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Description of metamorphic phases in the oyster *Crassostrea virginica* and effects of hypoxia on metamorphosis

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**ABSTRACT:** Four phases of metamorphosis in the eastern oyster *Crassostrea virginica* were characterized: 'settlers' have attached to the substrate but retain larval characteristics; metamorphosis and degeneration of the velum has begun in 'prodissococonch postlarvae'; in 'dissoconch postlarvae' shell growth beyond the prodissococonch has begun but the foot persists; and 'juveniles' have lost all larval organs and metamorphosis is complete. These phases were used in examining the metamorphic process during and following continuous and short-term exposures to hypoxia (1.5 mg O$_2$ L$^{-1}$, 20% of air saturation) and microxia (<0.07 mg O$_2$ L$^{-1}$, <1% of air saturation). We observed no abnormal development in the oysters, but development was delayed following 3 d exposures to hypoxia, and 2 and 3 d exposures to microxia. Under continuous exposure to microxia, oysters did not develop to the dissoconch postlarva or juvenile phases. Approximately 50% of the control oysters died within the 2 wk period following settlement. Mortality was virtually confined to the settler and prodissococonch postlarva phases. Short-term exposures to hypoxia (1 to 3 d) and microxia (1 d) had little effect on the median mortality time or final total mortality, compared to controls. Microxic treatments longer than 1 d did affect mortality and oysters continuously exposed to microxia had a median mortality time of 87 h. Short-term exposures to low oxygen did not have permanent effects on post-settlement growth rates. Oysters exposed to microxic treatments, however, appeared to have slower growth rates during the exposure period. We conclude that low oxygen conditions, in particular those that are microxic and last longer than 24 h, have detrimental effects on the development, growth, and mortality of post-settlement oysters.

**KEY WORDS:** Bivalve, Hypoxia, Larval settlement, Metamorphosis, Morphology

**INTRODUCTION**

Some coastal embayments and estuaries, such as Chesapeake Bay (USA), exhibit oxygen depletion of deep waters as the result of density stratification during the summer months (Taft et al. 1980, Officer et al. 1984). Wind forcing may tilt the pycnocline, thereby moving hypoxic or anoxic deep water into shallow areas where oyster reefs occur (May 1973, Malone et al. 1986). Irrigation of shallow areas may continue for several hours to 2 or 3 d (L. Sanford, K. Sellner & M. Bundy unpubl.). Oxygen depletion of deep waters occurs during warm summer months. Therefore, hypoxic intrusion onto the shallow flanks of the Chesapeake Bay often coincides with the period of settlement and subsequent metamorphosis of the oyster *Crassostrea virginica* (Gmelin, 1791).

Many morphogenetic processes, such as embryonic development and growth of larvae, are affected negatively by oxygen deprivation. Exposure to hypoxia causes delayed and abnormal embryonic development and reduced larval growth rates in both the clam *Mercenaria mercenaria* and the mussel *Mytilus edulis* (Morrison 1971, Wang & Widdows 1991). Metamorphosis is a morphogenetic process in which larval organs disappear and permanent organs reorganize and develop (Fioroni 1982); yet, it is not known what effects
low oxygen conditions have on bivalve metamorphosis.

Effects of low oxygen exposure on metamorphosis must necessarily be evaluated against normal metamorphic processes. Although several authors have observed the morphology of post-settlement oysters (Jackson 1888, Stafford 1913, Cole 1937, 1938, Hickman & Guffydd 1971), no description of metamorphosing *Crassostrea virginica* identifies specific morphogenetic phases in live oysters.

The objectives of this study included first developing a scheme of metamorphic phases that are easily identifiable in live *Crassostrea virginica*. Then, using this scheme, we examined the process of metamorphosis during continuous exposure to low oxygen, as well as during recovery periods following exposure.

**MATERIALS AND METHODS**

**Metamorphic phases.** Pediveliger larvae of the eastern oyster *Crassostrea virginica* were obtained from the Virginia Institute of Marine Science hatchery at Gloucester Point, Virginia, USA. Frosted Mylar acetaate settlement substrates were held in seawater for at least 2 d prior to each experiment to develop a settlement-inducing bacterial coating (Fitt et al. 1990). Larvae (290 ± 18 μm mean shell height, dorsal-ventral axis) were exposed to a conditioned settlement substrate unit for 1 h. Larvae that had not settled and attached within that time were rinsed from the substrate unit. Ten newly attached larvae were labeled by marking their location on the substrate unit with pencil. The substrate unit and attached oysters were maintained in a glass dish in filtered (0.45 μm) seawater of 21 ppt salinity at an ambient temperature of 25°C. Water was changed daily and oysters were fed *Isochrysis galbana* at a concentration of 20000 cells ml⁻¹.

The internal morphology of labeled oysters was examined and photographed using an Olympus SAH zoom stereo microscope (maximum magnification 64X) and illumination base equipped with a phototube and a Contax (137 MA Quartz) 35 mm camera. Examinations were made at 3 to 24 h intervals, beginning at 3 h post-settlement. The photographs and notes were used to develop a scheme of metamorphic phases based on the condition of the velum, foot, gills, labial palps and shell. Figures were made with a microscope drawing attachment.

**Continuous low oxygen experiments.** Treatment flasks of seawater, containing algal food (*Isochrysis galbana*) at a concentration of 20000 cells ml⁻¹, were continuously bubbled with either air, a mixture of oxygen and nitrogen, or nitrogen. The target oxygen concentrations were 7.3 mg O₂ l⁻¹ (100% of air saturation), 1.5 mg O₂ l⁻¹ (20% of air saturation) and <0.07 mg O₂ l⁻¹ (<1% of air saturation). These treatments are referred to as normoxic, hypoxic and microxic respectively. Oxygen concentrations were measured twice a day with a Strathkelvin Instruments (SI) oxygen sensor (1302) coupled to an SI oxygen meter (781) and chart recorder. The oxygen sensor was calibrated with air-saturated water (100% of air saturation) and a zero oxygen solution of sodium borate and crystalline sodium sulfite. Normoxic, hypoxic and microxic treatments were consistently maintained at 90 to 100, 17 to 24 and 0 to 1% of air saturation respectively.

The metamorphic phases of oysters were determined after 24 to 120 h of continuous exposure to the 3 oxygen treatments, as follows. Pediveliger oyster larvae were exposed to a conditioned substrate for 3 h. The substrate was cut into 1 × 2 cm pieces so that each substrate unit had at least 10 newly attached oysters. Five substrate units were suspended in each of three 250 ml flasks (one for each oxygen treatment). The flasks were covered with inverted beakers and continuously bubbled with gases. One substrate unit was removed from each treatment every 24 h without replacement so that different individuals were examined each day. The metamorphic phases to which 10 oysters had developed were determined immediately. Remaining substrate units were transferred daily to identical flasks of seawater and algae that had been bubbled with the appropriate gases for at least 1 h. The continuous exposure experiment lasted 120 h and was repeated 3 times (3 replicates) with different cohorts of larvae.

For each oxygen treatment-exposure time replicate, counts of individuals in the different metamorphic phases were converted to percentages of the total number of individuals observed. The percentages were arcsine transformed. A 1-way analysis of variance was performed to test the null hypothesis that there was no effect of oxygen treatment on the proportion of oysters completing metamorphosis to the juvenile phase by the end of 120 h of continuous exposure. Tukey's multiple comparison test was used to identify differences between specific oxygen treatments. Statistical analyses were conducted using Minitab software. Data from the continuous experiments are reported as the back-transformed means and standard deviations of the 3 replicates using hours as the time scale.

**Recovery experiments.** Metamorphic phases and growth of individual oysters were examined during recovery following 1, 2 or 3 d of exposure to the 3 oxygen treatments. On Day 0, pediveliger larvae were allowed to settle onto conditioned substrate that was then cut into 2 × 7 cm substrate units. Fifteen newly attached oysters on each substrate unit were labeled with pencil on the adjacent substrate. Substrate units
Growth data were log-transformed and analyses of variance were used to test the significance of linear regressions. Analysis of covariance was performed to test the null hypotheses that there were no differences in slope or elevation between the 9 linear regression lines. If a null hypothesis was rejected, a Tukey's multiple comparison test was used to determine which linear regression lines were different from each other. Statistical analyses were conducted using Quattro Pro software.

RESULTS

Metamorphic phases

Four distinct phases of metamorphosis were defined (Fig. 1).

Settlers

Settlers are oysters that have attached but otherwise retain characteristics of the pediveliger larva (Fig. 1A). The distinguishing feature of the settler phase is the presence of an intact and active velum and foot. In some larvae the velum is pigmented. The velum and/or foot sometimes protrude from between the valves and the velar cilia continue to beat in a coordinated manner. As in the pediveliger, the eyespots are prominent. The rudiment of the left gill, which was also present in the pediveliger, is visible. There is no shell growth beyond the prodissoconch. The shell height of settlers was the same as that of the unattached larvae (299 ± 18 μm).

Prodissoconch postlarvae

Metamorphosis has begun and the organs have started an anterior-dorsal revolution (counterclockwise if viewed with the right valve up; Fig. 1B). The distinguishing feature of prodissoconch postlarvae is the degeneration of the velum, leaving a larger visceral cavity. If the velum was pigmented in larval and settler phases, the pigment is now concentrated in the area of the velar remnant. Velar cilia sometimes beat in an irregular and uncoordinated manner. The foot is present and the right eyespot is still visible. The gill filaments are longer than in the previous phase. The posterior adductor muscle is visible. There is no shell growth beyond the prodissoconch but the mantle sometimes protrudes beyond the prodissoconch. Prodissoconch postlarvae, that later went on to metamorphose, first appeared an average of 18 h post-settlement.
**Dissoconch postlarvae**

Metamorphosis and revolution of the organs continues in this phase (Fig. 1C). The distinguishing characteristics of dissoconch postlarvae are shell growth beyond the margin of the prodissoconch and the persistence of the foot. The velum is completely gone. The foot or a foot remnant has rotated along with the rest of the body and is mid-ventral, located between the gills at their proximate end. The eyespot has submerged in the tissue and become a thin line of pigment located more dorsally than the eyespot; we call this line the 'eyestreak'. Both the right and left gills are visible but the right is smaller than the left. The gill filaments of the left gill are longer and new filaments have been added at the distal end. The outer and inner labial palps are visible as active lobes. If the velum is pigmented, the inner palps are also pigmented. New shell has grown in a thin line at the ventral edge of the prodissoconch. Dissoconch postlarvae first appeared an average of 29 h post-settlement.

**Juveniles**

In juveniles, all larval organs are lost and metamorphosis is complete (Fig. 1D). The eyestreak is usually gone but sometimes persists for several days. Both the descending and ascending limbs of the inner demibranch of the left gill are visible. (The outer demibranchs develop later.) The right gill is still smaller than the left. The mantle is fused at the distal end of the left gill. The outer palps form a hood over the mouth and around the proximate end of the gills. The adductor muscle extends past the edge of the prodissoconch with the intestine passing around it posteriorly. The dissoconch extends in wings on either side of the hinge. Both valves are approximately the same size and shape and the whole of the left valve is still attached. Juveniles appeared an average of 48 h post-settlement with a mean shell height of 410 ± 40 μm.

**Continuous exposure experiment**

Although the number of settlers decreased more slowly in the hypoxic treatments than in the normoxic treatments, the proportion of settlers remaining alive at 120 h was approximately 30% in both treatments (Fig. 2). Prodissoconch postlarvae were always observed in low numbers. In normoxic treatments the highest proportion (40%) of dissoconch postlarvae was observed at 48 h; after that the proportions declined steadily as they became juveniles. The highest proportion of dissoconch postlarvae observed in hypoxic treatments was 22% at 96 h. In microxic treatments,
only 1% of the oysters reached the dissoconch postlarva phase and they died within 24 h.

Major portions of the final juvenile counts in the normoxic and hypoxic treatments appeared by 48 to 72 h. Although the difference between proportions of juveniles at 120 h in normoxic and hypoxic treatments was large (51 and 18% respectively), they were not significantly different (p > 0.05) from each other. They were, however, both significantly different (p ≤ 0.05) from the microxic treatments in which no oysters reached the juvenile phase.

Mortality of all metamorphic phases was approximately 8% in both normoxic and hypoxic treatments at 120 h, although variation was higher in the hypoxic treatments. Oysters in the microxic treatments had a median mortality time (time to 50% mortality) of 87 h and mortality was 100% by 120 h. Nearly all oysters in microxic treatments died as settlers, without beginning metamorphosis.

Exposure and recovery experiment

The number of settlers declined fairly steadily in all treatments and the proportions of settlers remaining on Day 14 was between 3 and 13%. Prodissoconch postlarvae were always observed in low numbers and some were still present at the end of the experiment. Dissoconch postlarvae were observed in proportions of <21%.

The final proportions of juveniles were between 6 and 30%. Large portions of the final juvenile counts appeared by Day 3, the exceptions being 3 d exposures to hypoxia, in which the number of juveniles doubled between 6 and 10 d post-settlement, and 2 and 3 d exposures to microxia, in which juveniles appeared at 6 to 14 d post-settlement. No significant (p > 0.05) effect of exposure time on the proportion of juveniles at 14 d post-settlement could be detected. Although not statistically significant, in the microxic treatments there was a trend of fewer juveniles when exposure time was longer. There was no interaction of exposure time and oxygen treatment. There was, however, a significant difference (p ≤ 0.05) between the proportions of juveniles on Day 14 in the normoxic and microxic treatments but none could be detected between hypoxic and normoxic treatments. Variation between replicate experiments was large and was greatest in the microxic treatments.

Median mortality times were greater than 10.4 d except for the 2 and 3 d exposures to microxic treatments which had median mortalities of 3.6 d and less than 3 d, respectively (Fig. 3). Mean total mortality by Day 14 was between 46 and 61% except in treatments exposed to 2 and 3 d of microxia; these treatments had final mean mortalities of 79% and 88%, respectively. Virtually all oysters that died, did so as settlers or prodissoconch postlarvae; only 1 dissoconch postlarva was observed to die, and no juveniles died.

Linear regressions of log-transformed growth of dissoconch postlarvae and juveniles were significantly different from zero (p ≤ 0.05) (Fig. 4). The slopes of the 9 oxygen treatment/exposure time combinations were not significantly different (p > 0.05) from each other.

Fig. 2. Crassostrea virginica. Percentage (means + SD) of 4 metamorphic phases of oysters after different periods of continuous exposure to 3 oxygen concentrations: normoxia (7.3 mg O₂ l⁻¹), hypoxia (1.5 mg O₂ l⁻¹) and microxia (<0.07 mg O₂ l⁻¹). MMT: median mortality time
There were, however, significant differences ($p \leq 0.05$) in the elevations of the lines. The elevation of the growth regression of oysters that experienced 2 d exposures to microxia was significantly lower ($p \leq 0.05$) than that of the normoxic control. Oysters that experienced 3 d exposures to microxia had a regression elevation that was significantly lower ($p \leq 0.05$) than that of both the normoxic control and that of oysters exposed for 3 d to hypoxia. Within oxygen treatments, there were no significant differences ($p > 0.05$) in regression elevation between exposure times. The microxic treatments, and the 2 and 3 d exposures to hypoxic treatments, had a number of dissoconch postlarvae that appeared late and/or remained small throughout the experiments.

**DISCUSSION**

Previous descriptions of the morphology of metamorphosing bivalves have been based on specific organs. This method is inadequate for identifying phases of metamorphosis in live oysters because it is difficult to piece together concurrent events. We have, therefore, organized our observations into 4 distinct phases through which all *Crassostrea virginica* pass during metamorphosis. Our observations of metamorphosis are consistent with those of previous researchers. Jackson (1888) and Cole (1938) noted rotation of the organs in an anterior-dorsal direction during metamorphosis of *Ostrea edulis*. Several authors (Cole 1938, Galtsoff 1964, Hickman & Gruffydd 1971) have reported the reduction of the velum and location of the velum remnants in *C. virginica* and *O. edulis*. At no time did we observe the velum being cast off or swallowed as has been suggested by some authors (Sigerfoos 1907, Galtsoff 1964).

The persistence of the foot beyond that of the velum has also been observed previously (Cole 1938, Galtsoff 1964). Galtsoff (1964) stated that the eyespots broke up into irregular clumps of pigment. The dorsal movement and elongation of the eyespot into the eyestreak has not been reported previously. Our observation that the labial palps are pigmented in those oysters in
which the velum was pigmented, corroborates the conclusions of Cole (1938), Quayle (1951) and Galtsoff (1964) that the labial palps develop from the apical portion of the velum.

The speed at which metamorphosis occurs varies considerably between individuals. Some oysters may metamorphose to juveniles in less than 24 h while others of the same cohort may spend several days in each phase before becoming juveniles. On average, however, those oysters that complete metamorphosis successfully do so within the first 1 to 3 d post-settlement. Those oysters that fail to metamorphose may remain in the settler or prodissoconch postlarval phases for many days before dying.

We observed no abnormal development in oysters that survived exposure to either hypoxic or microxic treatments. Abnormal development has been reported in bivalve larvae grown in low oxygen. For instance, Morrison (1971) observed that eggs of Mercenaria mercenaria exposed to 0.34 mg O₂ l⁻¹ (5% of air saturation at 25°C and 28 to 30 ppt) develop to the trocho- phore stage but do not grow shells. Mytilus edulis larvae, developing to the prodissoconch larval stage following 60 h exposure to 0.6 mg O₂ l⁻¹ (1.38 kPa pO₂, 7% of air saturation at 15°C and 31 ppt), do not grow shell (Wang & Widdows 1991). Based on this observation it is suggested that low oxygen may interfere with the shell gland or shell secretion. If low oxygen does interfere with shell growth, and if shell growth and morphogenesis of organs are linked, this factor may have contributed to the lack of dissoconch postlarvae and juveniles in microxic treatments.

Although development was normal, we did observe arrested development after even short-term exposures to low oxygen. Oysters in normoxic conditions passed quickly through the first 3 phases of metamorphosis and juveniles appeared as early as 24 h post-settlement. Following 2 and 3 d exposures to microxia and 3 d exposures to hypoxia development to the juvenile phase was, on average, delayed until several days into the recovery period. Final proportions of juveniles, though, were the same for normoxic and hypoxic treatments, in both the continuous and recovery experiments.

Short-term low oxygen exposures also delay development in other species of bivalves. Wang & Widdows

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Fig. 4. Crassostrea virginica. Log of shell height of dissoconch postlarvae and juveniles following 1, 2 and 3 d exposures to normoxic (7.3 mg O₂ l⁻¹), hypoxic (1.5 mg O₂ l⁻¹) and microxic (<0.07 mg O₂ l⁻¹) treatments. *Elevation of regression significantly lower (p ≤ 0.05) than control; ** elevation significantly lower (p ≤ 0.05) than both hypoxic treatment and control.

Borsa, P., Jousselin, Y., Delay, B. (1992). Relationships between allozymic heterozygosity, body size, and survival to natural anoxic stress in the palourde Ruditapes decussa-

LITERATURE CITED

(1991) report delayed development of Mytilus edulis embryos to prodissococonch larvae during and following 60 h exposures to 0.6 and 1.3 mg O2 1-1 (1.38 kPa PO2, or 7% of air saturation, and 3.16 kPa PO2, or 15% of air saturation, respectively). Veliconch larvae of M. edulis had eyespot development delayed by 1, 2 and more than 6 d when exposed continuously to oxygen treatments of 2.4 (5.91 kPa PO2, 29% of air saturation), 1.3 and 0.6 mg O2 1-1 respectively.

The 2 wk period post-settlement appears to be one of the most critical in the life cycle of the oyster. We found that approximately 50% of the control oysters died by 14 d post-settlement with the majority of the mortality occurring during the second week. Treatments in which oysters were exposed to short-term hypoxia showed similar mortality trends. Initial mortality of Crassostrea virginica in the field is also high. Roegner (1991) reported 73% mortality within a week of settlement. Mortality rates of eastern oysters that survive the first 1 to 2 wk post-settlement, however, are less than 4% per week (Roegner 1991).

All exposures to hypoxia, both short term and continuous, and 1 d exposure to microxia had little effect on the median mortality time or final total mortality, compared to controls. Microxic conditions of longer than 1 d duration, however, did increase mortality. During continuous exposure to microxia, oysters had a median mortality time of 87 h. This time is in good agreement with the median mortality time of 84 h already reported (Baker & Mann 1992). Widdows et al. (1989) reported anoxic median mortality times of 11, 18 and 51 h for oyster prodissococonch, veliconch and pediveliger larvae, and 150 h for juveniles 16 mm in shell height. The median mortality times of post-settlement oysters is consistent with the trend of increasing tolerance of low oxygen conditions with developmental stage and body size. The median mortality times and relative insensitivity of post-settlement oysters to short-term exposures suggest that they, like larvae and adults, are capable of anaerobic metabolism, but only for a limited time.

There was high variation in speed of development and proportion of mortality among replicates both in the continuous and recovery experiments, especially in hypoxic and microxic treatments. Gallager et al. (1986) observed a positive correlation between egg quality, measured as lipid content, and the proportion of Crassostrea virginica and Mercenaria mercenaria larvae completing metamorphosis. Borsa et al. (1992) report a positive relationship between heterozygosity and survival of anoxic stress in the bivalve Ruditapes decussatus. If egg quality and/or heterozygosity of the several cohorts of larvae that we used differed, these factors may, at least partially, explain the variations observed between replicates.

Growth rates of dissoconch postlarvae and juveniles, during recovery from short-term exposures to low oxygen, were not different from the growth rates of control oysters, as shown by the lack of statistical difference between slopes of the regressions. Growth rates were between 11 and 20 μm d-1. Elevations of some of the regressions were significantly different from each other, indicating that growth rates prior to the recovery period varied. For instance, oysters that experienced 2 and 3 d exposures to microxia had lower growth progression elevations during recovery than did the controls. The oysters in microxic treatments, therefore, had slower growth rates during the exposure period.

Short-term exposures to low oxygen appear to have no permanent effect on growth rates of larvae of other bivalve species. Morrison (1971) observed that the growth rates of Mercenaria mercenaria larvae varied directly with dissolved oxygen level but that growth rates became normal when transferred to normoxia following exposures to 1.0 mg O2 1-1 (14% of air saturation) for up to 6 d. Growth rates of Mytilus edulis veliconch larvae equaled control growth rates after 2 d of recovery from 6 d exposures to 0.6 mg O2 1-1 (Wang & Widdows 1991).

Metamorphosis is a stage in the life cycle of bivalves that is rarely studied. This study demonstrates, however, that the 2 wk period following settlement is especially critical to recruitment. Low oxygen conditions, in particular those that are microxic and last longer than 24 h, exacerbate mortality and have detrimental effects on the development and growth of post-settlement oysters. Intrusions of microxic water onto oyster beds, therefore, may limit recruitment into the adult population by slowing development and growth, and by increasing mortality.


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