have been thermally stressful, or increased their susceptibility

426   to bacterial infection, resulting in the high mortality (Delaporte et al. 2006; Lokmer and Mathias

427   Wegner 2015; Wendling and Wegner 2013). In addition to thermal stress, mortality in the

428   7°C+high-food treatment could also be due to oxidative stress caused by high reproductive

429   effort. Just prior to spawning the 12-week trial broodstock, plumpness (a proxy for condition

430   index) increased in the 7°C+high-food treatment only (Figure 7). Condition index is positively

431   associated with post-spawning mortality in other oyster species, explained in part by high

432   glycogen reserves fueling rapid gamete proliferation, which elevates metabolism and causes

433   oxidative stress (Alonso-Alvarez et al. 2004; Chávez-Villalba, Villelas-Ávila, and Cáceres-

434   Martínez 2007; Huvet et al. 2010). High mortality in the 7°C+high-food broodstock may

435   therefore be associated with synergistic effects of thermal stress and oxidative stress. The

436   extended time in captivity likely compounded these effects, resulting in high mortality in the 12-

437   week trial during spawning.

438   4.2 Additional lessons learned for *O. lurida* reproduction and hatchery practices

439   *4.2.1 Gonad activity in winter*

440   It is commonly noted that temperate oyster species enter reproductive diapause in winter, when

441   water temperature drops below a thermal threshold for gametogenesis (Brian Leicester Bayne

442   2017). The majority of *O. lurida* literature concurs, and defines the thermal threshold for

443   gametogenesis as 12.5°C for *O. lurida* in Washington State. However, much of the literature is

444   from the early 20th century and is limited to field observations from a few populations (Hopkins

445   1936, 1937). Here, we find evidence for gonad activity at low temperatures based on gradual

446   changes in gonad stage and sex throughout the winter (Figure 2, Supplemental Figure 1).

447   Spencer et al. (2020) also observed that spermatogenesis can occur at lower temperatures

448   (~10°C) in three Puget Sound *O. lurida* populations, and Barber et al. (2016) recently found *O.*

449   *lurida* adults brooding embryos at 10.5°C in a northern Puget Sound lagoon. Taken together, the

450   concept that reproductive activity ceases below 12.5°C is no longer reliable for *O. lurida* in the

451   Puget Sound estuary. To inform Olympia oyster restoration and commercial operations, the

452   thermal limits for *O. lurida* spawning should be investigated, and in the context of other potential

453   reproductive drivers (e.g. food availability, tidal cycle, photoperiod). Furthermore, winter

454   conditions should not be overlooked when examining *O. lurida* reproductive cycles.

455   4.2.2 *Influence of overwintering time on larval production*

456   The amount of time broodstock were held prior to spawning substantially impacted larval

457   production. Broodstock held for 7 weeks produced nearly twice the number of larvae per animal

458   than those held for 12 weeks. Poor production following prolonged captivity could be explained

459   by stress associated with the unnatural hatchery conditions. Oyster plumpness consistently

460   decreased over time in most treatments regardless of algal densities (Figure 7), indicating that

461   broodstock may have utilized energy reserves despite access to ample food. The hatchery

462   conditions and regular handling during cleanings may have stressed the animals such that they

463   did not eat, and instead catabolized endogenous energy reserves which are commonly used

464   during gametogenesis (Brian Leicester Bayne 2017). Alternatively, the nutritional value of

21

465   hatchery-cultured microalgae may have been insufficient, such that the broodstock fed cultured

466   algae for longer prior to spawning depleted more endogenous reserves (Wilson, Chaparro, and

467   Thompson 1996). Helm et al. (1973) observed a similar temporal trend when experimenting with

468   *Ostrea edulis* broodstock, as larval yield, growth and competency decreased as their experiment

469   progressed. In the present study, varying mortality did result in fewer broodstock per tank in the

470   12-week trial, that should be considered. We normalized larval production by the number of

471   broodstock in each tank; however, due to the Olympia oyster's cryptic spawning behavior, the

472   correlation between the number of broodstock in a mass spawning tank and mating pairs is not

473   known, and may be nonlinear. To optimize *O. lurida* larval production and broodstock survival,

474   hatcheries should minimize the time that broodstock are held in captivity prior to spawning.

475   4.2.3 *No association between larval size upon release and survival*

476   Larval shell height and width, measured upon release from the brood chamber in 48 larval

477   groups, did not correlate with survival through metamorphosis (35 days post-release). This is

478   unexpected, as larval size upon release has been positively linked to larval "quality" in other

479   *Ostrea* species. In the Chilean flat oyster (*Ostrea chilensis*), pediveliger size upon release

480   positively correlates with larval growth and spat survival (Wilson, Chaparro, and Thompson

481   1996), in addition to oocyte size and biochemical properties (lipid, protein, carbohydrate).

482   Growth and competency through metamorphosis of *O. edulis* larvae positively relates to lipid

483   content upon release from the brood chamber (Helm, Holland, and Stephenson 1973). In other

484   bivalves larval size and/or growth rate is linked to oocyte size and larval competency, such as in

485   the Eastern oyster *Crassostrea virginica* and hard clam *Mercenaria mercenaria* (Gallager and

486   Mann 1986; Kraeuter, Castagna, and van Dessel 1981), the Japanese abalone *Haliotis discus*

487   *hannai* (Fukazawa et al. 2005), and bay scallop *Argopecten irradians* (Kraeuter, Castagna, and

488   van Dessel 1981). In Fukazawa et al. (2005) and Kraeuter et al. (1981) larvae were unfed, and

489   thus relied solely on endogenous resources provided by the mother. This was not the case in the

490   present study, as larvae had access to algae throughout the experiment. Had larvae been unfed it

491   is possible that survival may have correlated more closely with shell size upon release.

492   Nevertheless, under hatchery conditions *O. lurida* larval size upon release from the brood

493   chamber is not predictive of survival to spat. Hatcheries should therefore not presume that small

494   larvae are of poor quality.

495   4.3 Recommendations for Hatchery Production

496   Broodstock mortality is inefficient for hatcheries, can hinder larval production, and decrease

497   genetic diversity of offspring. To minimize broodstock mortality during spawning, we

498   recommend acclimating broodstock to hatchery conditions at a slightly warmer temperature,

499   rather than mimicking cooler winter temperatures (e.g. 10°C vs. 7°C). Hatcheries may also

500   consider testing slower rates of temperature increase if high mortality occurs during spawning

501   (e.g. 0.5°C/day), and minimizing the time broodstock are in captivity prior to spawning. We find

502   no evidence that restricting food during hatchery acclimation impacts larval production or

503   survival, given that broodstock are in good condition upon collection. When larvae are grown

504   with good husbandry practices, size upon release is not predictive of survival. Depending on the

505   geographic location and population characteristics, low level gametogenesis can occur in *O.*

506   *lurida* throughout the winter, thus the time that broodstock are collected in the winter may

507   influence spawn timing and larval release magnitude.

5. Conclusion

508

509   There are many gaps in our understanding of *Ostrea* spp. reproductive systems, which constrain

510   interpretation of data collected from *Ostrea* spp. For instance, in the absence of genetic testing,

511   the number of families produced in a batch spawn is based on estimates of brood size per female

512   (see Supplemental Materials), which may not reflect present-day populations (Hopkins 1936).

513   Studies needing to estimate the number of breeding females would benefit greatly from a model

514   of brood size against adult size and the number of oysters in a mass spawn across multiple

515   populations. Furthermore, many operating theories are based exclusively on early observations

516   of *O. lurida* (Hopkins 1936; W. R. Coe 1931a), which do not likely represent present-day

517   populations and may be unreliable, such as the concept that gametogenesis ceases in winter and

518   residual gametes are resorbed. We find little evidence of resorption in both treated and wild-

519   collected oysters throughout the winter, and theorize that many residual gametes remain viable.

520   Finally, a deeper understanding of the relative influence of environmental drivers (*e.g.*

521   temperature, food, tidal cycle, allosperm), and internal drivers (*e.g.* age, source population), on

522   reproduction, including what precisely triggers spawning, would greatly improve the outlook for

523   restoration and commercial aquaculture of *Ostrea* spp.

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8. Tables

Table 1: Impacts of treatments on gonad development. Gonad sex, spermatocyte stage, and oocyte stage were compared among treatments during exposures (Dec 20 - Feb 27) and during spawning (Mar 13 & 23) by Chi-square contingency table tests (unless otherwise noted). Treatments included four hatchery conditions (7°C+low-food, 7°C+high-food, 10°C+low-food, 10°C+high-food) and wild-conditions (at collection site, Mud Bay, Bremerton), where high-food was 50k cells/mL and low-food was 5k cells/mL. Significant differences are in bold (α=0.0056, adjusted due to multiple comparisons, 9 tests per gonad metric), and include treatments that differed according to pairwise Fisher Exact tests.

Date sampled	Gonad Sex	Spermatocyte Stage	Oocyte Stage
2 wks in treatment (Dec 20)	p=0.58 *(Fisher test)*	χ^2 = 11.1, p=0.86	χ^2 = 14.5, p=0.63
4 wks in treatment (Jan 4)	p=0.044 *(Fisher test)*	χ^2 = 18.8, p=0.29	p=0.63 *(Fisher test)*
7 wks in treatment (Jan 23) *End of 7-week trial*	p=0.76 *(Fisher test)*	χ^2 = 19.7, p=0.24	χ^2 = 12.7, p=0.84
9 wks in treatment (Feb 9)	χ^2 = 9.5, p=0.78	χ^2 = 16.0, p=0.48	p=0.98 *(Fisher test)*
12 wks in treatment (Feb 27) *End of 12-week trial*	χ^2 = 19.9, p=0.061	**χ^2 = 32.5, p=0.0047** *Pairwise treatment differences:* 7°C+low-food vs. 10°C+high-food 10°C+low-food vs. 10°C+high-food	χ^2 = 21.8, p=0.12
All treatment wks combined (Dec 20 - Feb 27)	χ^2 = 14.0, p=0.30	χ^2 = 31.0, p=0.012	χ^2=17.2, p = 0.37
2 weeks in common spawning conditions (Mar 13)	χ^2 = 9.2, p=0.43	χ^2 = 23.4, p=0.017	χ^2 = 8.6, p=0.51
3.5 weeks in common spawning conditions (Mar 23)	χ^2 = 9.7, p=0.39	χ^2=25.2, p=0.0090	χ^2 = 9.8, p=0.68
Both spawning dates combined (Mar 13 & 23)	χ^2 = 8.7, p=0.48	**χ^2 = 32.2, p=0.0010** *Pairwise treatment differences:* 7°C+high-food vs. 7°C+low-food 7°C+high-food vs. 10°C+high-food 10°C+low-food vs. 10°C+high-food	χ^2 = 11.7, p=0.49

Table 2: Influence of temperature, food level, and treatment duration on larval release timing and magnitude. F-statistics from 3-way Analysis of Variances indicate effects of temperature (7°C, 10°C), food level (5k cells/mL, 50k cells/mL), and duration (7-week exposure, 12-week exposure). Significant differences are in bold (α=0.05). Kruskal-Wallis Chi-Square Tests were used to assess the number of days until larval release onset.

Metric	Temp.	Food	Trial	Temp:Food	Trial:Temp	Trial:Food	Trial:Temp:Food
Days until larval release onset *Kruskal-Wallis Chi-Square Test*	χ^2 = 0.35 p=0.55	**χ^2 = 6.84 p=8.9e-3**	**χ^2 = 5.61 p=0.018**	χ^2 = 7.27 p=0.064	χ^2 = 6.11 p=0.11	**χ^2 = 18.1 p=4.1e-4**	**χ^2 = 18.9 p=8.6e-3**
Days until peak larval release	F=0.45 p=0.51	F=0.072 p=0.79	**F=6.14 p=0.022**	F=1.50 p=0.24	F=1.27 p=0.27	F=0.34 p=0.56	**F=5.95 p=0.024**
Average no. larvae released each day (~brood size)	F=6.0e-3 p=0.94	F=0.35 p=0.56	F=2.10 p=0.16	F=0.76 p=0.39	F=0.13 p=0.72	F=0.40 p=0.54	F=0.94 p=0.34
Cumulative no. larvae released over the 30-day collection	F=0 p=0.99	F=0.74 p=0.40	**F=29.4 p=2.65e-5**	F=1.2 p=0.29	F=0.21 p=0.89	F=0.11 p=0.74	F=2.81 p=0.11

Table 3: Larval survival through metamorphosis for each larval group, at 5 weeks after larval release. 12 larval groups were reared from each treatment, collected across four tanks per treatment (3 replicates per group). Spawning group replicates (e.g. 1-1) are sorted by date, with -1 collected earliest, and -3 collected latest during a 19-day period.

Broodstock treatment		7°C+low-food		7°C+high-food		10°C+low-food		10°C+high-food)	
Broodstock treat. replicate		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Spawning group and replicate *sorted by date collected*	1-1	0.3±0.3%	8.3±7.2%	3.9±2.9%	4.2±1.1%	5.4±2.8%	5.7±1.7%	1.0±0.4%	17.8±3.2%
	1-2	9.0±2.3%	10.4±5.0%	7.9±3.5%	1.9±1.8%	7.0±8.8%	13.9±3.3%	29.2±2.9%	4.7±0.7%
	1-3	46.1±5.6%	22.0±1.8%	2.1±1.7%	8.0±3.6%	9.3±3.6%	19.8±6.6%	6.9±5.3%	2.2±0.8%
	2-1	9.6±1.9%	2.7±2.3%	10.0±4.1%	2.0±2.1%	7.5±2.0%	8.5±1.6%	21.1±5.4%	1.4±0.9%
	2-2	9.1±3.8%	6.9±1.1%	2.7±1.3%	21.4±8.5%	25.3±5.9%	7.6±0.8%	33.0±10.6%	21.3±3.4%
	2-3	8.8±5.6%	4.1±3.6%	5.2±2.9%	35.6±3.2%	0.4±0.5%	7.3±1.8%	7.1±5.0%	4.4±2.2%
Grand Mean		**11.4±12.1%**		**8.7±10.1%**		**9.8±6.8%**		**12.5±11.4%**	

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9. Figure Legends

Figure 1: Experimental timeline.

Figure 2: Gonad sex, oocyte stage, and spermatocyte stage for each treatment. Frequency of Stage 0 oocytes and spermatocytes, indicating none present, are omitted. Pre-treatment samples were collected on November 30 (n=40), then treatments began on December 8 and oysters were sampled (n=10) every two or three weeks during treatment. Oysters were also sampled directly from the collection site ("Wild") through February 27. On January 24 the 7-week trial oysters were removed from treatments to spawn in common conditions (dashed line), and all subsequent samples were collected from the remaining 12-week trial broodstock. On February 28 the 12-week trial oysters were removed from treatments (solid line), and were sampled again on March 13 and 23 (n=12) while in common, spawning conditions. Oysters from both trials were induced to spawn by increasing temperature 1°C/day and feeding with live algae ad libitum.

Figure 3: Larvae released over 30-days from broodstock exposed for 7-weeks and 12-weeks to varying temperatures and food levels, where low-food=5k algal cells/mL, and high-food=50k algal cells/mL. Broodstock held for 7 weeks released more larvae and released larvae earlier than those held for 12-weeks (dashed lines highlight peak release, generated using a LOESS smoothing function). Larvae were also released earlier from broodstock exposed to low-food levels.

Figure 4: The number of larvae released (normalized by number of broodstock) during the 30-day collection period by broodstock previously exposed to varying temperature and algal densities for 7-weeks (✳) and 12-weeks (●). Low-food=5k algal cells/mL, and high-food=50k algal cells/mL. Broodstock released more larvae if they had been held for less time prior to volitional spawning. There was no effect of temperature or food treatments.

Figure 5: Left: Larval shell width upon release from brood chamber. Larvae tended to be larger from broodstock previously exposed to elevated temperature ($F_{(1,120)}=3.92$, $p=0.050$) and to elevated food levels ($F_{(1,120)}=3.00$, $p=0.086$), although these differences were not statistically significant. **Right**: Maximum oocyte length of ripe oocytes (stage 3) at termination of the 12-week treatments (February 27) and during volitional spawning (March 13 & 23). Broodstock fed high levels of food (50k algal cells/mL) contained larger ripe oocytes than those fed low-food (5k algal cells/mL, $F_{(1,33}=6.60$, $p=0.015$).

Figure 6: Larval survival (%) by larval shell width upon release from the brood chamber, and broodstock treatment where low-food=5k algal cells/mL, and high-food=50k algal cells/mL. Survival was independent of both size upon release and parental treatment. For each of the 12 spawning tanks (4 per treatment), three larval groups were reared in triplicate from larval pulses released on different days, for a total of 144 larval tanks (each point = one larval tank).
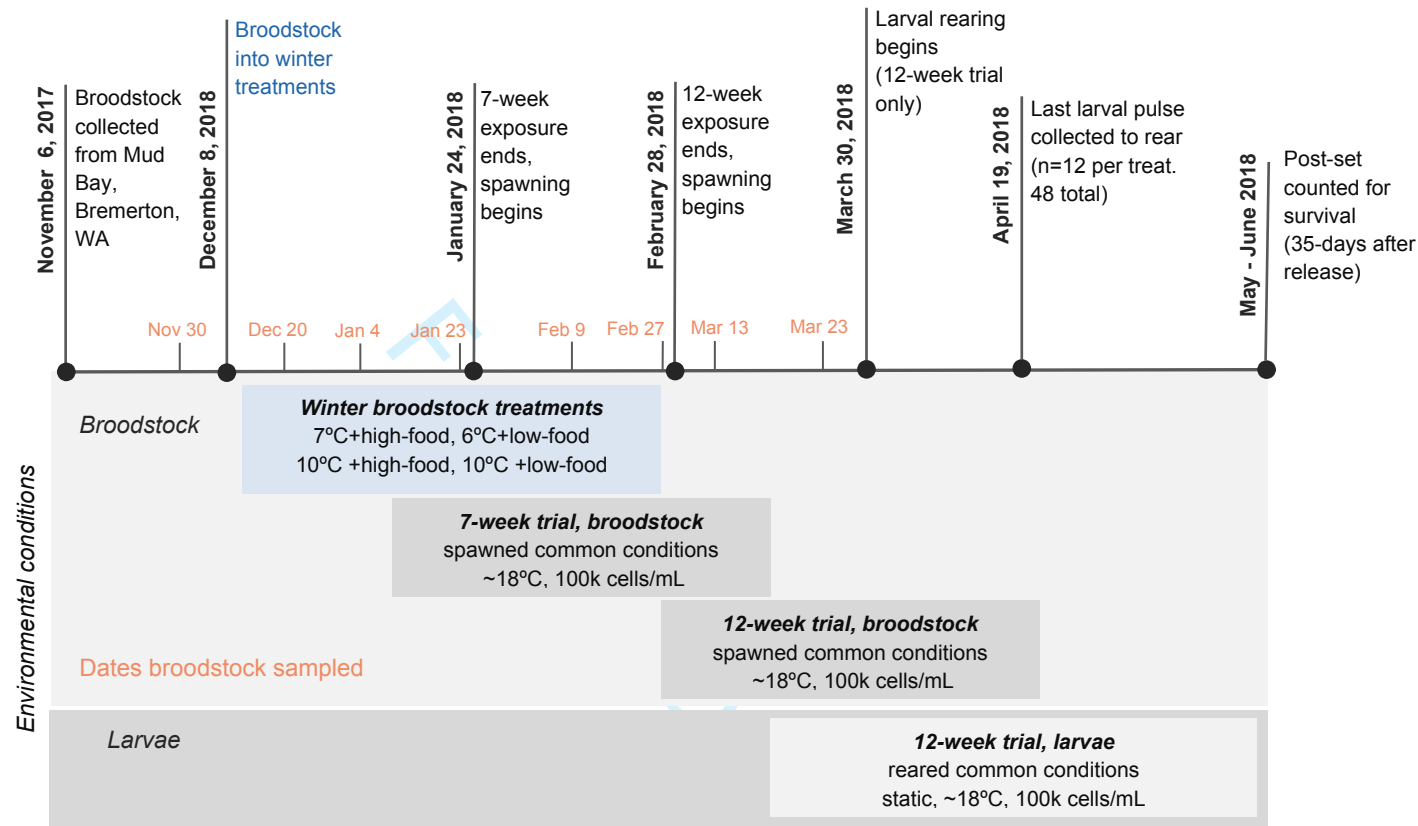
Figure 7: broodstock survival (top pane) and plumpness (wet tissue weight standardized by shell length, bottom pane) over time by treatment. The dashed lines indicate when broodstock were removed from treatments and entered common spawning conditions. There was higher mortality in broodstock exposed to overwintering treatments for 12 weeks, particularly in those exposed to 7°C. Broodstock plumpness decreased throughout overwintering treatments, with no effect of feeding level or temperature.
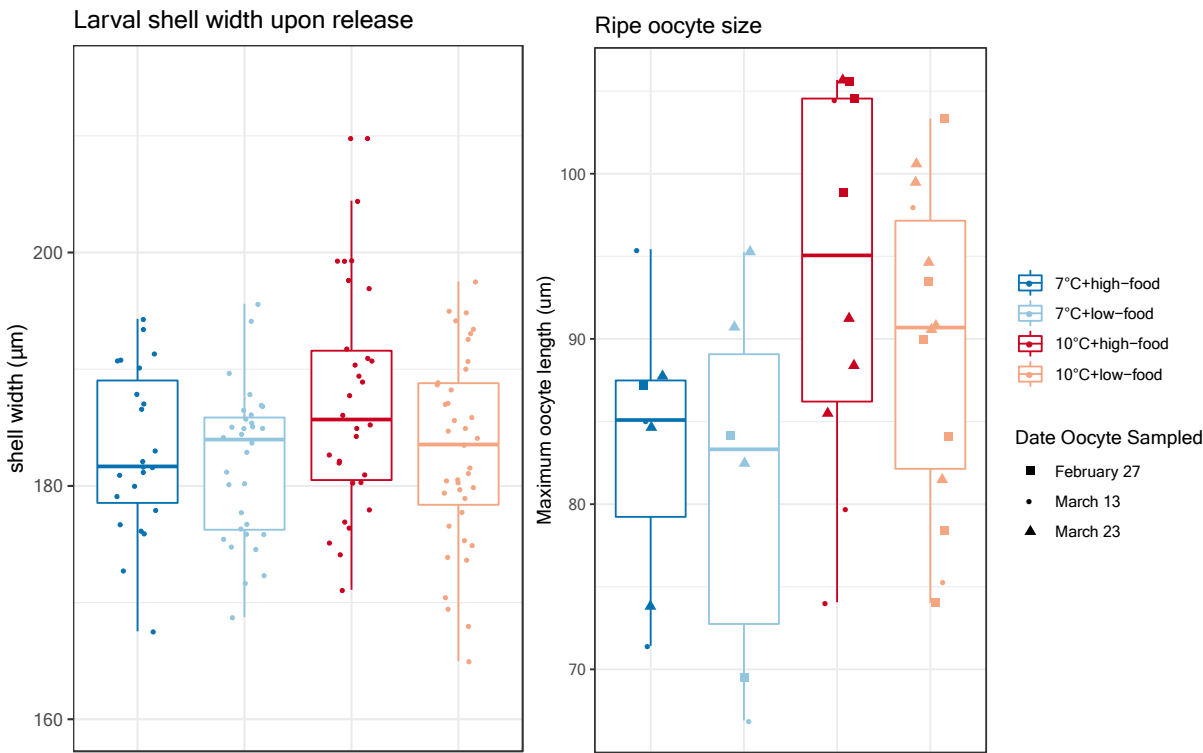
10. Figures

Figure 1.

Figure 2.
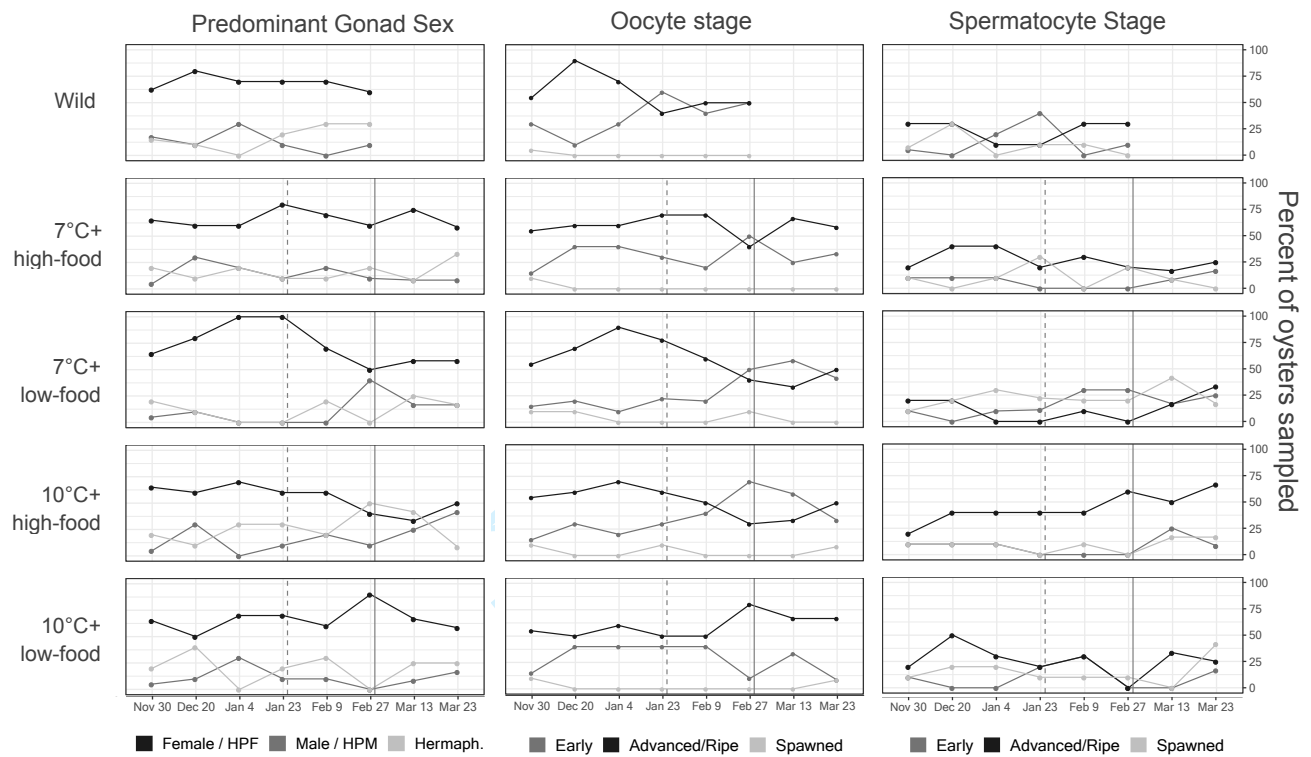
Figure 3.

Figure 4.
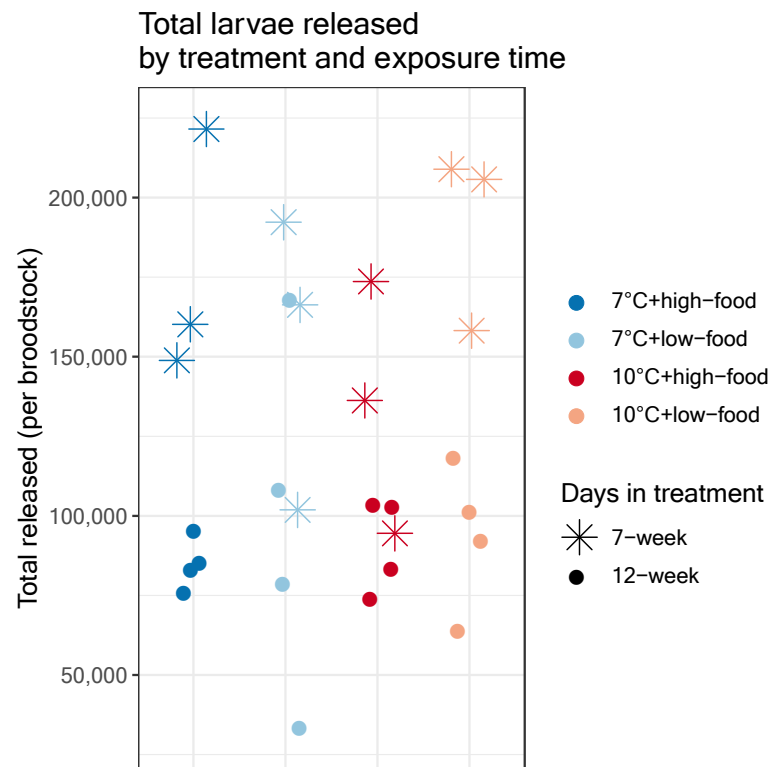
Total larvae released
by treatment and exposure time

Figure 5.

Figure 6.

Larval survival ~ shell width upon release
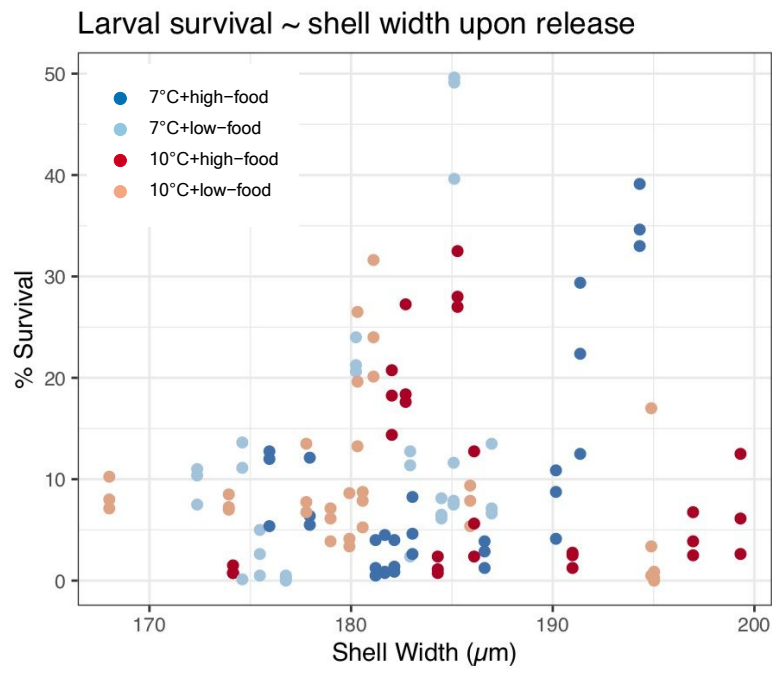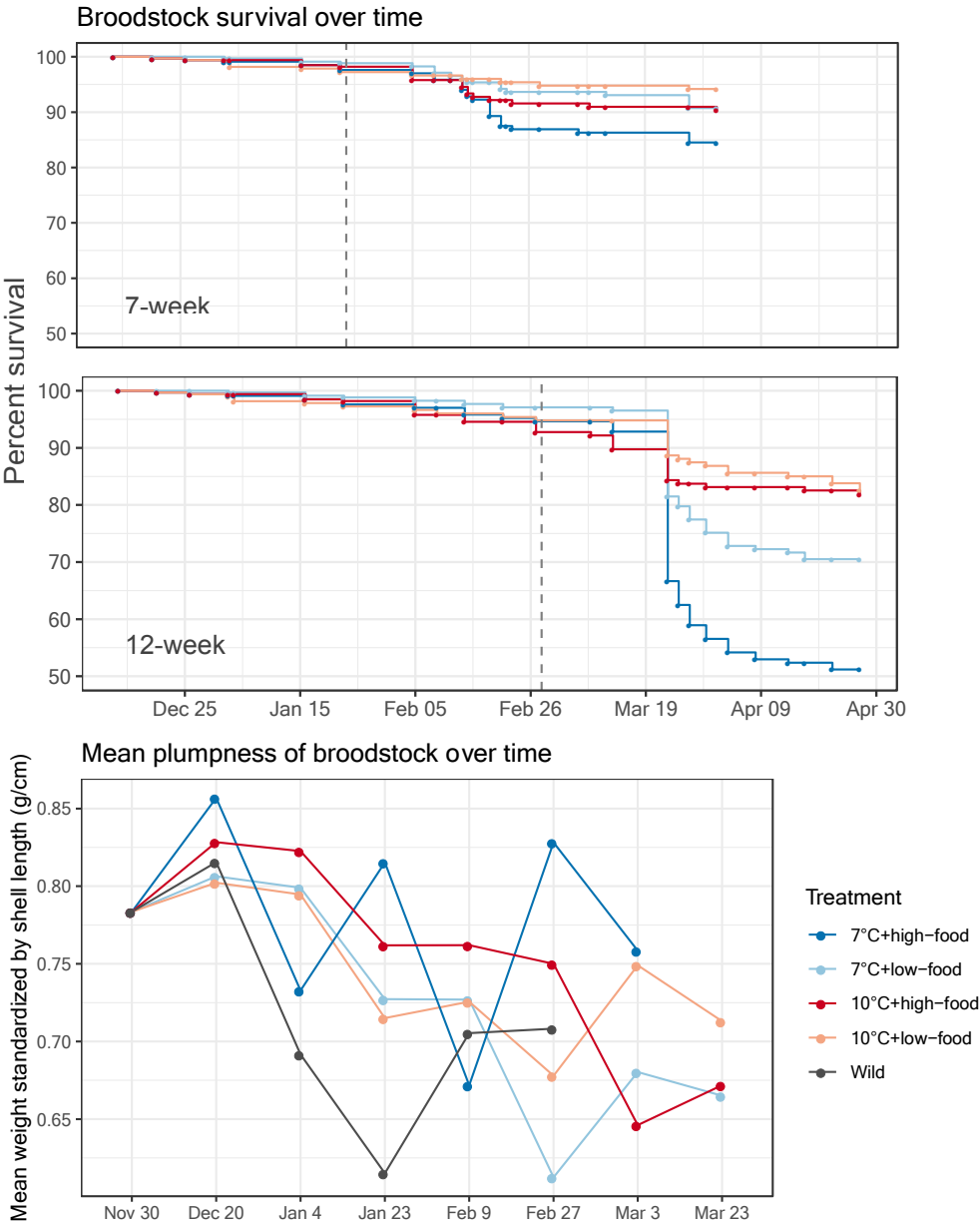
Figure 7.

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*Effects of winter conditions on Olympia oyster reproduction and larval yield*

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Supplemental Materials

Supplemental Figure 1: Gonad sex, male gamete stage, and female gamete stage over time by treatment. Broodstock were in treatments during weeks 0-13, and were in common conditions to induce spawning (high food and high temperature) on weeks 15 and 16.

1

10°C - High Nutrition

10°C - Low Nutrition
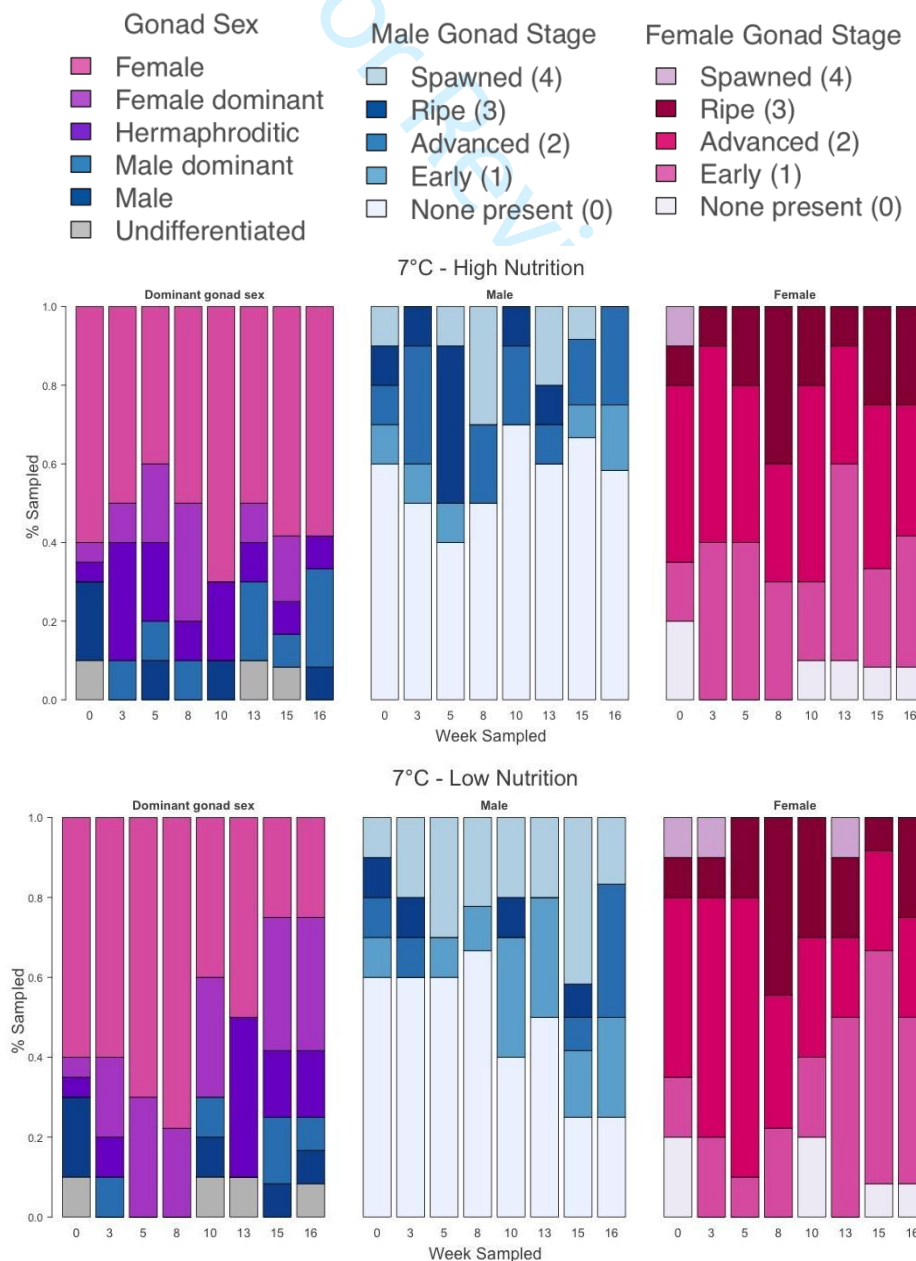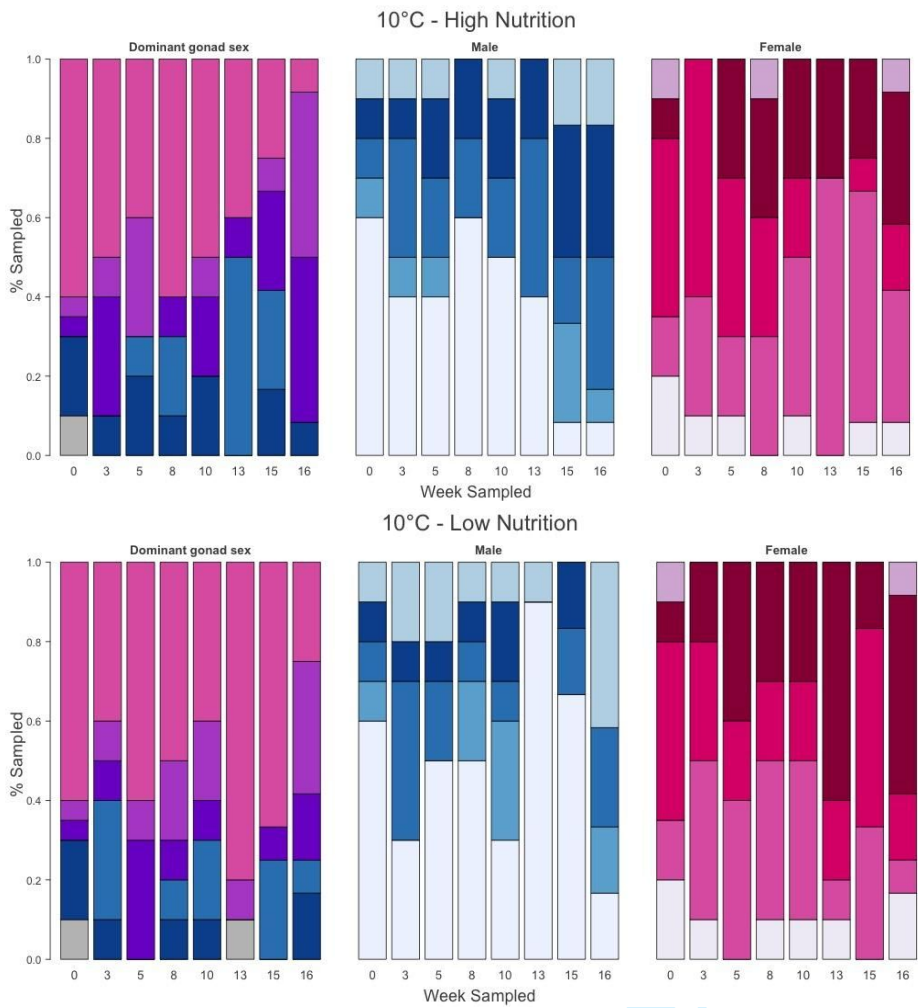
Supplemental Table 1: Male gonad stage at week 13 differed by treatment (final sample date prior to reproductive conditioning), $\chi^2 = 32.5$, p=0.0052.

Male stage, week 13 only	None present (0)	Early (1)	Advanced (2)	Ripe (3)	Spawned (4)
7C / LOW FOOD (A)	5	3	0	0	2
7C / HIGH FOOD (C)	6	0	1	1	2
10C / LOW FOOD (B)	9	0	0	0	1
10C / HIGH FOOD (D)	4	0	4	2	0

Supplemental Table 2: Male gonad stage during reproductive conditioning (weeks 15 & 16 combined) differed by treatment, $\chi^2 = 32.2$, p=0.0010

Male stage, weeks 15 & 16	None present (0)	Early (1)	Advanced (2)	Ripe (3)	Spawned (4)
7C / LOW FOOD (A)	5	5	4	1	7
7C / HIGH FOOD (C)	15	3	5	0	1
10C / LOW FOOD (B)	10	2	5	2	5
10C / HIGH FOOD (D)	2	4	6	8	4

Supplemental Table 3: Larval collection data by broodstock winter treatment (4 replicate spawning tanks per treatment).

	7C - Low Nutr.	7C - High Nut.	10C - Low Nutr.	10C - High Nutr.
Mean larvae collected / day	424,000 365,000 140,000 357,000 = 339,000 (overall)	219,000 349,000 448,000 269,000 = 312,000 (overall)	364,000 299,000 381,000 197,000 = 304,000 (overall)	395,000 261,000 280,000 295,000 = 304,000 (overall)
Median larvae collected/day	168,000 382,000 135,000 229,000 = 212,000 (overall)	186,000 457,000 256,000 262,000 = 256,000 (overall)	210,000 281,000 419,000 186,000 = 218,000 (overall)	319,000 125,000 164,000 184,000 = 195,000 (overall)
Max collected	2,073,000 1,274,000 698,000 248,000	1,116,000 610,000 567,000 426,000	1,238,000 915,000 871,000 561,000	1,190,000 962,000 918,000 677,000
Total larvae released	5,939,000 3,652,000 1,116,000 2,854,000 = 13,561,000	1,756,000 1,743,000 3,133,000 2,421,000 = 9,053,000	4,729,000 3,583,000 3,427,000 2,561,000 = 14,300,000	3,558,000 2,606,000 3,361,000 3,831,000 = 13,357,000
No. of Broodstock (at end of collection)	32, 31. 28, 34 = 125 (sum)	19, 19, 28, 23 = 89 (sum)	36, 33, 34, 37 = 140 (sum)	34, 32, 37, 35 = 138 (sum)
Total larvae released, normalized by # broodstock	185,600 117,800 39,900 83,900	92,400 91,700 111,900 105,300	131,400 108,600 100,800 69,200	104,700 81,400 90,800 109,500

3

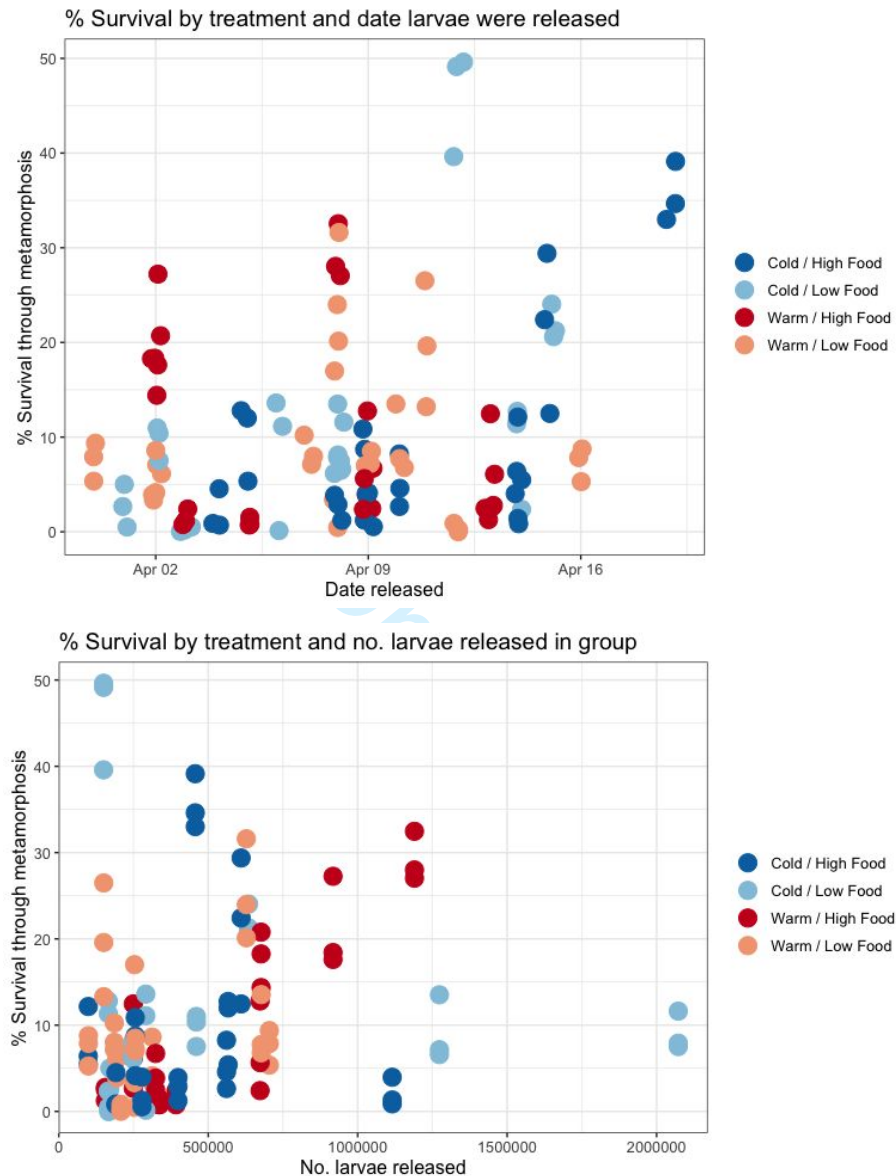|	= 106,800 (average)	= 100,300 (average)	= 102,500 (average)	= 96,600 (average)
Mortality during spawning	54	86	36	35
Date of first release	3/30, 3/30, 3/30, 4/2	4/4, 4/4, 4/4, 4/7	3/30, 3/30, 3/30, 4/1	4/1, 4/1, 4/2, 4/2
Date of max release	4/4, 4/8, 4/8, 4/8	4/5, 4/14, 4/15, 4/20	4/4, 4/7, 4/9, 4/25	4/2, 4/2, 4/7, 4/8
Date of last release	4/17, 4/22, 4/25, 4/25	4/18, 4/19, 4/20, 4/25	4/20, 4/22, 4/25, 4/27	4/18, 4/18, 4/22, 4/25
No. big larval release days (>10k)	13, 9, 7, 8 = 37 (sum)	8, 4, 7, 9 = 28 (sum)	13, 12, 9, 12 = 46 (sum)	9, 10, 11, 12 = 42 (sum)

Estimates of female reproduction rate

The number of broodstock that reproduced was estimated from the total number of larvae released during the collection period, divided by 215K larvae per female, which was based on prior estimates for *O. lurida* of shell height 35 mm (Hopkins 1936). Recent pair-mating experiments, however, indicate that brood size can range from 100,000 to 500,000 larvae from *O. lurida* of similar sizes (Ryan Crim, unpublished). We therefore did not include the rate of female mating estimates in the main text, but report it here for those who are interested.

Approximately 76% of the 7-week trial broodstock reproduced as female, which was significantly more than the 12-week trial (estimated ~43%) ($F_{(1,20)}$= 29.7 p=2.45e-05). The estimated percent of oysters that reproduced as female did not differ among treatments (for temperature, food, and temperature:food, respectively: $F_{(1,12)}$=0.015, 0.25, 0.11; p=0.91, 0.63, 0.75), and across the 16 spawning tanks was on average 43±13%.

Supplemental Table 4: Summary statistics of larval dimensions upon release/collection

	7°C+low-food	**7°C+high-food**	**10°C+low-food**	**10°C+high-food**
Mean width ± SD	182.0 ±6.4 µm	183.0 ±7.0 µm	183.0 ±8.1 µm	187.3 ±9.5 µm
Shell width range	168.7 - 195.6 µm	167.5 - 194.3 µm	165.0 - 197.5 µm	171.1 - 209.8 µm
Mean height ± SD	118.6 ±3.7 µm	117.2 ±4.7 µm	117.6 ±6.1 µm	119.1 ±5.1 µm
Shell height range	112.8 - 126.8 µm	104.7 - 124.6 µm	102.8 - 133.4 µm	106.3 - 126.8 µm

Supplemental Figure 2: Top: Larval survival (%) by date larvae were released from the brood chamber, color coded by broodstock treatment, showing a positive relationship. **Bottom**: Larval survival (%) by the number of larvae released/collected in that larval group (estimate of brood size), showing a negative relationship.
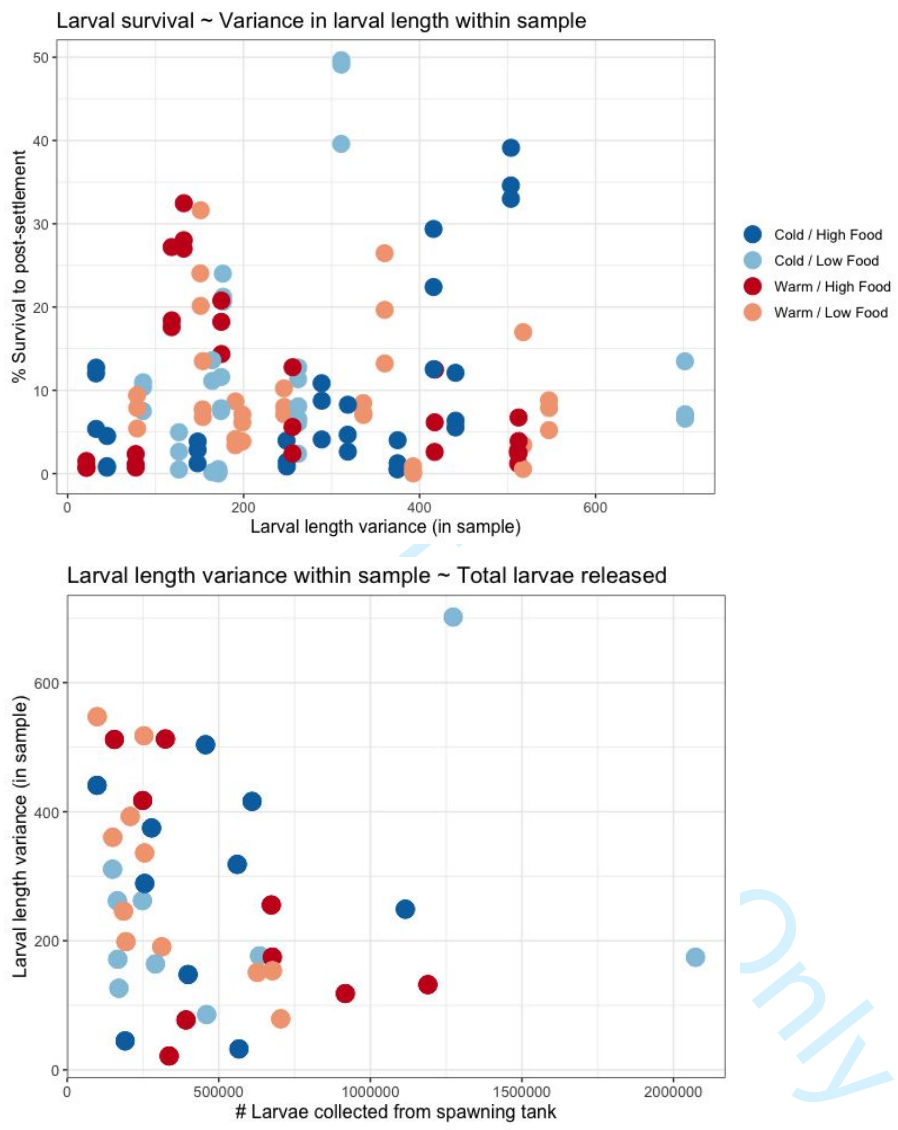
Supplemental Figure 3: **Top**: Larval survival to post-settlement stage ~ variation in larval length within a pulse (upon release). No significant associations or differences among treatments were found. **Bottom**: Variation in larval length within a larval pulse ~ total no. of larvae released.

6