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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.

Keywords	Temperature; nutrition; gametogenesis; climate change; <i>Ostrea lurida</i>
Taxonomy	Seasonality of Reproduction, Animal Rearing, Animal Physiology, Marine Biology, Animal Energetics
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Dr. Delbert Gatlin
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Dear Dr. Gatlin,

We are pleased to submit the enclosed manuscript, “Effects of winter conditions on Olympia oyster reproduction and larval yield,” for consideration in *Aquaculture*.

Temperature regulates many reproductive processes in marine invertebrates, particularly for those living in temperate regions. As global temperatures rise due to anthropogenic inputs, milder winters are anticipated due to increased sea surface temperature and more frequent marine heat waves. Elevated winter temperature has capacity to alter cultured stocks and wild populations through wide-scale shifts in reproductive timing and capacity, and offspring viability. Yet, we know little about how changes to winter conditions will impact critical reproductive processes in marine species.

Our novel study exposed adult Olympia oysters (*Ostrea lurida*) to two winter temperatures (7°C, 10°C) in the presence of two feeding regimes. We show that changes to winter conditions substantially alters bivalve physiology and gamete development, resulting in more developed sperm, larger oocytes, and larvae that tended to be larger. In the wild, more developed gametes and larger larvae following milder winters could greatly impact recruitment patterns. However, larval production timing and magnitude were unaffected, indicating that *O. lurida* reproduction may be relatively resilient to shifting winter temperatures.

This is the first study to assess reproductive impacts of winter warming in the presence of varying feeding regimes in an *Ostrea* spp, and to do so in a controlled environment in a marine bivalve. Now, the winter environment prior to spawning must be considered when predicting and examining marine invertebrate reproductive cycles.

This study also contributes valuable information pertinent to the commercial and restoration production of *O. lurida* and related flat oysters (*O. angasi*, *O. edulis*, *O. chilensis*), species of economic and ecological importance. Specifically, we show that larval size upon release does not predict larval survival in a hatchery setting, and to optimize larval production the amount of time broodstock are held prior to spawning should be minimized.

This manuscript is not under consideration by another journal, and all authors approve of the manuscript and its submission to *Aquaculture*.

Thank you for your consideration.

Sincerely,
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Effects of winter conditions on Olympia oyster reproduction and larval yield

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Highlights of the Manuscript

1. Milder winters may result in more developed *O. lurida* sperm, larger oocytes, and larger larvae, but will not likely impact larval production timing or magnitude, indicating that *O. lurida* reproduction is relatively resilient to shifting winter temperatures.
2. In a hatchery setting, *O. lurida* larval size upon release does not predict larval survival, and hatcheries should not presume that smaller *O. lurida* larvae are of poor quality.
3. When overwintering *Ostrea lurida* broodstock in the hatchery prior to spring production, chilling seawater to historic winter temperatures is not necessary, nor is feeding broodstock high algal densities, and the amount of time broodstock are held prior to spawning should be minimized.

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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.

Keywords

Temperature, nutrition, gametogenesis, climate change, *Ostrea lurida*

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.

Many temperate species are thought to enter reproductive diapause in the winter, until temperatures exceed the physiological minimums for breeding in the spring (Orton 1920; Giese 1959; Pearse 1968; B. L. Bayne 1976). Winter warming could therefore result in uninterrupted gametogenesis, precocious spawning, or asynchronous spawning if spermatogenesis and oogenesis respond differently to winter warming (Philippart et al. 2003; Chevillot et al. 2017). Warming could also impact offspring phenotype by direct impacts to gametes, or indirectly via impacts to progenitors. Egg size, which is associated with lipid content and egg quality, typically correlates negatively with maternal environmental temperature (Moran and McAlister 2009; Gosselin et al. 2019; Atkinson et al. 2001). Maternal RNAs and lipid composition, which are utilized by embryos and larvae during development, could differ if their structure or production is sensitive to winter temperature (Leroy et al. 2018; Krisher 2013). It is also possible that elevated metabolic demand during warmer winters will drain glycogen reserves, resulting in smaller or poor-quality oocytes in the spring (Mathieu and Lubet 1993). Elevated winter temperature therefore has capacity to alter wild populations and cultured stocks through wide-scale shifts in reproductive timing and capacity, and offspring viability.

Impacts of warming on aquaculture species is particularly concerning, given the increased global reliance on cultured fishes that is needed to meet growing demand for animal protein (Hall 2011; U.S. Department of Commerce 2015). Aquaculture industries that require reliable sources of wild-caught broodstock, larvae, or juveniles, or that grow their animals in natural settings, all may be impacted by warming-induced reproductive changes (Cochrane et al. 2009; Stickney and McVey 2002). Here, we explore the effects of elevated winter temperature on spring larval production in *Ostrea lurida*, the Olympia oyster. *Ostrea lurida* is native to the Northeast Pacific Ocean, is cultured for restoration and the commercial market, and is related to

the widely cultured European flat oyster *Ostrea edulis* and Chilean flat oyster *Ostrea chilensis* (Baker 1995). Adult *O. lurida* were overwintered at two temperatures (7°C, 10°C), both below the reported minimum breeding temperature for the region (12.5°C) and selected to represent historic and elevated winter temperatures, and larval production was subsequently monitored. Two feeding regimes were tested to assess effects of temperature under high (50k cells/mL) and low (5k cells/mL) algal density. To examine whether duration of temperature exposure impacts reproduction, the four treatments were applied for two exposure durations (7 weeks, 12 weeks). This study sought to examine whether winter warming will impact *O. lurida* reproduction and offspring viability, and to improve *O. lurida* broodstock pre-conditioning practices to optimize hatchery larval production.

1.2 Predictions

Observations of poor larval production in an *O. lurida* restoration hatchery following the anomalously warm winter (10°C) as experienced during 2014-2016 (Gentemann, Fewings, and García-Reyes 2017) suggested possible negative effects of elevated winter temperature on fecundity and larval vigor. This prediction was based on energetic models in marine ectotherms, which allocate energy for growth, reproduction, and maintenance (Sokolova et al. 2012). In theory, elevated winter temperature increases an organism's metabolic rate, and in the absence of increased food results in less energy available for reproduction (i.e. gamete development) and growth (i.e. glycogen/lipid storage, calcification). Additionally, it was previously understood that Olympia oysters enter reproductive quiescence in winter months due to cold temperatures (Oates 2013; Hopkins 1936; W. Coe 1932; W. R. Coe 1931b). Winter quiescence is presumably an important phase for resorption of remnant gametes from the previous season, needed to maintain 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 µm), 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 $p=0.0056$. 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

veliger larvae, which are released from the maternal brood chamber approximately 10-14 days after fertilization (Coe 1931a, Hopkins 1937). Once larval release began, larvae were collected daily for four weeks and counts were estimated by hand-counting larvae in triplicate subsamples. Twice weekly tanks were cleaned and broodstock were inspected for mortality.

Larval production timing and magnitude were compared among treatments (temperature x food level) and treatment duration (7-week, 12-week). Release timing was assessed by comparing the date of onset and date of maximum release using Kruskal-Wallis rank sum tests. Release magnitude was assessed by comparing the average number of larvae released each day and the total number of larvae released over the 4-week collection period by Analysis of Variance (ANOVA) of a fitted linear model with aov().

2.6 Larval size upon release

To assess impacts of broodstock treatment on larval size, larvae were measured upon release from the maternal brood chamber. After larvae were collected, counted, and aliquoted to rearing tanks, excess larvae were preserved directly in -80°C, then measured using a Nikon eclipse Ni microscope and the NIS-Elements BR imaging and measuring software (version 4.60). Mean shell height (distance from hinge to margin, perpendicular to hinge), and mean shell width (longest distance parallel to hinge) were estimated from at minimum 40 larvae per group. Mean height and width were compared among treatments using Two-Way Analysis of Variance.

2.7 Larval survival

To assess impacts of broodstock treatments on larval vigor, a subset of larvae collected from the 12-week trial were reared through settlement. In total, 48 pulses of larvae were reared, 12 per 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 μ m) 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 μ m) placed
 234 in individual containers with static seawater (800 ± 50 mL). Water was changed daily from a
 235 common mixture of filtered seawater (<1 μ m) 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 μ m) 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 $91\pm14\ \mu\text{m}$ and $83\pm13\ \mu\text{m}$ 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, $\eta^2 = 5.61$ $p=0.018$, 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 ($\eta^2 = 6.84$, $p=8.9\text{e-}3$, Figure 3). There was also
282 significant interaction between food level and exposure duration (Trial:Food, onset $\eta^2 = 18.1$
283 $p=4.1\text{e-}4$). 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 $\eta^2 = 18.9$ $p=8.6\text{e-}3$, peak $F=5.95$ $p=0.024$, Table

2). The 3-factor interaction was influenced predominantly by the 12-week 7°C+high-food treatment, which released larvae much later than the other groups (up to one week, Figure 3). Temperature did not influence onset or peak release timing as a sole factor.

3.2.2 Larval release magnitude

Broodstock held for 7-weeks released more larvae than those held for 12-weeks (Figure 4). The cumulative number of larvae, normalized by the number of broodstock and averaged across treatments and replicates, was $164,000 \pm 40,000$ from 7-week broodstock and $92,000 \pm 29,000$ from 12-week broodstock ($F(1,20)=29.4$, $p=2.65e-5$). The magnitude of larvae released did not differ among temperature or food treatments (Table 2).

3.3 Larval viability

3.3.1 Larval shell height and width at release

Broodstock exposed to elevated food levels produced larger larvae, with larger mean shell width ($F(1,120)=3.92$, $p=0.050$). Broodstock exposed to elevated temperature also tended to release larvae with larger shell width ($F(1,120)=3.00$, $p=0.086$, Figure 5). Of the larvae reared through settlement, those released from high-food exposed broodstock were larger (shell width: $F(1,36)=4.66$, $p=0.038$; shell height: $F(1,36)=4.63$, $p=0.038$).

3.3.2 Larval Survival

Larval survival through metamorphosis (assessed 5 weeks after release) was not significantly influenced by broodstock temperature ($\chi^2=2.7$, $p=0.098$) or food treatment ($\chi^2=0.012$, $p=0.89$), nor was there a significant interaction between the two treatments ($\chi^2=3.36$, $p=0.067$, evaluated using quasibinomial GLM). Larval survival was not associated with larval shell height ($\chi^2=2.65$,

p=0.10) or width ($\chi^2=0.29$, $p=0.59$, Figure 6) upon release, but survival was associated with the number of larvae released on the day of collection ($\chi^2=6.93$, $p=0.0085$), and the date of release ($\chi^2=15.5$, $p=8.5e-5$). Survival tended to be higher from larger pulses of larvae, and at later collection dates (Supplemental Figure 2).

Broadly, larval survival was highly variable among the 48 groups, which were collected from distinct larval pulses to maximize genetic diversity (3 groups from each of the 12 spawning tanks). Mean and median survival was 10.6% and 7.2% across all groups, and ranged from 0.3±0.3% to 46.1±5.6%. Survival exceeded 20% in 9 of the 48 groups (Table 3).

3.4 Broodstock survival and size

During reproductive conditioning, high broodstock mortality occurred in both 7°C groups. Cumulative survival at the end of the experiment was 50% and 70% in the 7°C+high-food and 7°C+low-food groups, and 81% in both 10°C+high-food and 10°C+low-food groups (Figure 7). Oyster plumpness (wet tissue weight normalized by shell height) decreased over time in all experimental broodstock ($F(1,280)=11.8$, $p=7.5e-4$), and in wild-collected oysters ($F(1,68)=29.5$, $p=8.3e-7$). Shell height, which was on average 38.0±5.0 mm, did not change significantly in experimental broodstock ($F(1,294)=3.55$, $p=0.061$), but increased in the wild-collected oysters ($F(1,68)=7.88$, $p=0.005e-3$). Broodstock shell height and wet tissue weight did not differ by temperature or feeding level during treatment.

4. Discussion

This study investigated impacts of over-wintering conditions on *O. lurida* larval production. Broodstock were simultaneously exposed to different temperatures (7°C, 10°C), and algal densities (low-food=5k cells/mL, high-food=50k cells/mL) for 7-weeks or 12-weeks in winter

prior to spawning. We predicted that elevated winter temperature would result in fewer larvae or poorly provisioned larvae (i.e. smaller, poor survival), which would be amplified in the presence of low food and after prolonged treatment (12-weeks). This hypothesis was based on observations of poor larval production in an *O. lurida* hatchery the spring following the winter 2015 marine heat wave in the Northeast Pacific Ocean (Gentemann, Fewings, and García-Reyes 2017). Elevated winter temperature was posited to interfere with seasonal reproductive quiescence, which is considered an important component of the *O. lurida* reproductive cycle, and deplete glycogen and lipid reserves due to increased metabolic demand.

While winter temperature and algal density did influence gamete development and larval release timing, treatments did not ultimately influence larval production. Counter to predictions, larvae tended to be larger from broodstock exposed to elevated temperature, however larval size did not correlate with larval survival. Surprisingly, high broodstock mortality occurred following exposure to low temperature, and fecundity was substantially lower after prolonged exposure to all treatments. We expand on these findings below, and include recommendations for the handling of *O. lurida* broodstock during the winter for restoration and commercial hatcheries.

4.1 Effects of overwintering temperature and algal density

4.1.1 Larval production

Larval production was unaffected by winter temperature and algal density, regardless of exposure time (7 or 12 weeks). The results are in contrast with a complementary study, Spencer et al. (2020), in which elevated temperature exposure prior to spawning resulted in more larvae. The present study specifically expands Spencer et al. (2020), to use a new *O. lurida* population collected from the wild rather than oysters that were bred in captivity. The response of *O. lurida*

reproduction to elevated winter temperature may be conditional upon gonad stage prior to treatment, and population-specific reproductive traits (Barber, Ford, and Wargo 1991; Silliman, Bowyer, and Roberts 2018). For instance, in this study the percentage of female broodstock was unusually high upon collection (63%), and many already contained late-stage oocytes (55% of females). In comparison, only 28% of all oysters sampled in Spencer et al. (2020) were female, 33% of which contained late-stage oocytes. Oysters that enter the winter with late-stage oocytes may be less influenced by winter conditions, and may require less time and energy for maturation in the spring. The developmental status of Olympia oyster oocytes entering the winter season may therefore influence how winter temperature affects spring reproduction. That larval production was unaffected by overwintering treatments suggests that *O. lurida* are capable of withstanding a range of winter conditions without compromising spring larval production.

4.1.2 Larval survival

Larval survival was not influenced by parental exposure to elevated winter temperature. This indicates that the quality of *O. lurida* larvae produced in the hatchery is not sensitive to winter conditions. The broodstock collected for this study may have had sufficient energy stores to endure winter treatments and subsequently reproduce. Alternatively, *O. lurida* may modulate energy allocation to prioritize reproductive processes over other processes such as growth. While these results contrast expectations they are in alignment with the related study, Spencer et al. (2020), which also did not detect temperature effects on larval survival. Together, these studies indicate that elevated winter temperature does not compromise larval quality when larvae are reared in a hatchery setting. Interestingly, broodstock that were exposed to elevated temperature contained larger oocytes, and tended to produce larger larvae. Both oocyte and larval size are 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.

4.1.4 *Broodstock survival*

Poor survival was observed in broodstock that had been exposed to low temperature (7°C) in the presence of high food, particularly those exposed for 12-weeks (Figure 7). The majority of mortality occurred when broodstock from all treatments were in common conditions and undergoing a 1°C/day increase to induce spawning. The added 3°C increase for the low temperature exposed oysters may have been thermally stressful, or increased their susceptibility to bacterial infection, resulting in the high mortality (Delaporte et al. 2006; Lokmer and Mathias Wegner 2015; Wendling and Wegner 2013). In addition to thermal stress, mortality in the 7°C+high-food treatment could also be due to oxidative stress caused by high reproductive effort. Just prior to spawning the 12-week trial broodstock, plumpness (a proxy for condition index) increased in the 7°C+high-food treatment only (Figure 7). Condition index is positively associated with post-spawning mortality in other oyster species, explained in part by high glycogen reserves fueling rapid gamete proliferation, which elevates metabolism and causes oxidative stress (Alonso-Alvarez et al. 2004; Chávez-Villalba, Villelas-Ávila, and Cáceres-Martínez 2007; Huvet et al. 2010). High mortality in the 7°C+high-food broodstock may therefore be associated with synergistic effects of thermal stress and oxidative stress. The extended time in captivity likely compounded these effects, resulting in high mortality in the 12-week trial during spawning.

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

4.2.1 *Gonad activity in winter*

It is commonly noted that temperate oyster species enter reproductive diapause in winter, when water temperature drops below a thermal threshold for gametogenesis (Brian Leicester Bayne

2017). The majority of *O. lurida* literature concurs, and defines the thermal threshold for gametogenesis as 12.5°C for *O. lurida* in Washington State. However, much of the literature is from the early 20th century and is limited to field observations from a few populations (Hopkins 1936, 1937). Here, we find evidence for gonad activity at low temperatures based on gradual changes in gonad stage and sex throughout the winter (Figure 2, Supplemental Figure 1). Spencer et al. (2020) also observed that spermatogenesis can occur at lower temperatures (~10°C) in three Puget Sound *O. lurida* populations, and Barber et al. (2016) recently found *O. lurida* adults brooding embryos at 10.5°C in a northern Puget Sound lagoon. Taken together, the concept that reproductive activity ceases below 12.5°C is no longer reliable for *O. lurida* in the Puget Sound estuary. To inform Olympia oyster restoration and commercial operations, the thermal limits for *O. lurida* spawning should be investigated, and in the context of other potential reproductive drivers (e.g. food availability, tidal cycle, photoperiod). Furthermore, winter conditions should not be overlooked when examining *O. lurida* reproductive cycles.

4.2.2 Influence of overwintering time on larval production

The amount of time broodstock were held prior to spawning substantially impacted larval production. Broodstock held for 7 weeks produced nearly twice the number of larvae per animal than those held for 12 weeks. Poor production following prolonged captivity could be explained by stress associated with the unnatural hatchery conditions. Oyster plumpness consistently decreased over time in most treatments regardless of algal densities (Figure 7), indicating that broodstock may have utilized energy reserves despite access to ample food. The hatchery conditions and regular handling during cleanings may have stressed the animals such that they did not eat, and instead catabolized endogenous energy reserves which are commonly used during gametogenesis (Brian Leicester Bayne 2017). Alternatively, the nutritional value of

hatchery-cultured microalgae may have been insufficient, such that the broodstock fed cultured algae for longer prior to spawning depleted more endogenous reserves (Wilson, Chaparro, and Thompson 1996). Helm et al. (1973) observed a similar temporal trend when experimenting with *Ostrea edulis* broodstock, as larval yield, growth and competency decreased as their experiment progressed. In the present study, varying mortality did result in fewer broodstock per tank in the 12-week trial, that should be considered. We normalized larval production by the number of broodstock in each tank; however, due to the Olympia oyster's cryptic spawning behavior, the correlation between the number of broodstock in a mass spawning tank and mating pairs is not known, and may be nonlinear. To optimize *O. lurida* larval production and broodstock survival, hatcheries should minimize the time that broodstock are held in captivity prior to spawning.

4.2.3 No association between larval size upon release and survival

Larval shell height and width, measured upon release from the brood chamber in 48 larval groups, did not correlate with survival through metamorphosis (35 days post-release). This is unexpected, as larval size upon release has been positively linked to larval "quality" in other *Ostrea* species. In the Chilean flat oyster (*Ostrea chilensis*), pediveliger size upon release positively correlates with larval growth and spat survival (Wilson, Chaparro, and Thompson 1996), in addition to oocyte size and biochemical properties (lipid, protein, carbohydrate). Growth and competency through metamorphosis of *O. edulis* larvae positively relates to lipid content upon release from the brood chamber (Helm, Holland, and Stephenson 1973). In other bivalves larval size and/or growth rate is linked to oocyte size and larval competency, such as in the Eastern oyster *Crassostrea virginica* and hard clam *Mercenaria mercenaria* (Gallager and Mann 1986; Kraeuter, Castagna, and van Dessel 1981), the Japanese abalone *Haliotis discus hannai* (Fukazawa et al. 2005), and bay scallop *Argopecten irradians* (Kraeuter, Castagna, and

van Dessel 1981). In Fukazawa et al. (2005) and Kraeuter et al. (1981) larvae were unfed, and thus relied solely on endogenous resources provided by the mother. This was not the case in the present study, as larvae had access to algae throughout the experiment. Had larvae been unfed it is possible that survival may have correlated more closely with shell size upon release. Nevertheless, under hatchery conditions *O. lurida* larval size upon release from the brood chamber is not predictive of survival to spat. Hatcheries should therefore not presume that small larvae are of poor quality.

4.3 Recommendations for Hatchery Production

Broodstock mortality is inefficient for hatcheries, can hinder larval production, and decrease genetic diversity of offspring. To minimize broodstock mortality during spawning, we recommend acclimating broodstock to hatchery conditions at a slightly warmer temperature, rather than mimicking cooler winter temperatures (e.g. 10°C vs. 7°C). Hatcheries may also consider testing slower rates of temperature increase if high mortality occurs during spawning (e.g. 0.5°C/day), and minimizing the time broodstock are in captivity prior to spawning. We find no evidence that restricting food during hatchery acclimation impacts larval production or survival, given that broodstock are in good condition upon collection. When larvae are grown with good husbandry practices, size upon release is not predictive of survival. Depending on the geographic location and population characteristics, low level gametogenesis can occur in *O. lurida* throughout the winter, thus the time that broodstock are collected in the winter may influence spawn timing and larval release magnitude.

5. Conclusion

There are many gaps in our understanding of *Ostrea* spp. reproductive systems, which constrain interpretation of data collected from *Ostrea* spp. For instance, in the absence of genetic testing, the number of families produced in a batch spawn is based on estimates of brood size per female (see Supplemental Materials), which may not reflect present-day populations (Hopkins 1936). Studies needing to estimate the number of breeding females would benefit greatly from a model of brood size against adult size and the number of oysters in a mass spawn across multiple populations. Furthermore, many operating theories are based exclusively on early observations of *O. lurida* (Hopkins 1936; W. R. Coe 1931a), which do not likely represent present-day populations and may be unreliable, such as the concept that gametogenesis ceases in winter and residual gametes are resorbed. We find little evidence of resorption in both treated and wild-collected oysters throughout the winter, and theorize that many residual gametes remain viable. Finally, a deeper understanding of the relative influence of environmental drivers (*e.g.* temperature, food, tidal cycle, allospem), and internal drivers (*e.g.* age, source population), on reproduction, including what precisely triggers spawning, would greatly improve the outlook for 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 ($p=0.0056$, 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)	$\chi^2 = 11.1$, $p=0.86$	$\chi^2 = 14.5$, $p=0.63$
4 wks in treatment (Jan 4)	$p=0.044$ (Fisher test)	$\chi^2 = 18.8$, $p=0.29$	$p=0.63$ (Fisher test)
7 wks in treatment (Jan 23) End of 7-week trial	$p=0.76$ (Fisher test)	$\chi^2 = 19.7$, $p=0.24$	$\chi^2 = 12.7$, $p=0.84$
9 wks in treatment (Feb 9)	$\chi^2 = 9.5$, $p=0.78$	$\chi^2 = 16.0$, $p=0.48$	$p=0.98$ (Fisher test)
12 wks in treatment (Feb 27) End of 12-week trial	$\chi^2 = 19.9$, $p=0.061$	$\chi^2 = 32.5$, $p=0.0047$ Pairwise treatment differences: 7°C+low-food vs. 10°C+high-food 10°C+low-food vs. 10°C+high-food	$\chi^2 = 21.8$, $p=0.12$
All treatment wks combined (Dec 20 - Feb 27)	$\chi^2 = 14.0$, $p=0.30$	$\chi^2 = 31.0$, $p=0.012$	$\chi^2 = 17.2$, $p = 0.37$
2 weeks in common spawning conditions (Mar 13)	$\chi^2 = 9.2$, $p=0.43$	$\chi^2 = 23.4$, $p=0.017$	$\chi^2 = 8.6$, $p=0.51$
3.5 weeks in common spawning conditions (Mar 23)	$\chi^2 = 9.7$, $p=0.39$	$\chi^2 = 25.2$, $p=0.0090$	$\chi^2 = 9.8$, $p=0.68$
Both spawning dates combined (Mar 13 & 23)	$\chi^2 = 8.7$, $p=0.48$	$\chi^2 = 32.2$, $p=0.0010$ 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	$\chi^2 = 11.7$, $p=0.49$

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 ($p \leq 0.05$). 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 <i>Kruskal-Wallis Chi-Square Test</i>	$\chi^2 = 0.35$ $p = 0.55$	$\chi^2 = 6.84$ $p = 8.9 \times 10^{-3}$	$\chi^2 = 5.61$ $p = 0.018$	$\chi^2 = 7.27$ $p = 0.064$	$\chi^2 = 6.11$ $p = 0.11$	$\chi^2 = 18.1$ $p = 4.1 \times 10^{-4}$	$\chi^2 = 18.9$ $p = 8.6 \times 10^{-3}$
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)	$F = 6.0 \times 10^{-3}$ $p = 0.94$	$F = 0.35$ $p = 0.56$	$F = 2.10$ $p = 0.16$	$F = 0.76$ $p = 0.39$	$F = 0.13$ $p = 0.72$	$F = 0.40$ $p = 0.54$	$F = 0.94$ $p = 0.34$
Cumulative no. larvae released over the 30-day collection	$F = 0$ $p = 0.99$	$F = 0.74$ $p = 0.40$	$F = 29.4$ $p = 2.65 \times 10^{-5}$	$F = 1.2$ $p = 0.29$	$F = 0.21$ $p = 0.89$	$F = 0.11$ $p = 0.74$	$F = 2.81$ $p = 0.11$

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 <i>sorted by date collected</i>	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%	

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.

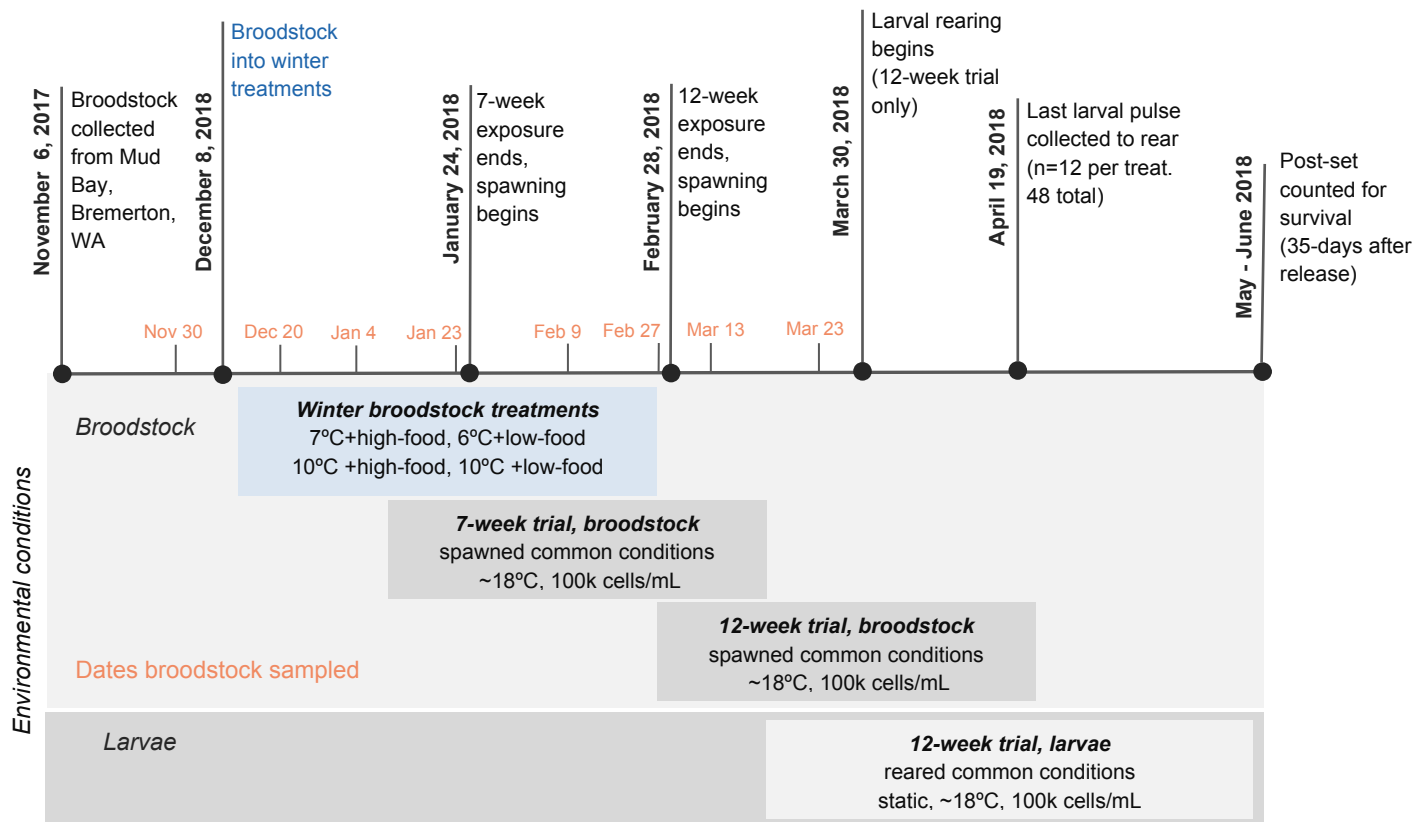


Figure 2.

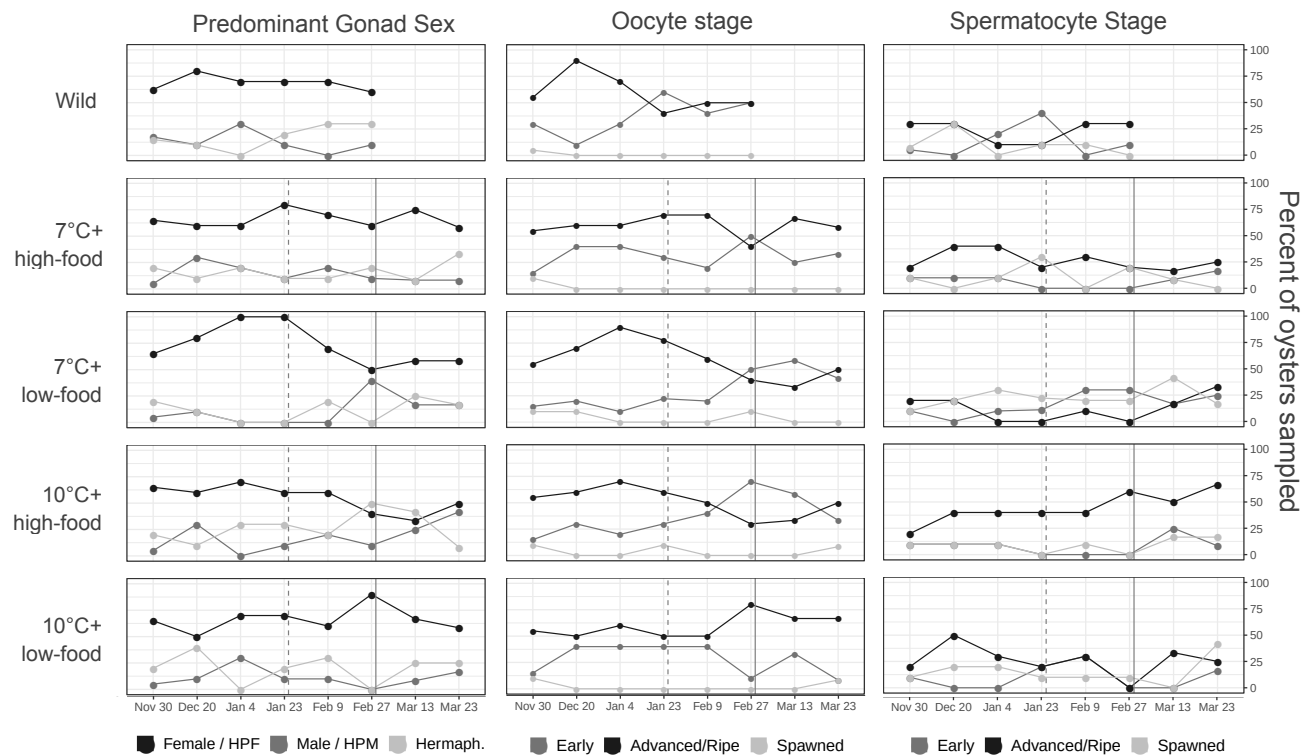


Figure 3.



Figure 4.

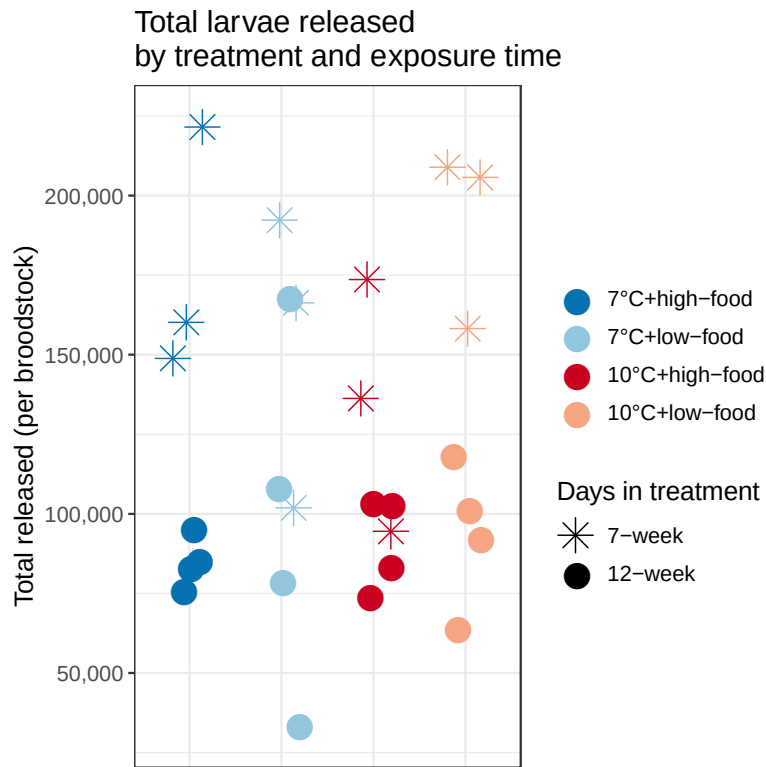


Figure 5.

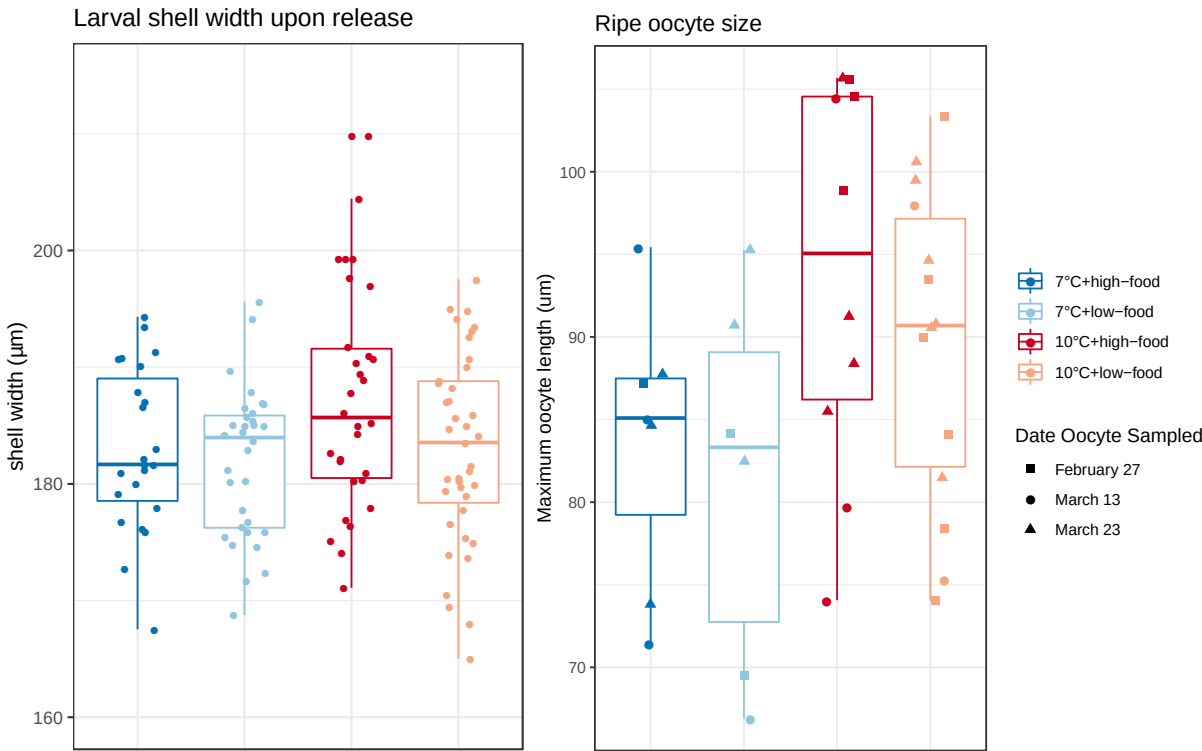


Figure 6.

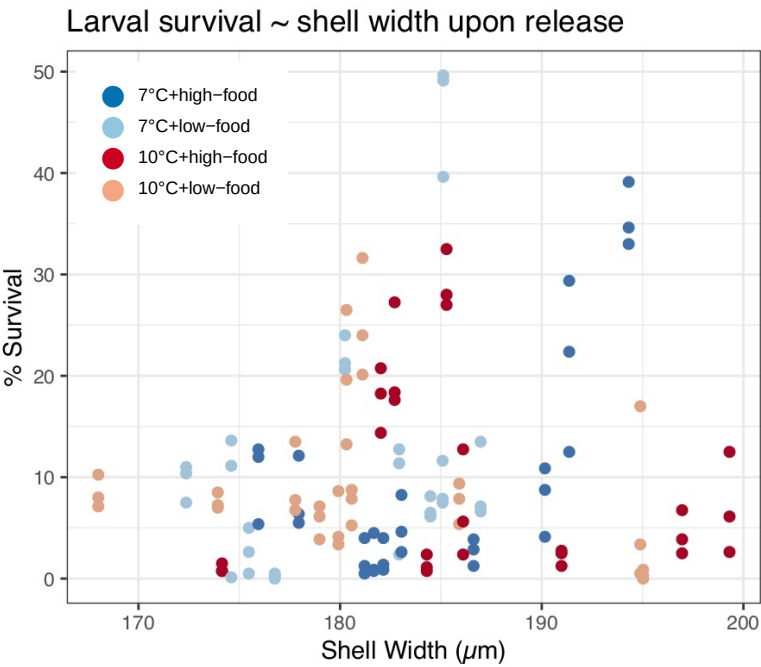
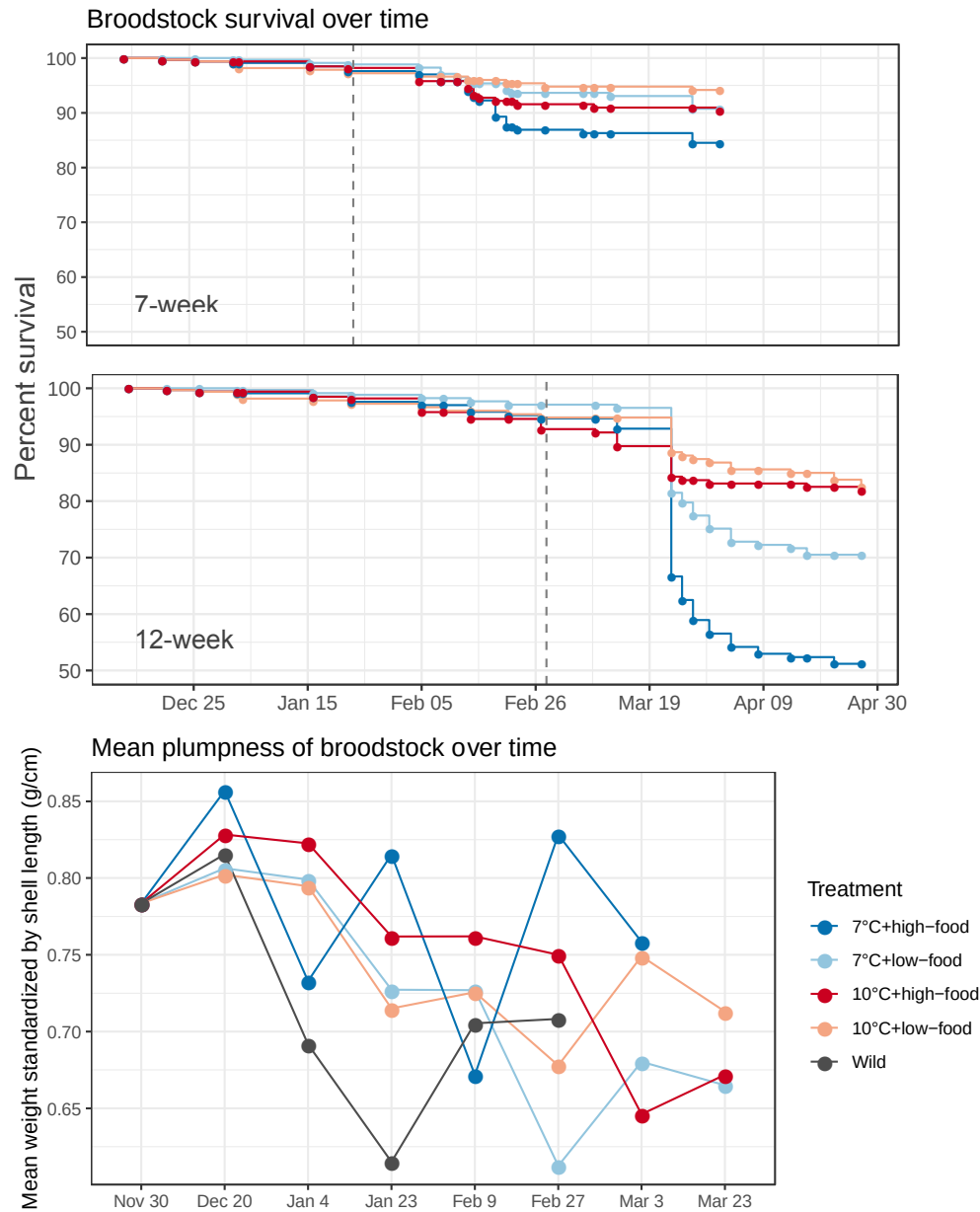


Figure 7.



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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

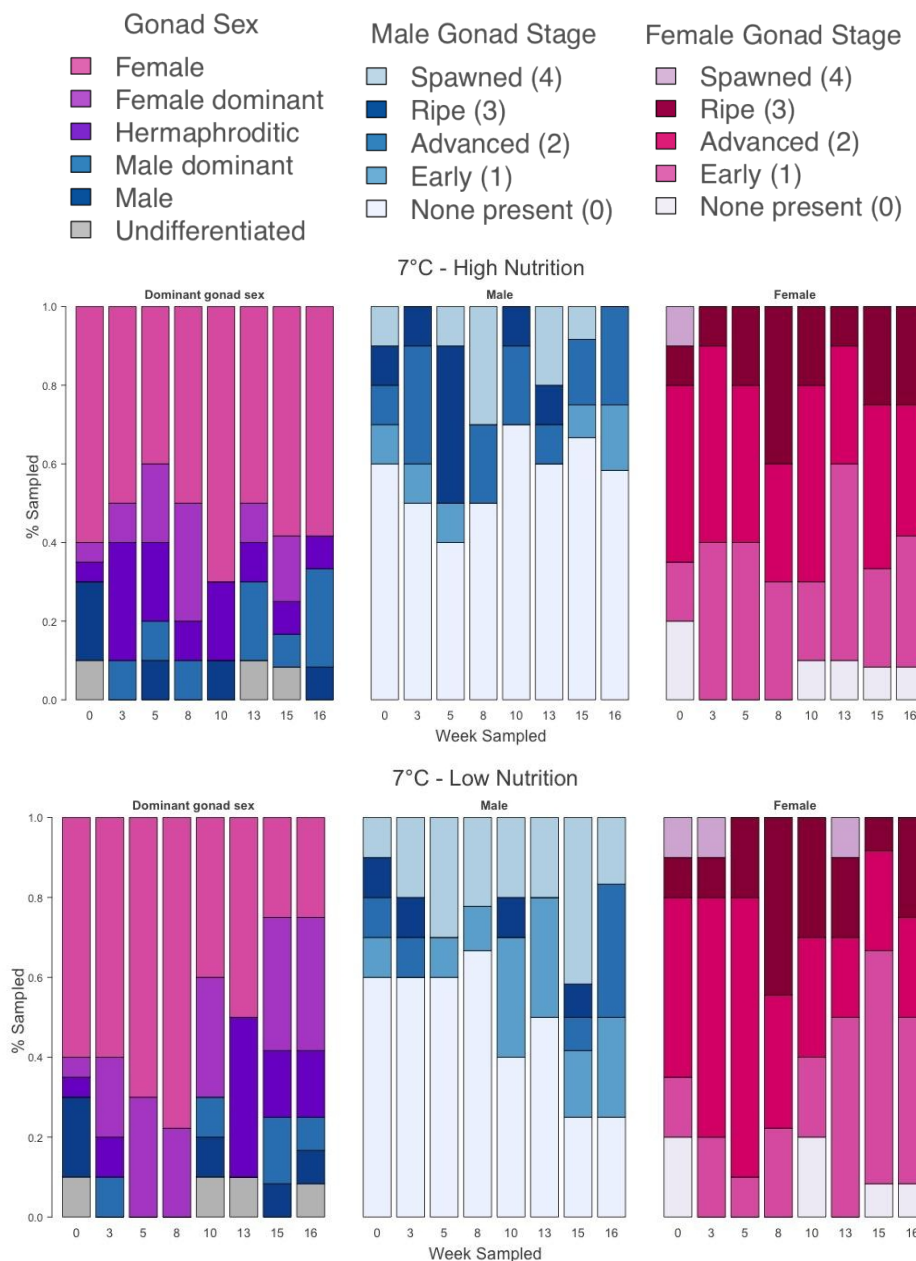
Authors: Laura H Spencer¹, Erin Horkan², Ryan Crim², Steven B Roberts¹

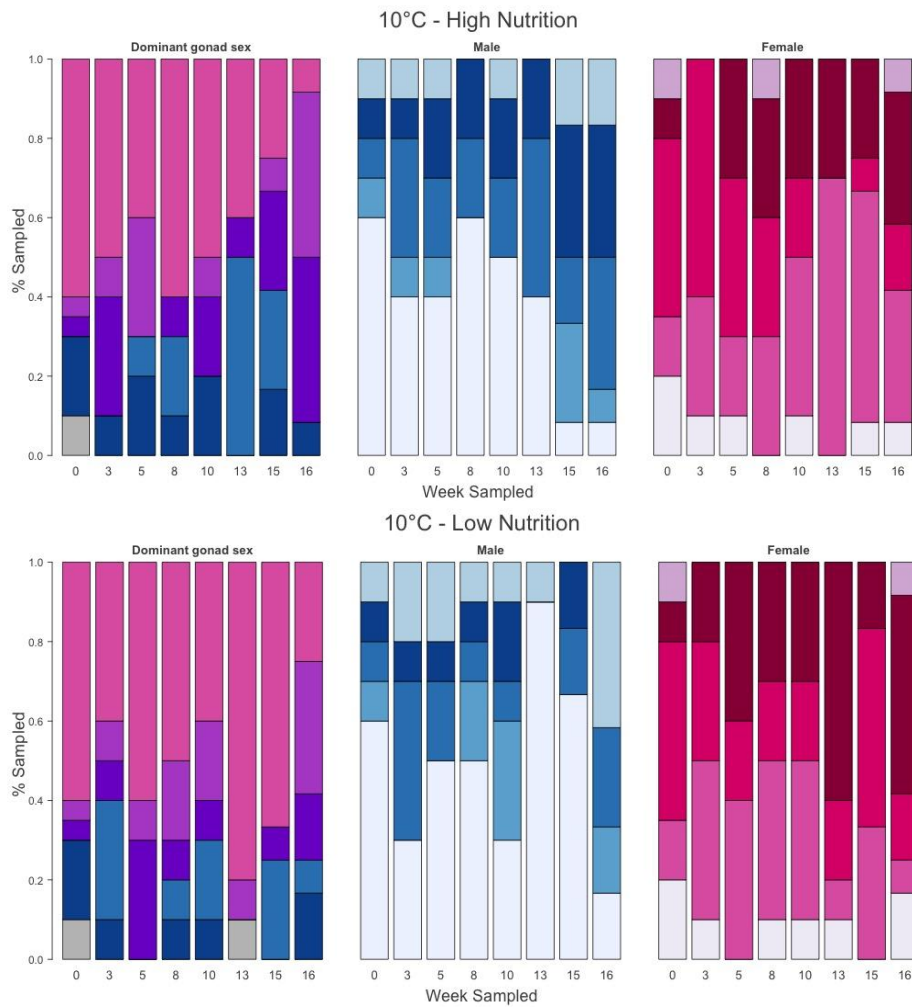
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Supplemental Materials

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.





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

	= 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)

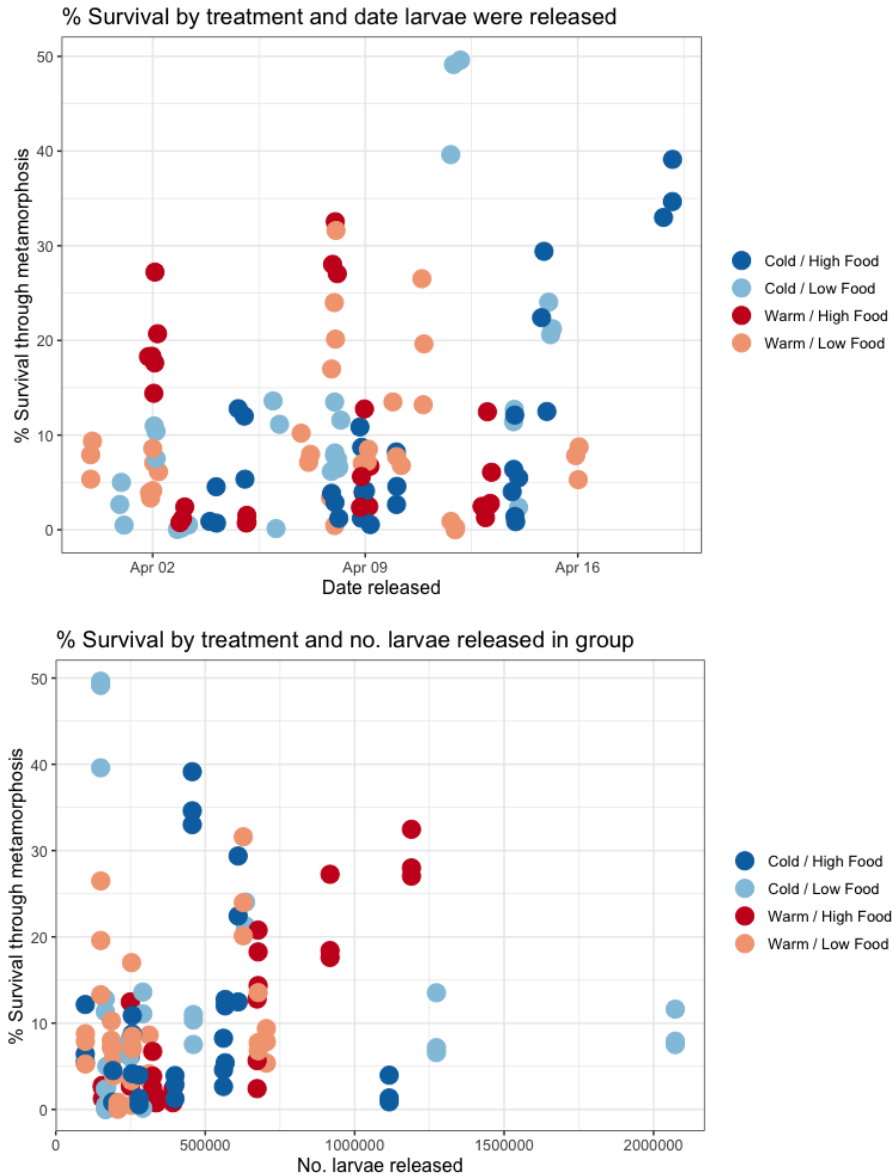
Estimates of female reproduction rate

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.

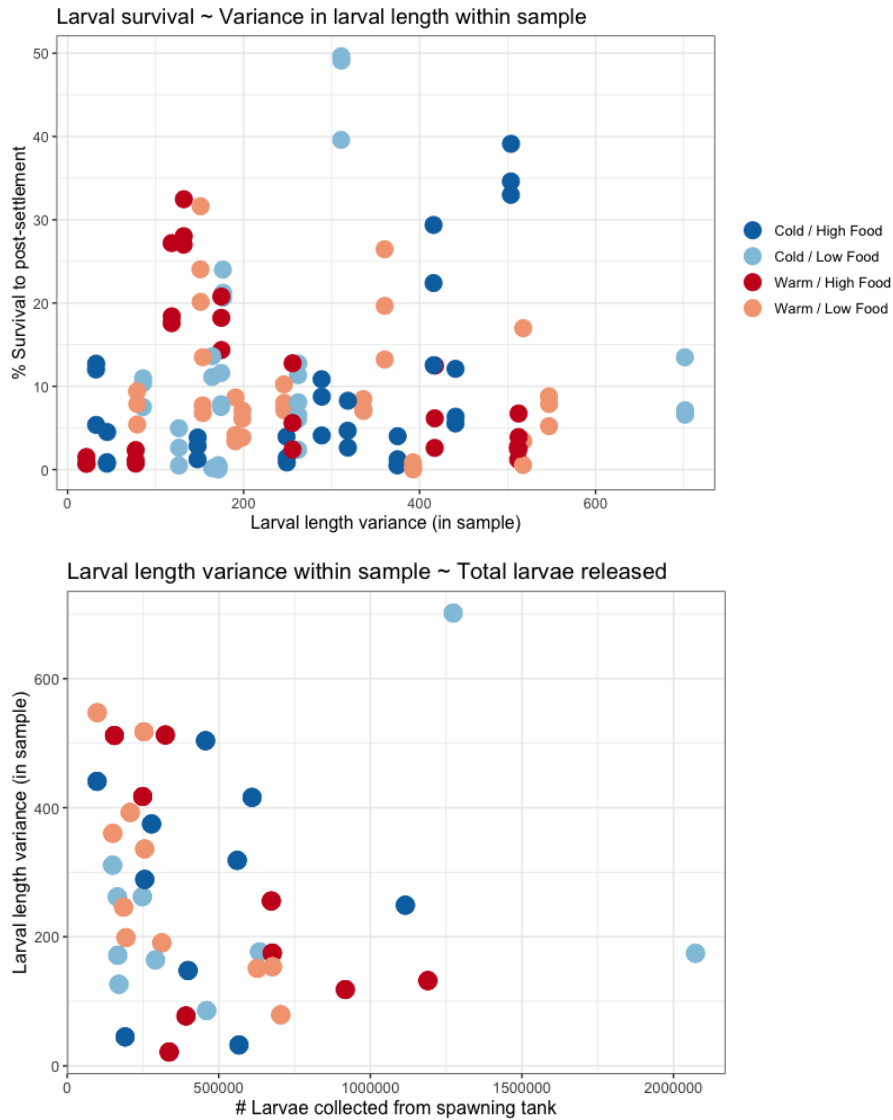
Approximately 76% of the 7-week trial broodstock reproduced as female, which was significantly more than the 12-week trial (estimated ~43%) ($F(1,20)=29.7$ $p=2.45e-05$). The estimated percent of oysters that reproduced as female did not differ among treatments (for temperature, food, and temperature:food, 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 $43\pm13\%$.

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.