Journal of Experimental Marine Biology and Ecology 407 (2011) 370–376

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Developmental consequences of association with a photosynthetic substrate for encapsulated embryos of an intertidal gastropod

Daniel A.O. Fernandes, Robert D. Podolsky *

College of Charleston, Grice Marine Laboratory, Charleston, SC 29412, United States

ARTICLE INFO

Article history:
Received 11 July 2011
Received in revised form 27 July 2011
Accepted 28 July 2011
Available online 19 August 2011

Keywords:
Egg mass
Embryo
Gastropod
Intertidal
Macrophyte
Oxygen

ABSTRACT

Aggregation of embryos in clutches that lack internal circulation can increase the risk of hypoxia by limiting gas exchange. As a result, limits on oxygen solubility and diffusion in water can constrain the size and embryo concentration of aquatic egg clutches. Hypoxia in egg masses can slow embryo development, increase mortality, and reduce size at hatching. The risk of hypoxia for embryos, however, can be reduced by association with photosynthetic organisms. We examined whether embryo development in egg ribbons of the cephalaspidean mollusk *Haminoea vesicula* is significantly influenced by oviposition on eelgrass (*Zostera marina*). Association with the photosynthetic substrate had marked effects on development relative to association with non-photosynthetic substrates, and the direction of these effects was mediated by light conditions. Under intermediate and high light levels, association with eelgrass accelerated embryo development, while under dim light, the presence of the macrophyte increased development rate and reduced hatchling shell size. Benefits of association with eelgrass at higher light levels likely result from oxygen production by eelgrass photosynthesis, while we attribute costs under low light to oxygen depletion by eelgrass respiration. Association with *Z. marina* also limited microphyte growth in egg ribbons of *H. vesicula*. In the field, measurements of light attenuation within an eelgrass bed showed that conditions under which benefits accrue to embryos are ecologically relevant and correspond to spatial patterns of oviposition on eelgrass in the field. The choice of a photosynthetic oviposition substrate under appropriate light conditions can improve embryo fitness by accelerating embryo development without compromising hatchling size and by reducing the potential for excessive and harmful fouling by microphytes.

1. Introduction

A major challenge to large multicellular organisms is to provide sufficient oxygen to internal tissues. This challenge is magnified in aquatic habitats, given the relatively low solubility and small diffusion coefficient of molecular oxygen in water (Benson and Krause, 1984). Furthermore, this principle applies to any mass of respiring tissue, including the egg clutches of many fishes, amphibians, and marine invertebrates, which can concentrate thousands of embryos but typically lack a circulatory mechanism for the delivery of oxygen. As a result, the distribution of oxygen in the aquatic environment plays a critical role in regulating embryonic development and in shaping the properties of egg clutches (Cronin and Seymour, 2000; Lardies and Fernandez, 2002; Lee and Strathmann, 1998; Seymour and Bradford, 1995; Strathmann and Chaffee, 1984).

Encapsulation of embryos in clutches has commonly been viewed as a reproductive mode that reduces risks to early development (Pechenik, 1979). Physical and chemical properties of egg masses can protect embryos against desiccation, osmotic change, UV radiation, microbial infection and predation (Benkendorff et al., 2001; Przeslawski, 2004; Rawlings, 1994; Rawlings, 1999). Nevertheless, aggregation of embryos can also increase risks by limiting the exchange of respiratory gases and the elimination of wastes (Booth, 1995; Cohen and Strathmann, 1996; Lardies and Fernandez, 2002; Segura et al., 2010). Theoretical models of the balance between oxygen consumption and diffusion suggest that gelatinous egg masses often approach limits of embryo concentration or mass thickness set by the availability of oxygen to central embryos (Lee and Strathmann, 1998; Moran and Woods, 2010; Strathmann and Strathmann, 1995). Thus, when encapsulated embryos rely on oxygen delivery strictly by diffusion from the surrounding water, the risk of hypoxia can be high (Brante et al., 2008; Cohen and Strathmann, 1996; Moran and Woods, 2007; Pinder and Friet, 1994).

Hypoxia toward the center of an egg mass can slow embryo development, increase mortality, and reduce size at hatching (Cancino et al., 2003; Cohen and Strathmann, 1996; Fernandez et al., 2006; Hassell et al., 2008; Mills and Barnhart, 1999; Shang and Wu, 2004; Wang and Widdows, 1991). However, several recent studies have demonstrated a reduction in hypoxia of central embryos for egg masses associated with photosynthetic organisms. Positive relationships between light and central oxygen level, for example, have been found

* Corresponding author at: Grice Marine Laboratory, 205 Fort Johnson Rd., Charleston, SC 29412, United States. Tel.: +1 843 953 9186; fax: +1 843 953 9199.
E-mail address: podolskyr@cofc.edu (R.D. Podolsky).

0022-0981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2011.07.033

in egg masses of amphibians, gastropods, and polychaetes fouled by microalgae (Cohen and Strathmann, 1996; Pinder and Friet, 1994; Strathmann, 2000). In some cases, photosynthesis by symbiotic microphytes can be the sole source of oxygen for late stage embryos (Pinder and Friet, 1994). Woods and Podolsky (2007) demonstrated higher oxygen levels under elevated light conditions not only for egg masses that contain microphytes (mostly diatoms) but also for those deposited on macrophytes (macroalgae and seagrass). Furthermore, when the latter egg masses were detached from macrophytes, oxygen levels failed to increase under elevated light, suggesting that the presence of the macrophyte may have limited the growth of microphytes (Woods and Podolsky, 2007). This hypothesis, however, has not been tested directly.

Although the contribution of microphytes to oxygen supply in egg masses is known for several taxa, measures of the developmental consequences for embryos have produced mixed results, with some studies showing consistent benefits (Tattersall and Spiegelaar, 2008) and others showing consequences that are condition-specific. For example, Peyton et al. (2004) found that high light conditions produced greater hatching success in egg masses of the gastropod *Haminoea elegans* fouled by microalgae, though only late in the reproductive season. Przeslawski and Benkendorff (2005) found that only 2 of 18 gastropod species they studied had faster development as a result of increased diatom loads. In fact, intense microphyte fouling can have detrimental effects on development and embryo survival (Biermann et al., 1992; Przeslawski and Benkendorff, 2005) by promoting the growth of protists (Przeslawski and Benkendorff, 2005), which can accelerate oxygen depletion (Cancino et al., 2000).

In contrast with studies of association with microphytes, relatively little is known about the developmental consequences of association with macrophytes. Although macrophytes can provide oxygen to central embryos even under well-stirred conditions (Woods and Podolsky, 2007), it is unclear whether such a change in oxygen supply is sufficient to alter embryo development, especially under natural light conditions. Studying the developmental consequences of deposition on macrophytes offers several advantages over studies of microphytes for addressing questions about the role of photosynthesis in metazoan development: association with macrophytes can be more easily manipulated, macrophytes cannot excessively "foul" an egg mass the way that microphytes can, and macrophytes may in fact help to regulate excessive microphyte densities (Woods and Podolsky, 2007).

Given the potential for macrophytes to alter oxygen conditions inside egg masses, selection of an oviposition site could influence embryo development. Some gastropod species, for example, deposit egg masses at locations that reduce exposure to desiccation, ultraviolet radiation, and predation (Benkendorff and Davis, 2004; Biermann et al., 1992; Przeslawski and Davis, 2007; Rawlings, 1999; Russell and Phillips, 2009a; Shimek, 1981). Whether photosynthetic potential also drives choice of oviposition substrate or position is unclear. In a field experiment, von Dassow and Strathmann (2005) found that the gastropod *Haminoea vesicula* favored macrophytes over discarded shells and certain macrophytes over others, but they did not evaluate whether light conditions influenced such choices. Some terrestrial insects prefer to oviposit on plants in high light (Grossmueller and Lederhouse, 1985; Rausher, 1979; Yang, 2006), although in these cases, light is more likely to provide an indirect indication of habitat quality for offspring (Yang, 2006) than a direct benefit in terms of oxygen supply. Given the limits on oxygen solubility and diffusion in aquatic systems, a tighter relationship would be expected between light level and the benefits of oviposition on a photosynthetic substrate. In particular, one would expect such an association to be beneficial above, but harmful below, compensation irradiance (the light level at which macrophyte photosynthetic activity would equal respiration).

We examined the developmental consequences of association with macrophytes for a common opisthobranch gastropod that can deposit egg masses on photosynthetic or non-photosynthetic substrates. This research had three main objectives: (1) to determine the effects of association with macrophytes on embryo development and growth under different natural light conditions, (2) to test the hypothesis that association with macrophytes reduces microphyte fouling, and (3) to measure light intensity at different depths within an eelgrass bed to gauge the ecological relevance of light exposures used in our lab experiments. We hypothesized that oxygen produced by the macrophyte under high light would be sufficient not only to increase oxygen levels inside egg masses but also to benefit embryo growth and development. In contrast, we predicted that under low light, respiration by the plant would worsen problems with oxygen supply and negatively affect development. Therefore, we expected a significant interaction between light level and macrophyte presence in their effects on embryo development and growth.

2. Materials and methods

2.1. Study organism

The cephalaspidean mollusk *H. vesicula* (Gould, 1855) ranges from southern Alaska to the Gulf of California and is abundant on tidal flats and in eelgrass beds around the San Juan Archipelago, WA (Strathmann, 1987). Adults oviposit conspicuous yellow egg ribbons (2–3 mm thick, up to 15 cm length) that are attached along one edge to hard substrates (Strathmann, 1987). Embryology and development of *H. vesicula* were described in detail by Gibson and Chia (1991). Thousands of individually encapsulated embryos are embedded in a gelatinous matrix and hatch as planktotrophic veligers at around 9 days of development at 12–15 °C (Gibson and Chia, 1991). Internal oxygen levels inside egg ribbons can drop to levels of hypoxia at which development is delayed (Cohen and Strathmann, 1996; Strathmann and Strathmann, 1995), and deposition on the eelgrass *Zostera marina* L. can increase oxygen levels at the center of egg ribbons under moderate light (Woods and Podolsky, 2007). Thus, egg ribbons of *H. vesicula* provide a useful system to determine whether photosynthetically-driven changes in oxygen availability have consequences for embryo development.

2.2. Development experiment

We used a factorial design to measure how embryo development and growth rates are influenced by association with eelgrass and by light level. Egg ribbons of *H. vesicula* deposited on the eelgrass *Z. marina* were collected at the mouth of False Bay, San Juan Island, WA. In the lab, ribbons containing embryos no older than 1 day, based on stage of development (i.e., early cleavage to blastula), were gently detached from eelgrass blades. Each egg ribbon ($N = 12$), a maternal half-sibship, was cut into fifteen 0.5 cm long pieces. Pieces were pinned individually into shallow wells on a mesh bottom tray and assigned to one of three treatments: on top of a photosynthetic substrate, on top of a non-photosynthetic substrate, or directly onto the mesh without an intervening substrate. They were then exposed to different levels of shading using natural light in outdoor flow-through tanks. Trays were checked twice daily until hatching was complete in order to measure the time to and size at hatching.

Trays were cut from half-inch thick polystyrene egg crate louver and fitted with chloroform-bonded nylon mesh (80 μm) bottoms to allow for water exchange, pinning of egg ribbon pieces using 000-size stainless steel insect pins, and retention of hatchlings. Each tray ($15 \times 9 \times 1.2$ cm) contained 12 square wells in a 3×4 array. The experiment involved 15 trays to accommodate the 180 egg ribbon pieces. A given tray included, for each of four ribbons, one piece pinned on top of a 2 cm long fresh *Z. marina* blade, one piece pinned onto a similarly sized (2×1 cm) rectangular strip of pre-soaked 0.45 μm Millipore® filter (Millipore Corporation, Billerica, MA) as a non-photosynthetic substrate, and one piece pinned directly onto the

mesh, all distributed randomly within the tray. The filter treatment was a control for any effects of the physical structure of the eelgrass.

The same setup for a given set of four ribbons was repeated on five trays in each of three outdoor tanks. Each of the five trays per tank was held at one of five light levels (100%, 75%, 50%, 25% and 0% treatments) by covering the tray with a clear Mylar® transparency sheet that had been printed with a particular gray level using a black-and-white laser printer (Xerox Phaser 4510 DT). The Mylar® sheet was supported on top of the tray by a clear Plexiglas® plate. The values of percent light of the different shading treatments are relative to the amount of light transmitted through the Plexiglas® and a clear Mylar® sheet. The Plexiglas® plates in the 0% sunlight treatment were covered by a piece of opaque black plastic. The shading level created by the laser printer that would block each percentage of light was calibrated by measuring light transmittance through Mylar® and Plexiglas® using a spectrophotometer (Model 1600, Shimadzu). These recordings showed that (1) clear Mylar® does not transmit wavelengths below about 420 nm, (2) the Mylar® and the laser printed gray patterns did not change the spectral qualities of the transmitted light, and (3) clear Mylar® and Plexiglas® together cut out about 13% of ambient light. Thus, the actual amounts of transmitted ambient sunlight in the five light treatments were: 87.4% (100% treatment), 65.5% (75% treatment), 43.7% (50% treatment), 21.8% (25% treatment), and 0% (0% treatment). For clarity, we refer to the five light treatments (100%, 75%, 50%, 25% and 0%) rather than the absolute percentage of ambient light when reporting results.

The 15 mesh-bottom plastic trays were placed in outdoor flow-through tanks with strong flow on top of plastic louver platforms to allow water circulation. Over the course of the experiment, seawater temperatures in the tanks averaged 14.4 ± 2.08 °C (mean ± SD) and temperatures did not differ systematically among treatments. Water level was adjusted to be just above mid-height of each well to keep the egg ribbon pieces fully submerged without risk of losing hatchlings. The tank was exposed during the day to natural light but was covered at night for protection.

Embryos were checked for hatching every 12 h. Newly hatched veligers were photographed on a stereo dissecting microscope (Olympus SZ-60) fitted with a digital camera (Nikon Coolpix 4500) as they were trapped on their sides in the surface tension of water. Time to hatching was recorded as the median time when 50% of the embryos in a given egg ribbon piece had hatched from capsules and was converted to its inverse, development rate ($days^{-1}$). The maximum length of the larval shell was measured from the captured digital images using image analysis software (ImageJ 1.43u, NIH, USA).

2.3. Measurements of chlorophyll content

In order to estimate the effect of the treatments used in the development experiment on fouling microphytes, we used the same collection procedure, experimental design and setup for a separate set of egg ribbons ($N = 16$). The amount of chlorophyll-*a* extracted from egg ribbons after several days of exposure was used as a measure of the photosynthetic potential of microphytes, which could reflect changes in the number of photosynthetic cells or chlorophyll content per cell (Janssen et al., 2001; Valladares and Niinemets, 2008). For 8 of the egg ribbons, we used 2×1 cm pieces of 0.45 μm Millipore® filter (Millipore Corporation, Billerica, MA) as the non-photosynthetic substrate, as in the previous experiment, while for the other 8 we used 2×1 cm pieces of Parafilm® paraffin film (Picheney Plastic Packaging Company, Chicago IL). The egg ribbon pieces were collected for chlorophyll measurement on day 6 of development, prior to any hatching. The pieces were blotted, massed to the nearest mg, transferred to darkened 1.5 mL polypropylene tubes and frozen at −80 °C for subsequent analysis.

For chlorophyll extraction (Parsons et al., 1984), egg ribbon pieces were thawed and one hundred percent acetone was added to each to adjust the final concentration of acetone to 90% v/v, assuming that egg ribbons were 100% water. Each piece was then sonicated briefly (approx. 5 s) on ice using a Branson Sonifier 250 (Danbury, CT). Tubes were capped, wrapped in aluminum foil, and left to extract in the dark at −20 °C for 24 h. Samples were centrifuged (IEC Centra CL3R refrigerated centrifuge, Thermo IEC, Needman Heights, MA, USA) at −9 °C at 4000 rpm for 4 min. The supernatant was removed and transferred to culture tubes, and additional 90% acetone was added to each tube to bring the volume to 4 mL. Chlorophyll content of samples was measured for *chl-a* in $\mu g\ L^{-1}$ in a fluorometer (Turner Biosystems TD700, Sunnyvale, CA). Concentration was then converted to $\mu g\ chl\text{-}a \cdot g^{-1}$ egg ribbon.

2.4. Field measurements of light levels

To estimate light exposure in the field for egg ribbons of *H. vesicula*, we measured the relative light illuminance inside a bed of *Z. marina* where the experimental egg masses had been collected. We deployed two HOBO® pendant light data loggers (Onset Computer Corporation, Cape Cod, MA) at each of four heights at the center of the eelgrass bed. Each logger was attached to the top of a floating foam disk (7.5×6.5 cm) tethered to a 5 kg dive weight by nylon cord. To choose positions within the eelgrass bed, we measured the average length of eelgrass blades (130 ± 10 cm, mean ± SD, $N = 15$) from the sediment to the tip of the blades and used lengths of nylon cord to position the loggers at the top (130 cm), mid-height (65 cm) or bottom (5 cm) of the eelgrass bed. To measure light illuminance at the water surface, a fourth logger was tethered by a four-meter-long nylon cord which was long enough to reach the surface at the highest tide. The light loggers took a reading each minute and remained in the field for 96 h during a spring tide series between August 8 and 11, 2010. The data from the loggers were processed using HOBOware Pro software (version 3.0.0, Onset Computer Corporation).

2.5. Data analysis

For the development experiment, we used a linear mixed model analysis in SPSS (ver. 17.0, SPSS, Inc.), with light level and substrate type as fixed effects and with tray and egg ribbon ID nested within tray as random effects. Tray was removed as a factor from the model as its removal yielded better information criteria scores (AIC and BIC, Burnham and Anderson, 2002). Separate analyses were performed using development rate ($days^{-1}$) and shell length (μm) as dependent variables. We were especially interested in the interaction between light level and eelgrass association given our prediction that eelgrass would have a net positive effect on embryo development under high light (as a net oxygen source from photosynthesis) but a negative net effect under low light levels (as an oxygen sink through respiration). To examine the nature of the interaction, we carried out planned contrasts between the data from the eelgrass treatment and those from the non-photosynthetic treatments. Post-hoc multiple comparison tests were performed using Fisher's least significant difference (LSD) when significant differences were found in main effects. Alpha levels were adjusted using Holm (1979) sequential Bonferroni method.

For the chlorophyll measurements, the data were square root transformed to meet assumptions of normality. We then performed a linear mixed model analysis, similar to that used in the development experiment, with the transformed chlorophyll-*a* concentration values ($\mu g\ chl\text{-}a \cdot g^{-1}$ egg ribbon) as the dependent variable.

A model II (reduced major axis) regression was used to provide estimates of light attenuation in the eelgrass bed. The relative illuminance at the three different heights within the eelgrass bed was estimated from the slope of the regression between the light illuminance at each height and the light illuminance at the water surface.

3. Results

3.1. Development experiment

Development rate for embryos of *H. vesicula* (Fig. 1) was significantly affected by substrate type ($F_{2,154}=5.212$, $P=0.006$), light level ($F_{4,154}=45.839$, $P<0.001$) and their interaction ($F_{8,154}=9.541$, $P<0.001$). No significant differences were detected between the filter and mesh treatments.

The planned contrasts showed that embryos attached to eelgrass, when compared with those in non-photosynthetic treatments, developed significantly faster under moderate and high light levels (50% treatment: $F_{1,22}=19.880$, $P<0.001$; 75% treatment: $F_{1,22}=28.654$, $P<0.001$; 100% treatment: $F_{1,22}=81.149$, $P<0.001$). However, at 0% light, the attachment to eelgrass significantly delayed embryo development relative to the non-photosynthetic treatments ($F_{1,22}=22.997$, $P<0.001$). In the 25% treatment, there was no significant effect of substrate type on developmental rate ($F_{1,22}=1.834$, $P=0.189$).

The effects of light on development rate followed a different pattern for the different substrate types. On the photosynthetic substrate, moderate light conditions (25% to 75% treatment) accelerated development of embryos relative to both high (100% treatment) and low (0% treatment) light levels. In both of the non-photosynthetic treatments, embryo development rate steadily decreased as light availability increased (above the 25% treatment).

Larval shell size of *H. vesicula* at hatching (Fig. 2) was significantly affected by substrate type ($F_{2,154}=13.305$, $P<0.001$), light treatment ($F_{4,154}=20.044$, $P<0.001$), and their interaction ($F_{8,154}=2.742$, $P=0.007$). No significant difference was found between the filter and mesh treatments. The planned contrast revealed that embryos attached to eelgrass, when compared with those in the non-photosynthetic treatments, hatched at significantly smaller sizes under low and intermediate light conditions (0% treatment: $F_{1,22}=20.706$, $P<0.001$; 25% treatment: $F_{1,22}=11.763$, $P=0.002$; 50% treatment: $F_{1,22}=10.492$, $P<0.001$). Larval size was not significantly affected by substrate type under high light (75% and 100% treatment). The effect of light on larval shell size was similar across all substrate types: hatchlings had significantly larger shells under intermediate and high light levels (50% to 100% treatment) compared with lower light levels.

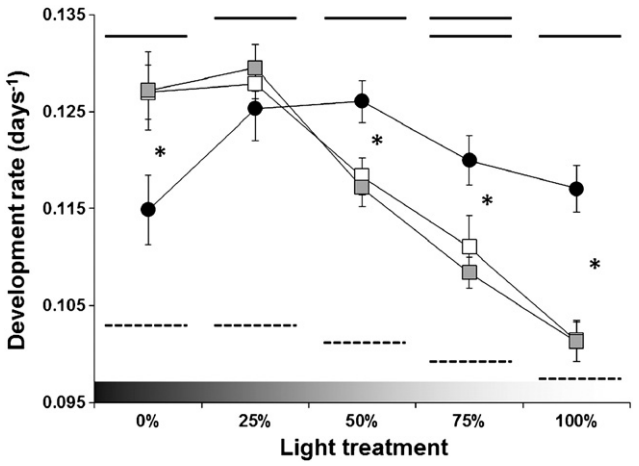

Fig. 1. Development rate (days to hatching^{-1}) of *Haminoea vesicula* embryos. Pieces of egg ribbon were pinned to the eelgrass *Zostera marina* (black circles), to Millipore® filter (gray squares) or directly to nylon mesh (white squares) and reared under different levels of shading. Each value represents mean ± SE for 12 replicates. The gradient bar is used as a visual aid for the light treatments. Asterisks (*) denote significant differences ($\alpha=0.05$) between the photosynthetic and the combined non-photosynthetic substrates at each light level. Above and below the data, lines indicate pairwise comparisons among light levels within the photosynthetic (solid) and the combined non-photosynthetic (dashed) substrates. Light treatments with lines at the same level do not differ significantly ($\alpha=0.05$).

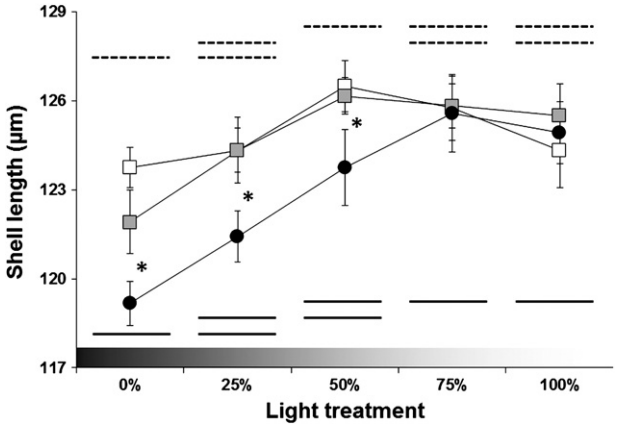

Fig. 2. Shell length of newly hatched larvae of *Haminoea vesicula*. Other details as in Fig. 1.

3.2. Measurements of chlorophyll content

Data on chlorophyll (*chl-a*) concentrations were similar between trials that used Millipore® filter (trial A) or Parafilm® (trial B) as the non-photosynthetic substrate (Fig. 3a, b). The amount of chlorophyll in egg ribbon pieces of *H. vesicula* was significantly affected by substrate type (trial A: $F_{2,98}=22.001$, $P<0.001$; trial B: $F_{2,98}=25.181$, $P<0.001$) and light treatment (trial A: $F_{4,98}=13.329$, $P<0.001$; trial B: $F_{4,98}=23.722$, $P<0.001$), but the interaction between substrate type and light was not significant (trial A: $F_{8,98}=1.385$, $P=0.213$; trial B: $F_{8,98}=1.195$, $P=0.310$). No significant difference in chlorophyll concentration was observed between egg ribbon pieces attached to the non-photosynthetic substrate and the mesh.

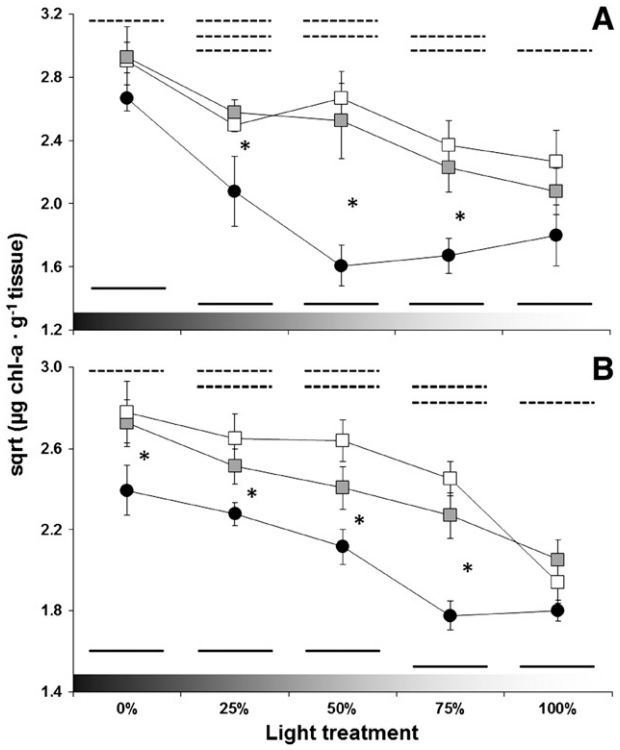

Fig. 3. Chlorophyll-*a* content per gram of tissue of *Haminoea vesicula* egg ribbons. Pieces of egg ribbon were pinned to the eelgrass *Zostera marina* (black circles), to Millipore® filter (trial A, gray squares), to Parafilm® (trial B, gray squares) or directly to nylon mesh (white squares) and reared under different levels of shading. Each value represents mean ± SE for 8 replicates. Other details as in Fig. 1.

In general, we observed an inverse relationship between light level and chlorophyll content across all substrate treatments, although the exact light level at which this drop occurred differed between substrate types and the two trials. The planned contrast revealed that, when compared with the non-photosynthetic treatments, the association with eelgrass reduced chlorophyll concentration in egg ribbons under intermediate light conditions (trial A — 25% treatment: $F_{1,21} = 4.764$, $P = 0.040$; 50% treatment: $F_{1,21} = 10.697$, $P = 0.004$; 75% treatment: $F_{1,21} = 7.792$, $P = 0.010$; trial B — 25% treatment: $F_{1,21} = 5.204$, $P = 0.033$; 50% treatment: $F_{1,21} = 10.410$, $P = 0.004$; 75% treatment: $F_{1,21} = 14.728$, $P < 0.001$). The main difference between the two trials was that chlorophyll concentrations at 0% light were significantly lower on eelgrass only in the Parafilm® trial ($F_{1,21} = 4.981$, $P = 0.036$).

3.3. Field measurements of light levels

From the slopes of the reduced major axis regressions, we estimated the reduction of light for egg ribbons positioned in the eelgrass bed relative to light at the water surface (Fig. 4). An egg mass deposited at the distal end of a *Z. marina* blade at the top of the bed would experience an illumination level around 68.0% of light at the water surface (corresponding to a light level between the 100% and 75% laboratory treatments). This relative light exposure would drop to 40.2% for egg ribbons attached at an intermediate height on an eelgrass blade and to 3.3% for egg ribbons attached near the bottom of the eelgrass bed.

4. Discussion

For marine organisms that oviposit rather than spawn, choice of oviposition substrate can have important consequences for reproductive success. Our results show that deposition of an egg mass on a photosynthetic substrate can influence embryonic development, and that the direction of such effects is mediated by light level. We found that association with the eelgrass *Z. marina* significantly affected embryo development rate, larval hatching size and the degree of microphyte fouling of attached egg masses of *H. vesicula*. At high light, attached embryos benefited from the association, whereas, under low light, embryos suffered a cost. Moreover, under all light treatments, the effect of a physically similar but non-photosynthetic substrate was no different from the effect of pinning the egg mass directly to mesh, further supporting the conclusion that changes in gastropod development were driven by the photosynthetic properties of the macrophyte rather than by physical effects of eelgrass (on water flux, for example). These results are the first to demonstrate that association with macrophytes under ecologically relevant light conditions can provide a measurable benefit to the development of metazoan embryos.

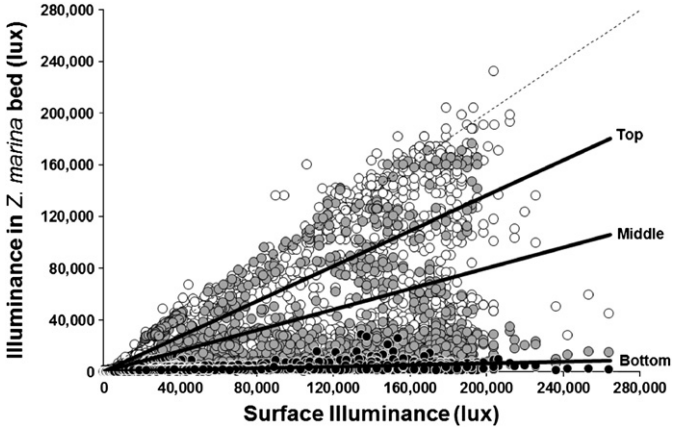

Fig. 4. Illuminance in a *Zostera marina* bed at the top (130 cm above the substrate; white circles), middle (65 cm; light gray circles) and bottom (5 cm; black circles) of the eelgrass canopy as a function of illuminance at the water surface. The drop in illuminance between the top of eelgrass and at the water surface represents the effect of light absorption in the water above the eelgrass canopy. Reduced major axis regression slopes: top = 0.681, middle = 0.402, and bottom = 0.033. Dotted line denotes isoline.

At intermediate and high light levels, the presence of eelgrass accelerated embryo development relative to the non-photosynthetic treatments by about 10%. This increase in development rate is likely related to the increase in oxygen availability provided by eelgrass photosynthesis (Woods and Podolsky 2007). In contrast, under dark conditions, we observed delayed development in egg masses attached to *Z. marina*, likely because the balance between photosynthesis and respiration results in eelgrass becoming a net oxygen sink (Ochieng et al., 2010). This positive relationship between oxygen availability and development rate is consistent with results from other gastropod species (Booth, 1995; Brante et al., 2009; Cancino et al., 2003; Chan et al., 2008; Fernandez et al., 2006; Strathmann and Strathmann, 1995). An alternative hypothesis is that some other light sensitive aspect of the physiology of macrophytes or associated organisms (e.g., biofilms, epibionts) is driving these patterns. For example, chemical signals associated with eelgrass photosynthesis could induce changes in gastropod development time as seen in with other external signals (Miner et al., 2010; Strathmann et al., 2010).

Our observation that development rate declined in the 100% light treatment is consistent with an expected decline in oxygen production in response to higher light intensities. Such a reduction in photosynthetic efficiency, termed photoinhibition, plays a protective role in dissipating excess light energy as heat (Touchette and Burkholder, 2000). Photoinhibition has been observed to peak in intertidal *Z. marina* at low tides in early afternoon (Hanelt, 1992; Touchette and Burkholder, 2000). In contrast, we observed that, as light levels increased, development rate of embryos on the non-photosynthetic treatments decreased. This effect on development must have been due to some other negative effect of light, such as a change in the contribution of oxygen by microphytes, a hypothesis that we address below. Regardless of which factors contribute to the particular shapes of these relationships, the crossing interaction between effects of light and oviposition substrate on development rate supports the hypothesis that the cost or benefit of association is context-dependent.

The interaction between light and oviposition substrate was also significant for hatchling size, but a difference between substrates was apparent only at low light levels, where hatchlings on eelgrass emerged with smaller shells than those in the non-photosynthetic treatments. It is possible that eelgrass reduced absolute growth rates at low light levels, resulting in smaller hatchlings, by acting as a net oxygen sink. This interpretation is backed by previous demonstrations of a negative relationship between hypoxia and gastropod shell length at hatching (Brante et al., 2009; Cancino et al., 2003; Chan et al., 2008; Strathmann and Strathmann, 1995). In addition, low levels of intracapsular oxygen can lower pH and impair shell calcification (Cancino et al., 2003). It is less clear why the reverse was not true at higher light levels, where we expected embryos on eelgrass to show a growth advantage. Because embryos also hatched earlier at high light, it is possible that any growth advantage was offset by the shorter intracapsular period, a tradeoff seen in diverse systems (Li, 2002; Warkentin, 1995; Yaro et al., 2006). The fact that embryos on eelgrass achieved similar sizes to those off eelgrass despite shorter development times suggests that one advantage of association with the macrophyte could be the ability to maintain a faster growth rate. A final possibility is that embryo size in both treatments reached an optimum for hatching, with growth in non-photosynthetic treatments aided by the greater photosynthetic contribution of microphytes (see below).

Whatever the mechanism, our results suggest that oviposition on a photosynthetic substrate can ultimately influence offspring fitness positively or negatively depending on light conditions. Embryo

development rate in the intertidal, for example, determines time of exposure to benthic predation and to several physical stressors (Deschaseaux et al., 2011; Gosselin and Chia, 1995; Pechenik, 1999; Podolsky, 2003; Russell and Phillips, 2009a; Russell and Phillips, 2009b; Spight, 1975). Likewise, offspring size can directly impact survival (Moran, 1999; Moran and Emlet, 2001; Spight, 1976), especially for hatchlings that enter the plankton, where size can affect vulnerability to predation (Allen, 2008; Pechenik and Levine, 2007), duration of planktonic period, growth rate, and time to competency and settlement (reviewed by Marshall and Keough, 2008).

Our data on chlorophyll content of egg masses provide direct support for Woods and Podolsky (2007) hypothesis that the presence of macrophytes can reduce the contribution of microphytes to oxygen supply for attached embryos. This hypothesis emerged from interspecific comparisons (Peyton et al., 2004; Woods and Podolsky, 2007) showing that the egg masses of species that oviposit on macrophytes tended to have lower chlorophyll concentrations than those ovipositing off macrophytes. A possible mechanism for this pattern involves the release of antifouling compounds by macrophytes – such as Zosteric acid, a sulphated phenolic acid produced by *Z. marina* – that could directly inhibit diatom growth (Callow and Callow, 1998; Todd et al., 1993). In addition, the demonstration of endogenous antifouling compounds against pathogenic (Benkendorff et al., 2001) and biofilm (Ramasamy and Murugan, 2005; 2007) bacteria in gastropod egg masses suggests that endogenous compounds could similarly modify the growth of microphytes, although such an effect has not been demonstrated. This apparent inhibition of microphyte growth could be even more general if conditions are made less favorable for microphyte growth simply by the change in respiratory gases local to the eelgrass blade (Pinckney and Micheli, 1998). Regardless of the mechanism, our results suggest that macrophytes can provide an ancillary benefit by controlling the kind of excessive fouling of egg masses (Przeslawski and Benkendorff, 2005) that has been shown to deplete oxygen, delay development, and increase mortality (Biermann et al., 1992; Cancino et al., 2000; Cohen and Strathmann, 1996).

While chlorophyll content was significantly lower in macrophyte-associated egg masses, it was also inversely related to light level. This inverse relationship is consistent with a common compensatory response of photosynthetic cells to increase chlorophyll content under low light conditions (Janssen et al., 2001; Valladares and Niinemets, 2008). Given that microphytes contribute a negligible amount of the photosynthesis-generated oxygen inside egg masses of *H. vesicula* that are deposited on eelgrass (Woods and Podolsky, 2007), it seems unlikely that microphytes played much of a role in driving patterns we observed for egg masses associated with eelgrass. For egg masses on the non-photosynthetic substrates, it is worth noting that chlorophyll content shows a direct relationship with development rate, but an inverse relationship with hatching shell size. As described earlier, these relationships could simply reflect a tradeoff between intracapsular growth period and final hatching size, with low light conditions favoring a shift toward shorter growth periods at the expense of hatching size. Nevertheless, the contribution of microphytes to these relationships in a species that preferentially lays on macrophytes (von Dassow and Strathmann, 2005) remains poorly understood.

Our measurements of light within eelgrass beds demonstrate that choice of oviposition site along an eelgrass blade could alter the light level, and therefore oxygen availability, experienced by embryos. The top half of the eelgrass bed was illuminated on average by 40% to 70% of the light available at the surface, a light level that matches those that were most beneficial to embryonic development in our experiments. Egg masses attached to eelgrass under light conditions between 44% and 66% of ambient light produced embryos that hatched faster and at larger sizes and also exhibited reduced fouling by microphytes. Thus, oviposition toward the distal end of a *Z. marina* blade would provide an advantage to embryonic development, whereas oviposition deeper in the bed would result in a cost. In addition, a more distal position can reduce benthic predation (R. Strathmann, unpubl. data) but also increase exposure to potentially damaging ultraviolet radiation (Biermann et al., 1992; Przeslawski et al., 2004), a factor that we eliminated from our experiments. Notably, at least five gastropods species that commonly oviposit on seagrasses – *H. elegans* and *H. antillarum* (Peyton et al., 2004), *H. vesicula*, *Lacuna vincta* and *L. variegata* (D. Fernandes, pers. obs.) – preferentially attach their egg masses to the distal half of blades.

We have shown that gastropod embryos can benefit or suffer costs from association with macrophytes, depending on oviposition conditions. The presence of the eelgrass *Z. marina* can significantly affect embryo development rate, larval hatching size and microphyte fouling on attached *H. vesicula* egg masses, and lighting conditions at which these shifts occur are ecologically relevant. It remains unclear how much adult oviposition behavior is influenced by these potential costs and benefits. Future studies addressing oviposition substrate choice and variation in maternal investment under different light conditions can expand our understanding of the selective advantages of the association between metazoan embryos and photosynthetic organisms.

Acknowledgments

We thank the director and staff of the Friday Harbor Laboratories for providing research facilities and logistical support. We also thank S. Smoot and S. Kacenas for assistance in carrying out experiments and A. Welch, E. Sotka, L. Burnett, C. Biermann, and an anonymous reviewer for comments. Financial support was provided by a Friday Harbor Laboratories Alan J. Kohn Fellowship to DAOF and NSF award OCE-0621467 to RDP. **[SS]**

References

Allen, J.D., 2008. Size-specific predation on marine invertebrate larvae. Biol. Bull. 214 (1), 42–49.

Benkendorff, K., Davis, A.R., 2004. Gastropod egg mass deposition on a temperate, wave-exposed coastline in New South Wales, Australia: implications for intertidal conservation. Aquat. Conserv. Mar. Freshw. Ecosyst. 14 (3), 263–280.

Benkendorff, K., Davis, A.R., Bremner, J., 2001. Chemical defense in the egg masses of benthic invertebrates: an assessment of antibacterial activity in 39 mollusks and 4 polychaetes. J. Invertebr. Pathol. 78 (2), 109–118.

Benson, B.B., Krause Jr., D., 1984. The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with the atmosphere. Limnol. Oceanogr. 29 (3), 620–632.

Biermann, C.H., Schinner, G.O., Strathmann, R.R., 1992. Influence of solar-radiation, microalgal fouling, and current on deposition site and survival of embryos of a dorid nudibranch gastropod. Mar. Ecol. Prog. Ser. 86 (3), 205–215.

Booth, D.T., 1995. Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. J. Exp. Biol. 198 (1), 241–247.

Brante, A., Fernandez, M., Viard, F., 2008. Effect of oxygen conditions on intracapsular development in two calyptraeid species with different modes of larval development. Mar. Ecol. Prog. Ser. 368, 197–207.

Brante, A., Fernandez, M., Viard, F., 2009. Limiting factors to encapsulation: the combined effects of dissolved protein and oxygen availability on embryonic growth and survival of species with contrasting feeding strategies. J. Exp. Biol. 212 (14), 2287–2295.

Burnham, K.P., Anderson, D.R., 2002. Model Selection and Multimodel Inference: a Practical Information–Theoretic Approach, 2nd ed. Springer-Verlag, New York.

Callow, M.E., Callow, J.A., 1998. Attachment of zoospores of the fouling alga *Enteromorpha* in the presence of zosteric acid. Biofouling 13 (2), 87–95.

Cancino, J.M., Gallardo, J.A., Torres, F., Leiva, G., Navarro, J.M., 2000. Effects of sessile Protozoa on intracapsular oxygen tension and embryonic shell calcification in the muricid *Chorus giganteus*. Mar. Ecol. Prog. Ser. 200, 141–148.

Cancino, J.M., Gallardo, J.A., Torres, F.A., 2003. Combined effects of dissolved oxygen concentration and water temperature on embryonic development and larval shell secretion in the marine snail *Chorus giganteus* (Gastropoda: Muricidae). Mar. Biol. 142 (1), 133–139.

Chan, H.Y., Xu, W.Z., Shin, P.K.S., Cheung, S.G., 2008. Prolonged exposure to low dissolved oxygen affects early development and swimming behaviour in the gastropod *Nassarius festivus* (Nassariidae). Mar. Biol. 153 (4), 735–743.

Cohen, C.S., Strathmann, R.R., 1996. Embryos at the edge of tolerance: effects of environment and structure of egg masses on supply of oxygen to embryos. Biol. Bull. 190 (1), 8–15.

Cronin, E.R., Seymour, R.S., 2000. Respiration of the eggs of the giant cuttlefish *Sepia apama*. Mar. Biol. 136 (5), 863–870.

Deschaseaux, E., Taylor, A., Maher, W., 2011. Measure of stress response induced by temperature and salinity changes on hatched larvae of three marine gastropod species. J. Exp. Mar. Biol. Ecol. 397 (2), 121–128.
Fernandez, M., Pappalardo, P., Jeno, K., 2006. The effects of temperature and oxygen availability on intracapsular development of *Acanthina monodon* (Gastropoda: Muricidae). Rev. Chil. Hist. Nat. 79 (2), 155–167.
Gibson, G.D., Chia, F.S., 1991. Contrasting reproductive modes in 2 sympatric species of *Haminaea* (Opisthobranchia, Cephalaspidea). J. Molluscan Stud. 57, 49–60.
Gosselin, L.A., Chia, F.S., 1995. Characterizing temperate rocky shores from the perspective of an early juvenile snail — the main threats to survival of newly-hatched *Nucella emarginata*. Mar. Biol. 122 (4), 625–635.
Grossmueller, D.W., Lederhouse, R.C., 1985. Oviposition site selection: an aid to rapid growth and development in the tiger swallowtail butterfly, *Papilio glaucus*. Oecologia 66 (1), 68–73.
Hanelt, D., 1992. Photoinhibition of photosynthesis in marine macrophytes of the South China Sea. Mar. Ecol. Prog. Ser. 82 (2), 199–206.
Hassell, K.L., Coutin, P.C., Nugegoda, D., 2008. Hypoxia impairs embryo development and survival in black bream (*Acanthopagrus butcheri*). Mar. Pollut. Bull. 57 (6–12), 302–306.
Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6, 65–70.
Janssen, M., Lars, B., Marquardt, J., Krumbein, W.E., Rhiel, E., 2001. Changes in the photosynthetic apparatus of diatoms in response to low and high light intensities. Int. Microbiol. 4 (1), 27–33.
Lardies, M.A., Fernandez, M., 2002. Effect of oxygen availability in determining clutch size in *Acanthina monodon*. Mar. Ecol. Prog. Ser. 239, 139–146.
Lee, C.E., Strathmann, R.R., 1998. Scaling of gelatinous clutches: effects of siblings' competition for oxygen on clutch size and parental investment per offspring. Am. Nat. 151 (4), 293–310.
Li, D.Q., 2002. Hatching responses of subsocial spitting spiders to predation risk. Proc. R. Soc. B Biol. Sci. 269 (1505), 2155–2161.
Marshall, D.J., Keough, M.J., 2008. The evolutionary ecology of offspring size in marine invertebrates. Adv. Mar. Biol. 53, 1–60.
Mills, N.E., Barnhart, M.C., 1999. Effects of hypoxia on embryonic development in two *Ambystoma* and two *Rana* species. Physiol. Biochem. Zool. 72 (2), 179–188.
Miner, B.G., Donovan, D.A., Andrews, K.E., 2010. Should I stay or should I go: predator- and conspecific-induced hatching in a marine snail. Oecologia 163 (1), 69–78.
Moran, A.L., 1999. Size and performance of juvenile marine invertebrates: potential contrasts between intertidal and subtidal benthic habitats. Am. Zool. 39 (2), 304–312.
Moran, A.L., Emlet, R.B., 2001. Offspring size and performance in variable environments: field studies on a marine snail. Ecology 82 (6), 1597–1612.
Moran, A.L., Woods, H.A., 2007. Oxygen in egg masses: interactive effects of temperature, age, and egg-mass morphology on oxygen supply to embryos. J. Exp. Biol. 210 (4), 722–731.
Moran, A.L., Woods, H.A., 2010. Limits to diffusive O_2 transport: flow, form, and function in nudibranch egg masses from temperate and polar regions. PLoS One 5 (8), e12113.
Ochieng, C.A., Short, F.T., Walker, D.I., 2010. Photosynthetic and morphological responses of eelgrass (*Zostera marina* L.) to a gradient of light conditions. J. Exp. Mar. Biol. Ecol. 382 (2), 117–124.
Parsons, T.R., M., Y., L., C.M., 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford.
Pechenik, J.A., 1979. Role of encapsulation in invertebrate life histories. Am. Nat. 114 (6), 859–870.
Pechenik, J.A., 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. Mar. Ecol. Prog. Ser. 177, 269–297.
Pechenik, J.A., Levine, S.H., 2007. Estimates of planktonic larval mortality using the marine gastropods *Crepidula fornicata* and *C. plana*. Mar. Ecol. Prog. Ser. 344, 107–118.
Peyton, K.A., Hanisak, M.D., Lin, J.D., 2004. Marine algal symbionts benefit benthic invertebrate embryos deposited in gelatinous egg masses. J. Exp. Mar. Biol. Ecol. 307 (2), 139–164.
Pinckney, J.L., Micheli, F., 1998. Microalgae on seagrass mimics: does epiphyte community structure differ from live seagrasses? J. Exp. Mar. Biol. Ecol. 221 (1), 59–70.
Pinder, A.W., Friet, S.C., 1994. Oxygen-transport in egg masses of the amphibians *Rana sylvatica* and *Ambystoma maculatum* — Convection, diffusion and oxygen production by algae. J. Exp. Biol. 197, 17–30.
Podolsky, R.D., 2003. Integrating development and environment to model reproductive performance in natural populations of an intertidal gastropod. Integr. Comp. Biol. 43 (3), 450–458.
Przeslawski, R., 2004. A review of the effects of environmental stress on embryonic development within intertidal gastropod egg masses. Molluscan Res. 24 (1), 43–63.
Przeslawski, R., Benkendorff, K., 2005. The role of surface fouling in the development of encapsulated gastropod embryos. J. Molluscan Stud. 71, 75–83.
Przeslawski, R., Davis, A.R., 2007. Does spawning behavior minimize exposure to environmental stressors for encapsulated gastropod embryos on rocky shores? Mar. Biol. 152 (4), 991–1002.
Przeslawski, R., Davis, A.R., Benkendorff, K., 2004. Effects of ultraviolet radiation and visible light on the development of encapsulated molluscan embryos. Mar. Ecol. Prog. Ser. 268, 151–160.
Ramasamy, M.S., Murugan, A., 2005. Potential antimicrobial activity of marine molluscs from tuticorin, southeast coast of India against 40 biofilm bacteria. J. Shellfish. Res. 24 (1), 243–249.
Ramasamy, M.S., Murugan, A., 2007. Fouling deterrent chemical defence in three muricid gastropod egg masses from the Southeast coast of India. Biofouling 23 (4), 259–265.
Rausher, M.D., 1979. Larval habitat suitability and oviposition preference in three related butterflies. Ecology 60 (3), 503–511.
Rawlings, T.A., 1994. Encapsulation of eggs by marine gastropods — effect of variation in capsule form on the vulnerability of embryos to predation. Evolution 48 (4), 1301–1313.
Rawlings, T.A., 1999. Adaptations to physical stresses in the intertidal zone: the egg capsules of neogastropod molluscs. Am. Zool. 39 (2), 230–243.
Russell, J., Phillips, N.E., 2009a. Synergistic effects of ultraviolet radiation and conditions at low tide on egg masses of limpets (*Benhamina obliquata* and *Siphonaria australis*) in New Zealand. Mar. Biol. 156 (4), 579–587.
Russell, J., Phillips, N., 2009b. Species-specific vulnerability of benthic marine embryos of congeneric snails (*Haminoea* spp.) to ultraviolet radiation and other intertidal stressors. Biol. Bull. 217 (1), 65–72.
Segura, C.J., Chaparro, O.R., Paschke, K.A., Pechenik, J.A., 2010. Capsule walls as barriers to oxygen availability: implications for the development of brooded embryos by the estuarine gastropod *Crepipatella dilatata* (Calyptraeidae). J. Exp. Mar. Biol. Ecol. 390 (1), 49–57.
Seymour, R.S., Bradford, D.F., 1995. Respiration of amphibian eggs. Physiol. Zool. 68 (1), 1–25.
Shang, E.H.H., Wu, R.S.S., 2004. Aquatic hypoxia is a teratogen and affects fish embryonic development. Environ. Sci. Technol. 38 (18), 4763–4767.
Shimek, R.L., 1981. *Neptunea pribiloffensis* (Dall, 1919) and *Tealia crassicornis* (Müller, 1776), on a snail's use of babysitters. Veliger 24, 62–66.
Spight, T.M., 1975. Factors extending gastropod embryonic development and their selective cost. Oecologia 21 (1), 1–16.
Spight, T.M., 1976. Ecology of hatching size for marine snails. Oecologia 24 (4), 283–294.
Strathmann, M.F., 1987. Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast. University of Washington Press, Seattle. 670 pp.
Strathmann, R.R., 2000. Form, function, and embryonic migration in large gelatinous egg masses of arenicolid worms. Invertebr. Biol. 119 (3), 319–328.
Strathmann, R.R., Chaffee, C., 1984. Constraints on eggs masses. II. Effects of spacing size and number of eggs on ventilation of masses of embryos in jelly, adherent group, or thinwalled capsules. J. Exp. Mar. Biol. Ecol. 84, 85–93.
Strathmann, R.R., Strathmann, M.F., 1995. Oxygen-supply and limits on aggregation of embryos. J. Mar. Biol. Assoc. U. K. 75 (2), 413–428.
Strathmann, R.R., Strathmann, M.F., Ruiz-Jones, G., Hadfield, M.G., 2010. Effect of plasticity in hatching on duration as a precompetent swimming larva in the nudibranch *Phestilla sibogae*. Invertebr. Biol. 129 (4), 309–318.
Tattersall, G.J., Spiegelaar, N., 2008. Embryonic motility and hatching success of *Ambystoma maculatum* are influenced by a symbiotic alga. Can. J. Zool. 86 (11), 1289–1298.
Todd, J.S., Zimmerman, R.C., Crews, P., Alberte, R.S., 1993. The antifouling activity of natural and synthetic phenolic-acid sulfate esters. Phytochemistry 34 (2), 401–404.
Touchette, B.W., Burkholder, J.M., 2000. Overview of the physiological ecology of carbon metabolism in seagrasses. J. Exp. Mar. Biol. Ecol. 250 (1–2), 169–205.
Valladares, F., Niinemets, U., 2008. Shade tolerance, a key plant feature of complex nature and consequences. Annu. Rev. Ecol. Evol. Syst. 39, 237–257.
von Dassow, Y.J., Strathmann, R.R., 2005. Full of eggs and no place to lay them: hidden cost of benthic development. Mar. Ecol. Prog. Ser. 294, 23–34.
Wang, W.X., Widdows, J., 1991. Physiological-responses of mussel larvae *Mytilus edulis* to environmental hypoxia and anoxia. Mar. Ecol. Prog. Ser. 70 (3), 223–236.
Warkentin, K.M., 1995. Adaptive plasticity in hatching age — a response to predation risk trade-offs. Proc. Natl. Acad. Sci. U.S.A. 92 (8), 3507–3510.
Woods, H.A., Podolsky, R.D., 2007. Photosynthesis drives oxygen levels in macrophyte-associated gastropod egg masses. Biol. Bull. 213 (1), 88–94.
Yang, L.H., 2006. Periodical cicadas use light for oviposition site selection. Proc. R. Soc. B Biol. Sci. 273 (1604), 2993–3000.
Yaro, A.S., Dao, A., Adamou, A., Crawford, J.E., Ribeiro, J.M.C., Gwadz, R., Traore, S.F., Lehmann, T., 2006. The distribution of hatching time in *Anopheles gambiae*. Malar. J. 5, 19.