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Low Oxygen Levels Slow Embryonic Development of *Limulus polyphemus*

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Abstract. The American horseshoe crab *Limulus polyphemus* typically spawns in the upper intertidal zone, where the developing embryos are exposed to large variations in abiotic factors such as temperature, humidity, salinity, and oxygen, which affect the rate of development. It has been shown that embryonic development is slowed at both high and low salinities and temperatures, and that late embryos close to hatching tolerate periodic hypoxia. In this study we investigated the influence of hypoxia on both early and late embryonic development in *L. polyphemus* under controlled laboratory conditions. Embryos were exposed to four different oxygen levels and their developmental stage was scored every second day. Embryos developed more slowly at both 5% O_2 and 10% O_2 than at the 21% O_2 treatment; late development was arrested when oxygen was reduced to 2%. Our study confims that *L. polyphemus* not only tolerates pronounced hypoxia in later embryonic developmental stages, but also in earlier, previously unexplored, developmental stages.

Introduction

The American horseshoe crab *Limulus polyphemus* (Linnaeus, 1758) is a marine chelicerate and one of only four extant horseshoe crab species in the world. *Limulus polyphemus* is distributed along the eastern coast of North America, from Maine to the Yucatan Peninsula (Shuster, 1982). The adults typically live offshore, but migrate in synchrony at high tide to shallow shore waters during spawning (Brockmann and Smith, 2009). Some males attach directly to females using their claspers during mating, but the nesting females are typically also surrounded by multiple satellite males. Thus, their eggs are often fertilized by several different males (Brockmann *et al.*, 2000; Brockmann, 2003; Botton *et al.*, 2010). Large females contain an average of 63,500 eggs, but the number varies with female size (Leschen *et al.*, 2006). Eggs are laid in discrete clusters of 960 to 5786 eggs per clutch (Leschen *et al.*, 2006; Weber and Carter, 2009), and individual females typically place their nests 10–20 cm from each other (Brockmann, 2003). The separation of nests at different beach elevations adds to a heterogeneous microenvironment for embryonic development. After spawning, the adults return to sea (Brockmann and Smith, 2009) whilst the embryos develop through the early stages in a clump, glued together with gravel and sand, at a 3.5–25.5 cm depth in the upper littoral zone (Weber and Carter, 2009). Developmental time of the embryos depends on various environmental factors, including temperature, salinity, and oxygen availability (Jegla and Costlow, 1982; Palumbi and Johnson, 1982). These factors are related to beach morphology and, therefore, change along with beach elevation (Penn and Brockmann, 1994).

Limulus polyphemus develops through four embryonic molts and 21 morphological stages before hatching (Sekiguchi, 1988; Shuster and Sekiguchi, 2003). The egg is surrounded by an outer egg membrane, the chorion, that persists through most of the embryonic development. An additional inner, extra-embryonic shell is formed from noncellular material secreted by the embryo from the epidermal layer at Stages 11–15 (Bannon and Brown, 1980). The first embryonic molt occurs at Stage 18, the second at Stage 19, and Stage 20 begins with the third embryonic molt. In Stage 20, an influx of water causes a swelling of the fluid compartment surrounding the embryo that ruptures the outer chorion, allowing the embryo to actively move around

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inside the inner extra-embryonic shell. Subsequently, at Stage 21 the fourth and final embryonic molt produces a trilobite larva. The following stage is the hatched trilobite larval stage (Brown and Clapper, 1981; Shuster and Sekiguchi, 2003; Botton *et al.*, 2010).

Embryonic development of the horseshoe crab is affected by abiotic conditions in the gravel and sand, and development is arrested below 15 °C (Brown and Clapper, 1981). Jegla and Costlow (1982) showed that embryonic development is slower at salinities of 10 and 40 ppt than at 20 and 30 ppt, and that development was fastest at temperatures of 25–30 °C. Ehlinger and Tankersley (2004) tested development and survival of *L. polyphemus* embryos and larvae at different salinities and temperatures, and showed that embryonic development was completed at salinities below 60 ppt, but failed when temperatures exceeded 35 °C. They also tested the osmolarity of the perivitelline fluid surrounding the developing embryo over a wide range of salinities, and showed that the fluid changed rapidly and became almost isosmotic with the surrounding medium. Botton *et al.* (1988) showed that spawning activities of *L. polyphemus* occurred at sandy beaches with peat in Delaware Bay, and that these breeding sites were characterized by periodic hypoxia and reducing conditions. Jackson *et al.* (2008) studied viability and development of *L. polyphemus* embryos in an estuarine beach in Delaware Bay, and also measured the dissolved oxygen level of the pore water. In that study embryos developed at moderate hypoxia corresponding to oxygen levels between 7% and 13%.

Arthropods that naturally experience hypoxia tend to regulate oxygen uptake under hypoxia. Spicer and El-Gamal (1999) compared development of brine shrimps (*Artemia franciscana*), cultured under normoxia with cultures under chronic moderate hypoxia (10% O_2), and showed that hypoxia accelerated development of respiratory regulation, stimulated early growth, and increased developmental rate.

Palumbi and Johnson (1982) showed that late embryonic stages of *Limulus polyphemus* under a nitrogen atmosphere in water continuously bubbled with nitrogen gas, tolerated extreme hypoxia for 4 days, but that development was halted. When the period of extreme hypoxia was extended to 9 days, mortality was around 50% and none of the embryos could complete the last embryonic molt. They concluded that late embryos showed slightly greater tolerance to hypoxia than did trilobite larvae. How hypoxia affects early development and how different oxygen levels affect development of *L. polyphemus* are unknown. Hence, the aims of this study were to examine the hypothesis that hypoxia slows early embryonic development in *L. polyphemus* and the effect of different oxygen levels on the rate of development.

Materials and Methods

Collection and storage of animals

Adult horseshoe crabs were obtained from the Marine Biological Laboratory, Woods Hole, MA (41°3′36″ N, 70°39′47″ W) in May 2012, and transported to Denmark, where they were maintained at the public aquarium Kattegatcentret in tanks with constant seawater flow and sand covers. The adult horseshoe crabs were fed with mussels and krill every day.

Artificial insemination

Fertility of the adult horseshoe crabs was tested by stimulating the gonopores, using a teaspoon. One female and one male that readily released eggs and sperm, respectively, were chosen as donors for gametes. Microscopy confirmed mobility of the spermatozoa. The two adults were dissected in order to obtain enough gametes. By making cuts near the basal parts of the prosomatic appendages, we removed hemolymph from the female to avoid inhibition of fertilization (Sekiguchi, 1988). Pieces of the soft parts of the ventral cuticle were cut away and eggs were released into a container with seawater (25 ppt salinity), then washed carefully. Immature eggs were removed and excluded from the experiment. Mature eggs were submerged in seawater in a net, within a plastic tray. Sperm was collected by making 2–3-cm cuts into the soft part of the prosomal ventral cuticle from the base of the appendages toward the lateral margin. The body fluid containing semen was collected in a 1-liter beaker and diluted in seawater to obtain a >10% sperm concentration (Brown and Knouse, 1973; Mowbray and Brown, 1974). The diluted sperm fluid was then stirred briefly, poured over the eggs in the net within 5 min, and incubated with the sperm for 1 h, followed by several gentle washes in seawater to avoid inhibition of fertilization by hemolymph remnants (Sekiguchi, 1988).

All fertilized eggs were maintained together in one container containing 0.5 l of aerated seawater (25 ppt) until most embryos reached Stages 6–7. This was done because the first developmental stages are difficult to discern under the stereomicroscope (Shuster and Sekiguchi, 2003; Botton *et al.*, 2010). Developmental stages of embryos were classified according to Sekiguchi (1988).

Experimental design

Fifty individual embryos were placed in each of 12 cylindrical, 390-ml plastic (polypropylene) containers with 250 ml of filtered, 25 ppt seawater. The containers were covered with lids with a hole drilled in the center and maintained under a 12 h:12 h light:dark cycle and a treatment temperature of 30 °C. This temperature allowed comparison of the development of embryos according to the classification scheme of Sekiguchi *et al.* (1982), who used

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the same temperature in their study of *L. polyphemus* development. Furthermore, the chosen salinity and temperature are known to shorten developmental time (Jegla and Costlow, 1982).

In the experiment four different treatments were compared, each replicated three times. The four treatments were atmospheric air (control; 21% O_2), 10% O_2, 5% O_2, and 2% O_2. These oxygen levels were chosen because a pilot experiment had shown that embryonic development halted below 1% O_2. An aquarium pump delivered atmospheric air while Wösthoff gas mixing pumps delivered 10% O_2, 5% O_2, and 2% O_2 from mixtures of oxygen and nitrogen. Atmospheric air and gas mixtures were supplied *via* airline tubing and air stones to the containers with seawater, through the drilled holes in the lids. Measurements recorded with an oxygen meter with automatic calibration (Handy Delta Portable DO Meter; OxyGuard, Farum, Denmark) in the beginning and intermittently during the experiment confirmed that the gas mixing worked as expected. Six hundred embryos at Stages 6–7 (Sekiguchi, 1988) were included in the experiment; 50 individuals were randomly assigned to each of the 12 containers in the various treatment conditions mentioned above. The experiment began 6 days after fertilization and continued for 18 days.

Experimental procedures

At Days 8, 10, 12, 14, 16, and 18, the water was changed and all 600 developmental stages were scored according to the classification scheme of Sekiguchi (1988). During embryo staging the containers were covered with an airtight lid that captured a 140-ml gas space above the water, thereby minimizing oxygen changes in the treatment water. The containers were transported and kept in an insulated box to avoid sudden shifts in temperature. Four different people, who were unaware of the treatment but trained to recognize the embryonic stages, scored them; the containers were distributed randomly among people at each scoring. Embryos and seawater from the treatment container were transferred by 3.5-ml transfer pipette with the tip cut off, to a petri disc with lid, examined, and scored under a stereomicroscope, then returned to the treatment container. It should be noted that during this examination the embryos in all but the control treatments experienced a slight rise in oxygen for a few min. If an embryo were between stages, the earlier stage was chosen. During scoring representative pictures of the different stages were taken (Fig. 1). Some embryos failed to develop and could not be scored to a particular stage. These were scored as unknown and kept in the containers during the whole experiment.

Data analysis and statistics

The numbers of individuals of the different developmental stages were noted for each container, and medians of the triplicates were calculated. Medians were used because they are not affected by outliers, which do affect the means. The medians were tested for differences between the different oxygen levels with a non-parametric Friedman test. The non-parametric test was applied since the data tested were count data that follow a Poisson distribution and, therefore, violate the assumption of normal distribution required by

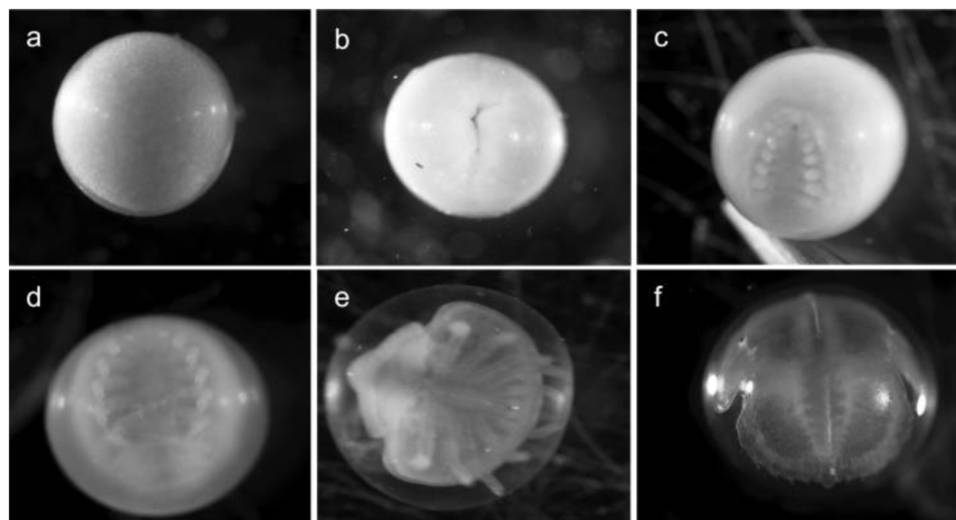

Figure 1. Different embryonic stages of *Limulus polyphemus* (stereo microscope). (a) Stage 5, showing a smooth surface with nuclei distributed equally over the surface; (b) Stage 8.1, in which a germ disk is being formed; (c) Stage 16, with rudimentary appendages; (d) Stage 19.2, after the second embryonic molt; (e) Stage 20.2, after the third embryonic molt, with the embryo moving inside; and (f) Stage 21, before hatching and with the embryo moving inside.

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the parametric tests (Bortz *et al.,* 2000). The tests with repeated measurements were chosen, as the data utilized are not independent of each other. In addition, a post hoc test (pairwise Wilcoxon, exact test) was performed to detect if the different treatments were significantly different from each other.

Chi-square tests (Preacher, 2001) were utilized for testing for pairwise differences of the medians of the different developmental stages in the four different oxygen levels on a specific day. Due to the large number of tests performed in this investigation, a sequential Bonferroni correction was performed on all of the tests conducted (Rice, 1989). Following Miller's (1981) suggestions, a separate probability statement was made for each day in which the numbers of developmental stages after exposure to different oxygen levels were counted. Therefore, the starting K of the sequential Bonferroni correction was (K = 9).

Results

The results of the Friedman test indicated that oxygen has a significant effect on the rate of development of horseshoe crab embryos (Friedman test: $\chi^2 = 11.05$, $P = 0.011$). The pairwise Wilcoxon, exact test was significant only for comparison between the 2% and 5% O_2 treatments (Wilcoxon $W = 21$; $P < 0.05$), while the pairwise chi-square tests showed no significant differences among treatments 8 days after fertilization. The tests were performed between the treatments at any stage where embryos were present.

Ten days after fertilization, no embryos from the 2% O_2 treatment had developed further than Stage 15. Most embryos at higher O_2 treatments had developed further; for example, the number of Stage 17 embryos in the atmospheric air treatment was almost doubled that of the 10% O_2 treatment, and were 10 times higher than those in the 5% O_2 treatment (chi-square test, $\chi^2 = 30.47$, $P < 0.0001$).

Twelve days after fertilization, embryos from the 2% O_2 treatment were the least developed. There were no embryos at Stages 13 and 14 in treatments with 5% O_2 and higher oxygen levels, but most embryos from 2% O_2 had developed to these stages (Stage 13; chi-square test, $\chi^2 = 30.00$, $P < 0.0001$; Stage 14; $\chi^2 = 18.00$, $P < 0.005$). There were almost three times as many Stage 17 embryos from the 5% O_2 treatment as there were in the 10% O_2 treatment, and five times as many as in the control (atmospheric air) (Stage 17; chi-square test, $\chi^2 = 21.00$, $P < 0.0001$); most embryos from the control and the 10% O_2 treatments had reached later stages. There were 10 times as many Stage 20.1 embryos from the control group as in the 10% O_2 treatment, and the other two treatments had none (Stage 20.1; chi-square test, $\chi^2 = 25.73$, $P < 0.0001$).

Fourteen days after fertilization, there was a clear difference between the developmental stages when comparing embryos from the 2% O_2 treatment with those of the other three treatments. No embryos in the 2% O_2 treatment had developed further than Stage 17 (Fig. 2), and there were 10 times as many embryos at Stage 17 (from 2% O_2) as there were from the 5% O_2 treatment (Stage 17; chi-square test, $\chi^2 = 25.73$, $P < 0.0001$). Embryos from the other treatments had developed further. There were almost twice as many Stage 19 embryos from the 5% O_2 treatment as there were in the 10% O_2 treatment, and almost four times as many as in the atmospheric air treatment (Stage 19; chi-square test, $\chi^2 = 18.19$, $P < 0.0001$). The atmospheric air treatment had about twice as many Stage 20.1 embryos as the 10% O_2 treatment, and about three times as many as in the 5% O_2 treatment (Stage 20.1; chi-square test, $\chi^2 = 27.17$, $P < 0.0001$).

After 16 days' exposure to the different oxygen levels, most of the embryos from the 2% O_2 treatment were at

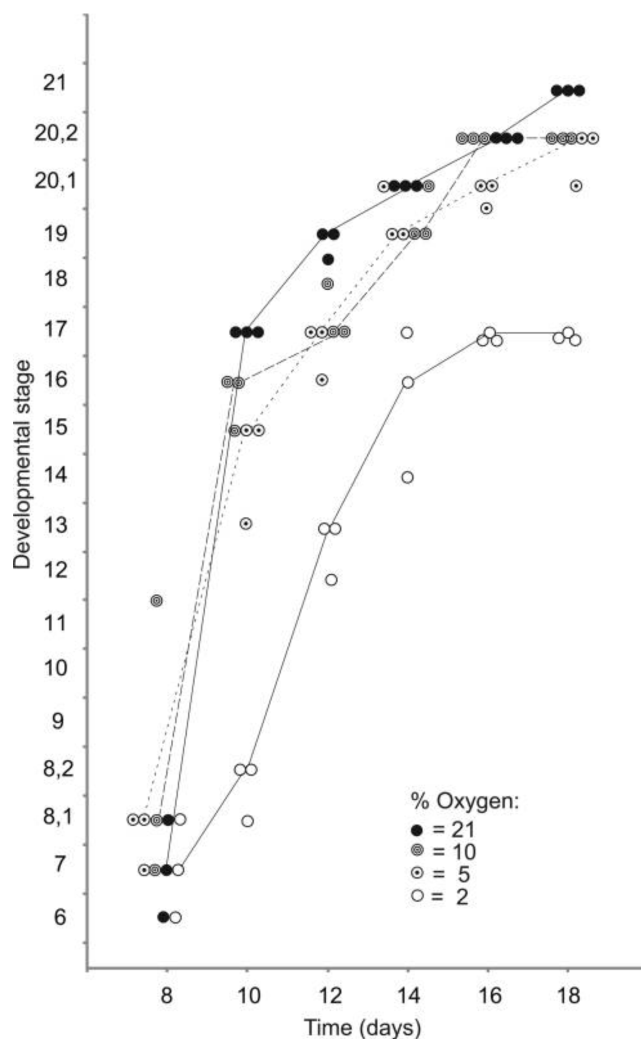

Figure 2. Relationship between the median developmental stage and time after fertilization in the four study treatments (2%, 5%$_2$, 10% O_2; and atmospheric air (control; 21% O_2)). Each treatment was tested in triplicate (3 × 50 embryonic stages) and scored every second day. Lines are drawn between the median of the medians for each treatment and time of scoring.

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Stage 17 (Fig. 2), while there were no Stage 17 embryos in the other treatments (Stage 17; chi-square test, $\chi^2 = 54.00$, $P < 0.0001$). There were more than twice as many Stage 20.1 embryos in the 5% O_2 as there were in the 10% O_2 treatment, and five times as many as in the atmospheric air treatments (Stage 20.1; chi-square test, $\chi^2 = 14.00$, $P < 0.005$). A highly significant result between the treatments was also found for Stage 20.2 (Stage 20.2; chi-square test, $\chi^2 = 35.13$, $P < 0.0001$). There were about five times as many Stage 20.2 embryos in the atmospheric air treatment as there were in the 5% O_2 treatment.

Most embryos from the atmospheric air treatment had hatched and were free trilobite larvae 18 days after fertilization. However, those from the 2% O_2 treatment never developed further than Stage 19; most were at Stages 17 and 18. There were almost five times as many Stage 18 embryos in the 2% O_2 treatment as in the 5% O_2 treatment (Stage 18; chi-square test, $\chi^2 = 19.91$, $P < 0.001$). In the 5% and 10% O_2 treatment groups, there were almost three times as many Stage 20.1 embryos as there were in the normoxic (control) group (stage 20.1; chi-square test, $\chi^2 = 14.00$, $P < 0.01$). The same applies to Stage 20.2 embryos: there were nearly three times as many Stage 20.2 embryos in the 5% and 10% O_2 treatments as there were in the control group (Stage 20.2; chi-square test, $\chi^2 = 21.4$, $P < 0.0001$). The number of embryos at Stage 21—the stage just before hatching—was twice as high in normoxia as in the 10% O_2 treatment; there were no Stage 21 embryos in the lower O_2 treatments (Stage 21; chi-square test, $\chi^2 = 14.67$, $P < 0.01$). Finally, the median number of embryos from atmospheric air that hatched to trilobite larvae was 14 compared to none in the other treatments (hatched larvae; chi-square test, $\chi^2 = 42.00$, $P < 0.0001$).

Our study showed a clear effect that oxygen levels had on the embryonic development in *Limulus polyphemus.* Embryos exposed to 2% oxygen developed more slowly than did embryos receiving higher oxygen levels. Moreover, it is clear that embryos exposed to atmospheric air from 10 days after fertilization developed more quickly than any of the three hypoxic treatment groups (Fig. 2).

Discussion

We studied the influence of different oxygen levels on embryonic development in *Limulus polyphemus* under controlled laboratory conditions at 2% O_2, 5% O_2, 10% O_2, and 21% O_2. These oxygen levels are ecologically relevant; Jackson *et al.* (2008) measured 7% to 13% O_2 in the pore water of horseshoe crab nests in Delaware Bay, and at 7% O_2 in surrounding pore water, it is likely that some embryos in the center of the egg clutch are exposed to even more severe hypoxia than those closer to the periphery. This could explain why embryos in the center develop more slowly than embryos located in the periphery, and also why embryos in small batches develop more quickly than those in larger clutches (Brockmann, 2003).

Our study confirms that *Limulus polyphemus* tolerates pronounced hypoxia not only as adults and juveniles (Towle and Henry, 2003), but also as late embryos just before hatching (Palumbi and Johnson, 1982). This study also provides the first documentation of hypoxia tolerance in early stages of development. We found that embryonic development ceased at 1% O_2 in our pilot experiment, but at 2% embryos developed normally through the early stages. Adults and juveniles survived 1% O_2 for up to 60 h; this remarkable tolerance to hypoxia involves coordinated slowing of the heart rate and the respiratory movements of the book gills (Watson and Wyse, 1978; Towle and Henry, 2003). Embryos develop book gills late at Stages 20.1–20.2 and these gills move rapidly at Stage 21 (Botton *et al.*, 2010), but how ventilation and heart rate are affected in hypoxia remains to be clarified in future studies.

There were no significant differences in development between hypoxia and normoxia 8 days after fertilization in our study, and it seems likely that these early and small embryos could meet their oxygen requirements in severe hypoxia. We hypothesized that oxygen demand would be higher from Stage 18 and onward because of the onset of embryonic molting, which is energetically expensive. By the end of our experiment, most embryos remained in Stage 17 at severe hypoxia, which confirms our hypothesis and supports evidence that the molting stages are particularly sensitive to environmental stress (Jegla and Costlow, 1982).

Sekiguchi (1988) reared *L. polyphemus* in the laboratory at normoxia, 30 °C, and at salinity of 34–35 ppt, and found that embryos reached Stage 17 in about 5 days after fertilization, which is faster than any treatment in our study. Here it took at least 10 days at normoxia, and developmental time was 40% slower in the most severe hypoxia. This difference in developmental rate became even more pronounced in the later embryonic stages. At Stage 20.1 the rupture of the chorion could increase sensitivity to changing environmental conditions (Sekiguchi, 1988) and explain why the effect of salinity and temperature exert smaller effects during early development (Jegla and Costlow, 1982; Ehlinger and Tankersley, 2004). We also expected a higher demand for oxygen from embryos at Stage 20 and onward, because of the onset of rotational locomotion in these late embryos. A higher demand for oxygen in late embryos was supported by our result that the only embryos to hatch were from the normoxic treatment. Late embryos arrest development under extreme hypoxia, but retain the ability to resume normal development for as long as 11 days (Palumbi and Johnson, 1982).

Horseshoe crab embryos are ecological important as food for several species of shorebirds and gulls, but most horseshoe crabs nests are buried at depths of 15–20 cm in the

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beach, which is too deep for shorebirds to reach. Some embryos are translocated to the surface layers of beach sand by wave action and by spawning activities from other horseshoe crabs. Horseshoe crabs perform mass spawnings in synchrony and, if spawning densities are high, then embryos already placed in the buried nests risk being brought to the surface, where they are eaten or desiccate (Botton *et al.*, 1994; Niles *et al.*, 2009). These mortality risks probably increase with lower oxygen levels since developmental time is prolonged.

Since most populations of *Limulus polyphemus* have declined due to historic climatic events and more recent anthropogenic pressures (Faurby *et al.*, 2010), protection of suitable beaches for spawning is an important conservation issue for both *L. polyphemus* and the migrating birds that prey on horseshoe crab eggs (Botton *et al.*, 2010; Funch (in press)). The hypoxia encountered by *L. polyphemus* during development (Jackson *et al.*, 2008) is likely to be exacerbated by increased anthropogenic nutrient loads on coastal waters. In addition, the prolonged developmental time shown in this study may be even more pronounced when global warming increases oxygen demand. Warmer water has a lower oxygen content (Harrison *et al.*, 2015) and hence will reduce the larval hatching success of *L. polyphemus*.

In conclusion, oxygen has considerable impact on developmental time in *L. polyphemus*. Embryos exposed to hypoxia developed more slowly than embryos exposed to higher oxygen levels. Adequate oxygen levels are therefore essential for embryos of *L. polyphemus*.

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